LABELING KIT

FISH*Bright*™
Nucleic Acid Labeling Kit

For 10 labeling reactions
(Labels 10µg)

Product code
FLK-001/FLK-002/FLK-003/FLK-004
FLK-005/FLK-006/FLK-007

For laboratory use only
Research purposes only

KREATECH
BIOTECHNOLOGY

Instruction manual

In case of emergencies check SDS for medical advice

Labelling According Regulation (EC) No 1272/2008

Code #	Description	Signal Word	Pictogram	Hazard Statements
LK-025B, LK-026B, LK-027B, LK-028B, LK-030B, LK-031D	ULS hapten/dye	Danger		H330
LK-017C	10x labeling solution)	N.A.	N.A.	N.A.
SP0005C	Krea*pure*™	N.A.	N.A.	N.A.
LK-084B	Cell hybridization buffer	Danger		H319, H360D
LK-087A	Paraffin Hybridization Buffer	Danger		H360D
LK-072B	KREA*boost*	Warning		H315, H319

KREATECH's FISH*Bright*™: Nucleic Acid 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.

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Table of Contents

A. Assay Materials

I. Components and Storage - Important

The contents of this FISH*Bright* kit are sufficient for labeling max. 10 µg input nucleic acid template according to the standard labeling protocol.

Materials Provided	Quantity	Storage Conditions
ULS hapten/dye	20 µl	2-6°C
10 x Labeling solution	100 µl	2-6°C
KREA*pure*™	10 pcs	2-6°C
Cell Hybridization Buffer	1 ml	2-6°C
Paraffin Hybridization Buffer	1 ml	2-6°C
KREA*boost*	100 µl	-20°C*

*KREA*boost* is shipped at 4°C, store at -20°C upon delivery

Reagents and Buffers not included in Kit

DNase I, nicking buffer, nuclease free H_2O, NH_4Ac, Ethanol and FISH-grade *cot*

II. Quality Control

In the standard QC procedure 1 µg of non-labeled, fragmented, repeat DNA, Unique DNA and Whole Chromosome Paint DNA are labeled with ULS following the standard protocol. Fluorescent in situ hybridization (FISH) is carried out followed by direct detection, except with the biotin-ULS labeled probes where detection is indirect with streptavidin-Cy3.

B. General Information

I. Product Description and Background

Fluorescent *in situ* hybridization (FISH) identifies, or labels, target genomic sequences so that their location can be studied. DNA sequences from appropriate, chromosome-specific probes are first labeled with reporter molecules. The labeled DNA probe is then hybridized to the metaphase chromosomes or interphase nuclei on a slide. After washing the specimen is screened for the reporter molecules by fluorescence microscopy.

KREATECH's FISH*Bright*: nucleic acid labeling kits are intended for the non-enzymatic labeling of FISH probes. They offer the opportunity to label 10 µg target nucleic acid material.

Fluorescent dyes/haptens available within the FISH*Bright* nucleic acid labeling kits

Catalog #	Product name	ULS dyes/ hapten	Abs (nm)	Em (nm)	Ext coefficient (ε_{max})
FLK-001	FISH*Bright*™415 Blue	ULS-Dy415	429	464	56 000
FLK-002	FISH*Bright*™495 Green	ULS-FLU	495	517	83 000
FLK-003	FISH*Bright*™505 Green	ULS-D-Green	501	526	78 000
FLK-004	FISH*Bright*™550 Red/Orange	ULS-RHO	553	576	95 000
FLK-005	FISH*Bright*™547 Orange	ULS-Dy547	547	565	150 000
FLK-006	FISH*Bright*™647 Far red	ULS-Dy647	647	665	250 000
FLK-007*	FISH*Bright*™Biotin	Biotin (Hapten)	-	-	-

* requires additional reagents (Avidin or Streptavidin) coupled to reporter molecules.

