PROTEIN LABELING KIT

ULS™

Two-color ULS™ Cell Lysate Protein Labeling and Fluorescent Detection Kit

4 dual FLU/BIO labeling and detection reactions for expression profiling on antibody arrays

Product code
PLK-002

For research use only
Not for diagnostic purposes

KREATECH BIOTECHNOLOGY

Instruction manual

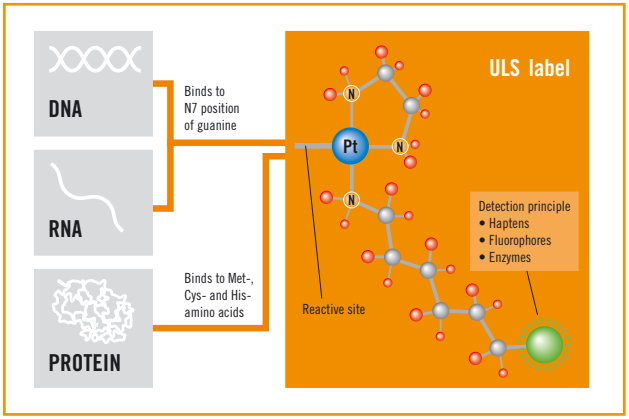

Figure 1. ULS, the Universal Linkage System that labels your DNA, RNA and proteins.

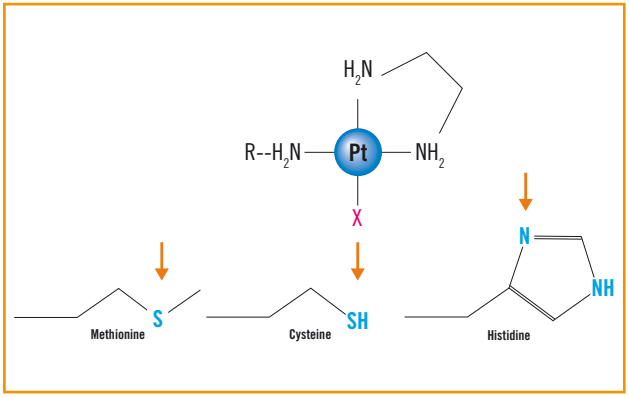

Figure 2. ULS labels proteins by forming a coordinative bond on:

- *Sulphur atoms of Methionine and Cysteine*
- *Nitrogen atom in Histidine (pH>4)*

KREATECH®'s Two-color ULS™ Cell Lysate Protein Labeling and Detection Kit

For cell lysate profiling on high-content antibody arrays

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

Important

Upon receipt, store the kit at 4°C

- 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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Table of Contents

A. Assay Materials

I. Components and Storage

All components should be stored at 4°C

Component	Description of function	Amount	Color Code
10 x UPEL buffer	10 x ULS Protein Extraction & Labeling buffer	20 ml	Transparent
FLU-ULS	Fluorescein-ULS label	10 µl	Green
BIO-ULS	Biotin-ULS label	10 µl	Red
PB agent	Protein Blocking agent (powder)	0.2 g	Blue
10 x PB agent solvent	Solvent for Protein Blocking agent	2 ml	Purple
ULS-Trap columns	Column for removal of non-reacted ULS	8	Yellow
2 ml sample collection tubes	Use in combination with ULS-Trap	16	-
PAB buffer	Protein Array Blocking buffer	2 ml	Black
PAW buffer	Protein Array Washing buffer	20 ml	Transparent
Rabbit anti-Flu-DY547	Fluorescein detection conjugate	20 µl	Green (brown vial)
Streptavidin-DY647	Biotin detection conjugate	2 µl	Red (brown vial)
Manual		1	

II. Materials Needed but Not Included in Kit

- 2-pad antibody microarray slides, spotted with antibodies
- FAST® Frame Multi-Slide Plate (Whatman Schleicher & Schuell #10486001)
- FAST® Dual-well Incubation Chambers (Whatman Schleicher & Schuell #10486087)
- Rocking platform shaker (e.g. IKA Labortechnik HS250 basic)
- Tabletop microcentrifuge (e.g. Eppendorf)
- 1.5 ml reaction vials (e.g. Eppendorf)
- Protease inhibitor cocktail (Complete™ Roche #1836153)
- Phosphatase inhibitor cocktails (Sigma P2850 and P5726)
- BCA Protein Assay Kit (Pierce #23225)
- 37°C incubator or waterbath
- PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4)
- Purified water (e.g. MilliQ)
- Rubber policeman (for adherent cells)
- Vortex
- Array scanner (e.g. Axon Instruments, GenePix™ Personal 4100A)
- Analysis software (e.g. GenePix™ 4100A)
- Data analysis software (e.g. Microsoft® Excel)
- 50 ml tubes with cap (e.g. Greiner)