II. Principle of the Universal Linkage System (ULS)

The proprietary ULS technology is based on the stable binding properties of platinum 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 the 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 nonenzymatic labeling of nucleic acids to be achieved within 30 minutes.

III. Critical Parameters for a Successful Labeling

- All nucleic acid samples need to be free from divalent cations (e.g. Mg2+), 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
- Assessment of the purity and yield of your DNA should be carried out by
  - Running your DNA on a 1% agarose gel
  - Determining the OD260
- Labeling efficiency is optimal at concentrations above 50 ng/µL (See Trouble Shooting on page 13)
- The labeling volume is flexible as long as the DNA concentration is not lower as 50 ng/µL and the amount of ULS added is adjusted to the amount of input DNA
- High Tris-HCl concentrations (>40 mM), EDTA (>5 mM), Mg-acetate (>1 mM) or NaCl (> 100 mM) concentrations and restriction enzyme digestion buffers have a rate limiting effect on the labeling reaction and should therefore be removed prior to labeling
- For in situ hybridization, the size of input DNA is recommended to be between 100 and 300 base pairs (of chromosome specific libraries: 200 - 2000 base pairs). This can be achieved by sonication or DNase treatment (DNA), prior to labeling
- Volume of ULS-labeled material added to KREA*pure* column should not exceed 50µl (higher volumes will result in a lower purification efficiency)

C. Protocols

I. Suggested Fragmentation Protocols

If DNA fragmentation by DNase I will be carried out then this must be done before ULS labeling. If fragmenting DNA by sonication then this may be carried out before or after ULS labeling

For in situ hybridization the optimal probe fragment size is between 100-300 bp. Probes over 1 kb will generate a spotted background in in situ hybridization. Because most PCR generated probes are smaller than 1 kb, these probes can be labeled without fragmentation. Other probes such as plasmids, cosmids, YACs, PACs and BACs must be fragmented prior to labeling with ULS. Fragmentation can be performed by sonication or DNase I treatment.

a. Suggested Fragmentation by Sonication

1. Prepare at least 100µL of DNA solution in 10 mM Tris- HCl, pH 8.0, 0.3 mM EDTA (TE) or just nuclease –free water, with a minimal DNA concentration of 50 ng/µL

2. Sonicate for at least 5 cycles of 1 minute each at amplitude 5 µm, keep sample on ice. Cool the DNA solution on ice for 1 minute after each cycle. Take a look on our website (www.kreatech.com) for protocols for different sonication apparatus

3. Check the fragment length by electrophoresis on a 1% agarose gel

4. Label the DNA according to the labeling protocol

b. Suggested Fragmentation by DNase I Treatment

1. Prepare DNase I stock solution: dissolve 1 mg of DNase I (Roche # 104 159, approx. 2000 units/mg) in 1 mL of 5 mM NaAc pH 5.2, 1 mM $CaCl_2$, 50% glycerol. Make sure buffer is ice cold before adding the lyophilized DNase I. Mix by inversion. Do not vortex. Store stock DNase I solution at -20°C; avoid freeze-thaw cycles
2. Prepare 10 x nicking buffer: 50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$

3. Dilute the DNase I stock 1:5000 in 1x nicking buffer. The buffer solution should be cold before the addition of the enzyme. These dilutions should be prepared immediately before use
4. Setup nicking reaction on ice as follows:

    - 1 µg template DNA
    - 2.5 µL 10 x nicking buffer
    - 3-5 µL diluted DNase I
    - H_2O to 25 µL

5. Incubate at 37°C for 10 minutes. Stop reaction by placing on ice for at least 1 minute and precipitate with ¼ volume of 10 M NH_4Ac and 2.5 volumes of 100% EtOH. Resuspend in the Nuclease-free Water
6. Check the fragment length by electrophoresis on a 1% agarose gel