B. General Information

I. Introduction and Product Description

Protein expression profiling using antibody microarrays has been growing rapidly over the last few years since they enable the study of protein expression and modification in a multiplex format (1). In antibody microarrays essentially two formats exist. One being the sandwich format, where antibodies capture analytes and these are subsequently detected with a second, labeled antibody. This method is sensitive, but has the disadvantage that the amount of antibody pairs that can be used simultaneously is limited to approximately 30, due to cross reactivity of antibodies and analytes. This maximum amount is often referred to as Gold's wall. In the second array format, called target labeling or direct labeling, the analytes are pre-labeled and, antibodies capture the pre-labeled analytes. This format has several advantages: there is no need for antibody pairs, (which are often not available), there is no Gold's wall, (thus an unlimited amount of antibodies can be used) and the format enables the use of two different labels for differential analysis. The Two-color ULS Cell Lysate Protein Labeling and Detection Kit is designed to perform target labeling for antibody arrays.

For target labeling to work, a protein labeling technology is needed that is robust, sensitive and that has minimal interference with the capture of the analytes by antibodies. The Universal Linkage System ULS is a labeling system that is highly suitable for target labeling. It is robust, stable in aqueous solutions, compatible with high temperatures and with all kinds of buffers, salts and detergents. It targets Methionine, free Cysteine and Histidine residues. These residues are less involved in protein-protein interactions than Lysine (which is targeted by isothiocyanate labeling or N-hydroxysuccinimide labeling). Therefore, when using ULS, there is a lower risk of epitope destruction than when labeling Lysine residues.

ULS labeling is compatible with most biochemical buffers including Tris and glycine, with detergents including SDS, Triton X-100, and with many salts, including EDTA. Incompatible substances are guanine, (thio)cyanides, thiourea, thioethers, thiols, and strongly reducing agents like Dithiothreitol (DTT), β-Mercaptoethanol, Tris(2-Carboxyethyl) Phosphine (TCEP) and Tributylphosphine (TBP).

This kit has especially been designed for cell lysate labeling and detection for profiling protein expression on antibody arrays. In the Two-color ULS Cell Lysate Protein Labeling and Detection Kit, the proteins are Fluorescein and Biotin labeled with FLU-ULS or BIO-ULS, respectively. Detection takes place in an indirect way using an anti-Fluorescein-DY547 conjugate to detect FLU-ULS and a Streptavidin-DY647 conjugate to detect BIO-ULS.

Removal of free label after protein labeling is essential, since non-used labels might label the antibodies spotted on the array and thus lead to false positive results, and/or lead to high background signals on the slide surface.
This kit contains ULS-Trap columns that specifically remove the excess ULS label.

Since in principle all proteins become labeled, it is very important to block non-specific binding to the surface of antibody arrays. This kit provides a proprietary non-proteinous array blocker – Protein Array Blocking buffer – that effectively prevents non-specific binding to the array surface.
The signal to noise ratios are further enhanced by adding a

blocker – Protein Blocking solution (Prot*block*) – to the sample, just prior to the removal of unreacted ULS reagent.

Commercial antibody arrays are available in several different formats: often they are sold as one array pad per slide, but multiple array pads per slide are also commercially available, like 2-pad, 8-pad, 12-pad and 16-pad arrays. The Two-color ULS Cell Lysate Protein Labeling and Detection Kit has enough reagents to perform 4 standard labeling reactions for both FLU-ULS and BIO-ULS. This is sufficient to profile 2 samples against a reference control sample in a two-color format on two 2-pad arrays. This manual has been written for 2-pad antibody arrays, but can be easily adjusted for use on single pad or multiple pad slides. Incubation volumes can be adjusted from 400 µl down to 100 µl. *See appendix I: Changing the array format.*

This labeling and detection kit is compatible with several types of surfaces e.g. nitrocellulose-based slides, hydrogel slides, aminosilane-coated slides, epoxysilane-coated slides, and CodeLink™ slides. Two-Color ULS Cell Lysate Protein Labeling and Detection Kit can be used in combination with the buffers and arrays in commercially available Clontech antibody array kits and Sigma Panorama™ antibody microarrays.

II. Principle of ULS™ Labeling

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 coordinative bond, firmly coupling the ULS to the target. In proteins, ULS binds to sulphur and nitrogen containing side chains of Methionine, Cysteine and Histidine and thereby enables a robust one-step chemical labeling of proteins. ULS is available coupled to a variety of labels and haptens, including fluorochromes, biotin and dinitrophenol (DNP).
See Figure 1 and 2 inside front cover.