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II. ULS Labeling

For labeling 1 µg nucleic acid sample

1. Briefly spin all required reagents to collect contents of tubes
2. Take 1 µg of nucleic acids sample
3. Add 2 µL of ULS label per 1 µg nucleic acid sample
4. Add 1/10 volume of 10x Labeling solution
5. Adjust with nuclease-free water to final volume and mix by pipetting (see example set-up below)
6. Label sample by incubation for 30 minutes at 85°C
7. Place samples on ice, spin down to collect contents of tube before proceeding with purification using the KREA*pure* columns

Example of ULS labeling of 1 µg nucleic acid sample in 20 µL

Nucleic acid sample in Nuclease-free Water	16 µL
ULS dye	2 µL
10 x Labeling solution	2 µL
Total volume	20 µL

Dye removal 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 nuclease-free water
7. Discard collection tube and flow-through
8. Put column in a new (nuclease free) 1.5 mL micro centrifuge tube
9. Add ULS-labeled nucleic acid on to column bed
10. Spin column for 1 minute at 20800 x g
11. Flow through is purified labeled nucleic acid sample. At this point the degree of labeling (DOL) can be measured

$$X = \text{ng (nucleic acid) / µl} = \frac{OD260 * \text{dilution factor} * 50}{\text{cuvet length (cm)}}$$

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

$$\textbf{Degree of Labeling (DoL)} = \frac{340 * Y * 100\%}{X * 1000}$$

ε dye ULS-Dy415 = 56 000
ε dye ULS-FLU = 83 000
ε dye ULS-D-Green = 78 000
ε dye ULS-RHO = 95 000
ε dye ULS-Dy547 = 150 000
ε dye ULS-Dy647 = 250 000

III *In situ* Hybridization Protocol

Slide Pretreatment:

For use on metaphase and interphase cells from peripheral blood cultures or direct preparations prepared by standard cytogenetic methods, see: The ACT cytogenetics laboratory manual. 2nd ed. New York: Raven Press; 1991.
Pre-treat dry sample slide in 2 x SSC, pH 7.0 at 37°C for 2 minutes. Dehydrate in 70%, 85% and 100% Ethanol for 1 minute each. Air dry at room temperature.

For paraffin embedded tissue sections refer to the Appendix at page 15
For slides with cytoplasmic background or difficult samples refer to Appendix at page 16.

Probe preparation:

Add to the labeled probe C_0T-1 DNA ,for example 25 x excess (FISH-grade *cot* available from Kreatech), when using a human unique sequence clone targeted to a human sequence. Precipitate the DNA mixture with ¼ 10 M NH_4Ac and 2.5 volumes abs. Ethanol. Cool the precipitate for 30 minutes at -80°C or overnight at -20 °C. Spin at 20.800 g for 30 minutes (cooled if possible). Dry pellet and dissolve in the appropriate hybridization solution, use CHB for metaphase spreads, bloodsmears and bone marrow. Use 9:1 THB and KREA*boost* for use on paraffin sections.
Combine different labeled probes in one hybridization, but note that C_0t-1 will suppress repeats as α-satellites in combination hybridizations, increase the concentration of such probes at least 5 times.

Co-denaturation:

Apply 10 µl of probe or probe-mix per 22 x 22 mm field. Cover with glass cover-slip and seal with Fixogum or rubber cement. Denature sample and probe on a hot plate at 75°C for 5 minutes (or 10 min for pre-treated tissue sections, see Appendix I). Continue with hybridization.
Or Separate Slide Denaturation, Optional: Denature slide in 70% Formamide/ 2x SSC, pH 7.0 at 72ºC (±1ºC) for 2 minutes. Dehydrate in ice cold (-20ºC) 70%, 80%, and 95% Ethanol for 2 minutes each. Air dry. Denature probe mix at 75°C for 10 minutes. Apply probe to denatured slide, cover with glass cover-slip, seal with Fixogum or rubber cement and continue with hybridization.

Hybridization:

Incubate overnight at 37ºC in a humidified chamber or as mentioned in the table on page 12

Post-Hybridization Wash:

Remove rubber cement, slide off cover-slips. If the coverslip does not come off incubate slides in 1 x Wash Buffer II (2 x SSC/ 0.1% NP-40)at room temperature. Wash slides in 1 x Post-Wash Buffer I (0.4 x SSC/ 0.3% NP-40) for 2 minutes at 72°C (±1ºC) without agitation. Wash slides in 1 x Wash Buffer II for 1 minute at room temperature without agitation. Proceed to counterstaining.

Counterstain:

Apply 15 µl DAPI/Antifade (final concentration 0.02 µg/ml or undiluted at 0.1 µg/ml for tissue) and apply glass cover-slip. Squeeze out remaining buffer by applying a paper tissue over the cover-slip and press gently. Proceed with Microscopy.

Procedural recommendations:

Temperature and buffer concentration (stringency) of hybridization and washing are important, as lower stringency can result in non-specific binding of the probe to other sequences, and higher stringency can result in a lack of signal. Incomplete denaturation of target DNA can result in lack of signal.

Recommendations for Fluorescence Microscopy

For optimal visualization use a well maintained and regularly calibrated microscope equipped with a 100 W mercury lamp and a x 63 or x 100 fluorescent objective is recommended. Triple band-pass filters (DAPI/FITC/Texas Red or DAPI/FITC/Rhodamine) are used to view multiple colors, single band-pass filters are used for individual color visualization.

Probe type	Concentration (ng/µL)	Time
PACs/BACs	4-10 ng	O/N
YACs	10-40 ng	O/N to 72 hours
PCR fragments	1-4 ng	O/N
Repeats	0.1-1 ng	2 hrs to O/N

D. Trouble Shooting

Problem	Possible Cause	Suggestions
Labeling of template too low	Amount of ULS added is not correct	Check the ratio of ULS/nucleic acid
	Concentration of template is lower than measured	Use a concentration of at least 50ng/µL of nucleic acid sample
	Reaction temperature is too low	Make sure that the reaction temperature is 85°C
	Template preparation is insufficiently pure	Purify DNA by phenol extraction and/ or ethanol precipitation
	Sample contains too much NaCl, TRIS, EDTA or proteins	Purify DNA by phenol extraction, ethanol precipitation or a commercial nucleic acid purification column **Note:** with silica based columns it is important to add an 80% ethanol wash step to insure the absence of buffer contaminants are in the sample
No hybridization signal	Hybridization and wash conditions are too stringent	Reduce temperature of hybridization Lower stringency of the wash buffer
	Labeled fragments are too large	Reduce size of probe by fragmentation prior to labeling to ensure proper hybridization of the probe (see fragmentation protocols)

	Labeling density is too low	Check the labeling density and if necessary add more ULS per µg nucleic acid
	Pretreatment of target is not sufficient	Treat next target longer or in another way.
Unspecific hybridization signal (background)	Hybridization temperature is too low	Increase hybridization temperature and/or increase temperature during washing step
	Probe concentration is too high	Reduce probe concentration
	Pretreatment of target is not sufficient	Treat next target longer or in another way.
	Labeling density is too high	Check the labeling density and if necessary add less ULS per µg nucleic acid

E. Appendix

I. Pretreatment of paraffin embedded tissue sections for FISH

Recommended for use with KB-60001 POSEIDON Tissue Pretreatment Kit

1. Bake 4-5 µm formalin fixed paraffin embedded tissue sections for 2-16 hours at 56ºC.
2. De-paraffinize slides by soaking in (regular refreshed) xylene for 10 minutes two times. Rehydrate by soaking in 100%, 85% and 70% Ethanol for 3 minutes each. Wash with dH2O for 3 minutes.
3. Pre-treat with 0.2 M HCl for 20 minutes, then wash in dH2O for 3 minutes.
4. Place slides in 8% sodium thiocyanate in dH_2O at 80ºC for 30 minutes. Rinse in 2 x SSC for 3 minutes.
5. Digest in 0.025 – 0.5 % pepsin in 0.2 M HCl at 37ºC for 30 minutes. (Time depending on tissue, fixation and pepsin activity). Wash in dH_2O for 1 minute and in 2 x SSC for 5 minutes.
6. Dehydrate slides by soaking in 70%, 85%, and 100% Ethanol for 1 minute each time. Air dry.