III. Procedural Overview and General Considerations

The procedure of protein expression analysis with the Two-color ULS Cell Lysate Protein Labeling and Detection Kit is as described below. See also the flow scheme in figure 3 (page 16-17). The entire procedure from sample preparation to antibody array scanning takes one day to complete.

The protocol contains the following steps:

1. Cell lysis and protein extraction
2. Determination of protein concentration
3. ULS labeling of proteins
4. Removal of free label using ULS-Trap columns
5. Incubation of labeled proteins on the antibody array
6. Washing to remove unbound proteins
7. Detection of FLU-ULS and BIO-ULS label with anti-Flu and Streptavidin fluorescent conjugates.
8. Scanning and analyzing the microarray slides to measure bound antigen

1. Cell lysis and protein extraction

It is strongly recommended to use freshly prepared extracts. Using frozen extracts, old extracts or cell lines with low viability may give inadequate results.
A wide variety of cell lysis protocols is available, most of them are based on lysis buffers containing detergents. The lysis conditions determine the amounts and types of proteins and thus the proteome that is going to be analyzed.

This kit contains a detergent-based gentle and non-denaturing protein extraction and labeling buffer that extracts both cytosolic- as well as membrane-bound proteins and that has been optimized for ULS labeling. Therefore, we strongly recommend using the protein extraction and labeling buffer provided in the kit to ensure optimal protein labeling. It is recommended to add protease inhibitors cocktail and phosphatase inhibitors cocktail to the extraction and labeling buffer when preparing the extracts. The cocktails that KREATECH has tested and found to be ULS compatible are listed in "Materials needed but not included in the kit". It is possible to use other extraction buffers as long as no ULS interfering substances are present e.g. reducing agents as DTT, β-mercaptoethanol. When using a stringent lysis buffer containing SDS, the use of Benzonase® nuclease is recommended to remove the DNA. In order to compare results from different experiments it is important to use always the same protein extraction and labeling buffer since different buffers may extract a different part of the proteome of the cells.

2. Determination of protein concentration

Adequate determination of the protein concentration is important since the correct ratio of protein to label is important to the success of the experiment. We recommend using the BCA protein assay reagent since this assay is detergent compatible and has been tested by Kreatech's scientists. The recommended amounts of ULS for labeling are based on the determination of protein with the BCA assay.

3. ULS labeling of proteins

Since ULS is very stable, FLU-ULS and BIO-ULS are provided as an aqueous stock solution. This makes time consuming weighing of precious labeling agents obsolete.

ULS labeling is robust under many conditions and very reproducible. The labeling time is optimized to 3-4 hours at 37°C, but may be extended to yield higher labeling densities. In that case, it is recommended to add fresh protease inhibitors. Determining the labeling density by measuring label to protein ratio is not necessary. Therefore, it is not a standard procedure. A method is provided in the appendix for determining label to protein ratios in case one wishes to measure it.

4. Removal of free label using ULS-Trap columns

Removal of free label after protein labeling is essential, since non-reacted labels might label the antibodies spotted on the array and thus lead to false positive results and/or lead to high background levels on the slide surface. To this end, many researchers first inactivate the excess label with a soluble target and use gel filtration spin columns to remove the free label. These methods remove 95-98% of free label, however small proteins or peptides may be lost.
KREATECH has developed a new spin column, ULS-Trap, for free label removal that specifically binds ULS, and removes free label very efficiently (>99.5%) with minimal loss of protein. The method is based on immobilized ULS targets and is specific for ULS. In this procedure ULS is therefore not inactivated with a soluble target prior to removal with the column. Since the column is not based on gel filtration even small proteins and peptides will flow through. The method is rapid and purification is performed in less than 5 minutes.

5. Incubation of labeled proteins on the antibody array

The labels FLU-ULS and BIO-ULS may have slightly different labeling characteristics towards individual proteins, and since the fluorochromes in the conjugates differ in fluorescence

intensity, the fluorescence intensities must be normalized. This protocol uses the dye-swap method for normalization. Each Perturbed sample and Control sample is labeled with a different label and in a parallel experiment the labels are reversed.
This method is especially attractive for antibody arrays because it takes into account label-specific differences in antigen antibody interactions. Thus, prior to incubation the labeled samples are mixed and incubated on two different antibody array pads.
The incubation time recommended in this protocol is 1.5 hours, at room temperature. Since diffusion of the analytes to the antibodies is often the rate-limiting factor, longer incubation times may be tried to increase the signal. In that case, it is recommended to add fresh protease inhibitor and phosphate inhibitor cocktail during the incubation. A rocking platform is recommended during incubation to increase diffusion. An Advalytix ArrayBooster™ incubation chamber may also be used to enhance the diffusion and thus obtain better signal to noise ratios and shorten the incubation times, but it is not essential.