Note: Check protein digestion and pre-treatment by applying 15 µl DAPI counterstain and evaluate slides using a fluorescence microscope equipped with a DAPI filter. 30 minutes protein digestion is normally sufficient for a wide range of breast tumors. Remove cover-slip and soak tissue in 2 x SSC for 2 minutes and increase protein digestion by 2-20 minutes if sample is under digested. Use a fresh sample and cut protein digestion time to 20 minutes if sample is over digested.

7. Apply 10 µl of probe or probe-mix per 22 x 22 mm field. Seal with Rubber Cement. Denature sample and probe on a hot plate at 75°C for 10 min.
8. Incubate overnight in a 37ºC humidified chamber.
9. Remove rubber cement and slide off cover-slips.
10. Wash slides in 1 x Post-Wash Buffer I (0.4 x SSC/ 0.3% NP-40) for 2 minutes at 72°C (±1ºC) without agitation.
11. Wash slides in 1 x Wash Buffer II (2 x SSC/ 0.1% NP-40) for 1 minute at room température without agitation. Proceed to counterstaining.
12. Apply 15 µl counterstain (0.1 µg/ml DAPI in Antifade) and cover tissue with a 22 x 50 mm glass cover-slip.

II. Pretreatment for slides with cytoplasmic background or difficult samples
Recommended for use with KB-60003 POSEIDON FISH & Digestion Kit

1. Pre-treat dry sample slide in 2 x SSC, pH 7.0 at 37°C for 2 minutes.
2. Incubate the slides 3-10 minutes in 0.005% Pepsin solution in 0.01 M HCl at 37°C.
3. Wash slide for 3 min in 1 x PBS at room temperature.
4. Post-fixate in 1% buffered formaldehyde in 1 x PBS/20 mM $MgCl_2$ for 10 min at room temperature.
5. Wash slide for 3 min in 1 x PBS at room temperature.
6. Dehydrate in 70%, 85% and 100% Ethanol for 1 minute each. Air dry at room temperature.

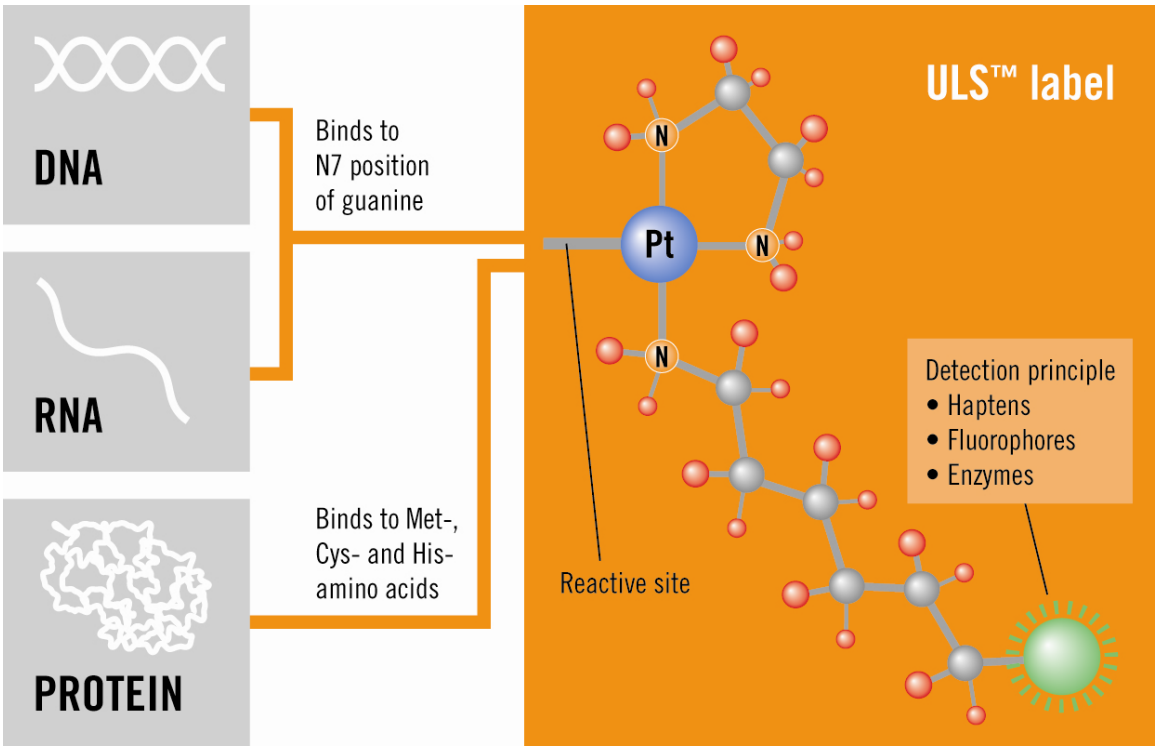
Proceed with Co-denaturation as on page 11.

F. Related products

- KB-60001: Poseidon FISH Kit
- KB-60002: Poseidon Tissue Pretreatment Kit
- KB-60003: Poseidon FISH & Digestion Kit
- LK-071A: Fixogum
- KB-cot: FISH-grade *cot*
- LK-093A: DAPI/ANTIFADE 10 x conc.

G. References

1. Jelsma T. *et al*., Increased labeling of DNA probes for in situ hybridization with the Universal Linkage System (ULS™), *Journal of NIH Research*, Vol. 5, p. 82, 1994
2. Kottler M.L. *et al*., Subregional mapping of the human Gonadotropin-Releasing Hormone Receptor (GnRH-R) Gene to 4q between the markers D4S392 and D4S409, *Human Genetics,* Vol. 96, No. 4, p. 477-480, 1995
3. Van Belkum A. et al., Application of a new universal DNA labeling system in the PCR mediated diagnosis of Chlamydia trachomatis and human papillomavirus type 16 infection in cervical lesions, *Journal of Virological Methods*, Vol. 45, p. 189-200, 1993
4. Van Belkum A*. et al*., Non-isotopic labeling of DNA by newly developed hapten-containing platinum compounds, *BioTechniques*, Vol. 16, No 1, p. 148-153, 1994
5. Van Es R.M. *et al*., High sensitivity of in situ hybridization using sheep Fab fragments conjugates. *Journal of NIH Research*, Vol. 6, p. 88, 1994
6. Van Eijsden R. et al., Development of a rapid and highly sensitive labeling method of DNA and RNA probes for (fluorescent) in situ hybridization, *Abstract Book, San Diego AACC Conference*, 1996
7. Tanke H. *et al*., New strategy for multicolor fluorescence in situ hybridization: COBRA: Combined Binary Ratio labeling. *European Journal of Human Genetics*. 1999 Jan: 7(1):2-11
8. Szuhai K et al., COBRA: combined binary ratio labeling of nucleic-acid probes for multi-color fluorescence *in situ* hybridization karyotyping. *Nat. Protocol*. 2006; 1 (1): 264-75
9. Universal Linkage System: versatile nucleic acid labeling technique. *Expert Rev Mol Diagn.* 2001 May;1(1):81-91


DNA
RNA
PROTEIN
Binds to
N7 position
of guanine
Binds to Met-,
Cys- and His-
amino acids
ULS™ label
Pt
N
N
N
Reactive site
Detection principle
• Haptens
• Fluorophores
• Enzymes

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