6. Washing to remove unbound proteins

The prime reason for washing is to remove non-specifically bound labeled proteins from the array surface. In addition, the specially designed wash buffer serves another important purpose. Since the protein extraction and labeling buffer is gentle and non-denaturing it is expected that functional protein complexes still exist to a certain extent. When an analyte would be captured, the entire labeled protein complex may be pulled out the cell lysate, yielding high fluorescent signals. This kit contains a specially developed wash buffer that leaves the antibody antigen interaction intact but is stringent enough to disrupt protein complexes. Thus, only the analyte of interest remains bound to the antibody. If one is interested in studying protein-protein interactions using this kit, a less stringent wash buffer is recommended, for instance PBS-T, which leaves the protein complexes intact.

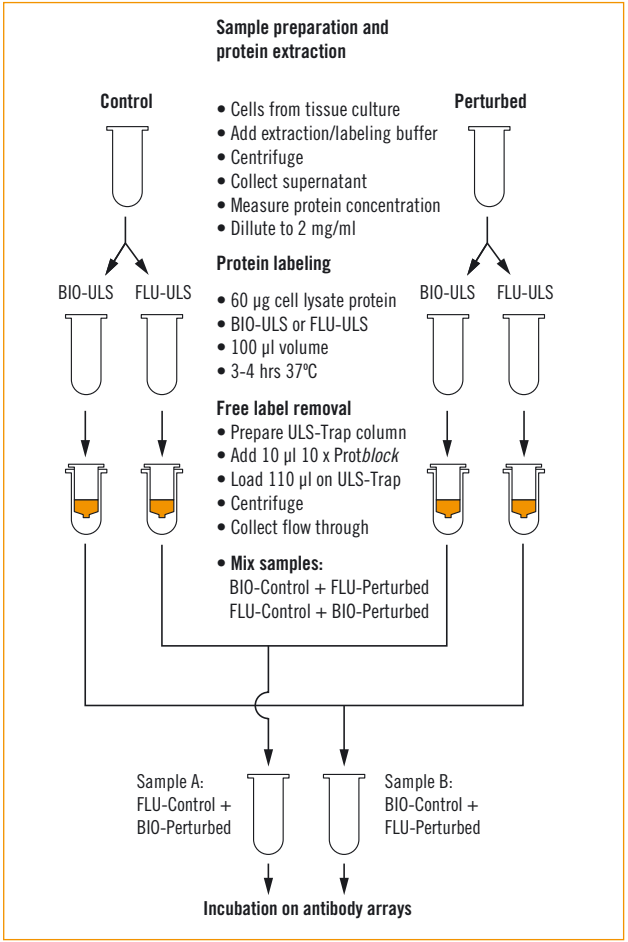
7. Detection of FLU-ULS and BIO-ULS label with anti-Flu and streptavidin fluorescent conjugates.

The FLU-ULS and BIO-ULS -labeled proteins are detected by fluorescent conjugates: rabbit anti-Fluorescein-DY547 for FLU-ULS and Strepavidin-DY647 for BIO-ULS. DY547 and DY647 have the same spectroscopic characteristics as Cy3 and Cy5, respectively.
This indirect method of detection has two major advances over direct labeling with fluorochromes: (i) there is much less background, especially on nitrocellulose-based surfaces and thus higher signal to noise ratios and (ii) the signal is amplified by the conjugates, since each conjugate carries multiple fluorochromes.

8. Scanning and analyzing the microarray slides to measure bound antigen

Scanning can be performed on any microarray scanner. It is recommended to scan the arrays within 24 hours after completing of the experiment. Meanwhile, protect the arrays from light. Normalization of the data is to be performed using the dye-swap method. The normalized ratios are calculated and the ratio represents the abundance of an antigen in the Perturbed sample over the Control sample. See also a review article for details on this subject (2).

IV. Schematic overview of ULS cell lysate protein labeling and detection process



Incubation on antibody arrays (continued)
- Adjust volumes with 1 x Prot*block*
- Add samples on pre-blocked array slides
- Incubate 1.5 hours at room temperature while gently rocking
- Wash the slides several times with washing buffer

↓

Probing of FLU-ULS and BIO-ULS with fluorescent conjugates
- Add detection solution: anti-Flu-DY547 and Streptavidin-DY647
- Incubate 45 min. at room temperature in the dark while gently rocking
- Wash the slides several times with washing buffer
- Wash the slides with PBS
- Wash the slides with purified water
- Dry the slides

↓

Scan slides

Figure 3. Schematic overview of ULS cell lysate protein labeling and detection process.

C. Protocol

Note: Spin all tubes briefly before use
Please read the entire protocol before starting

1. Cell lysis and protein extraction

- Prepare 2 ml 1 x UPEL buffer (dilute 200 µl 10 x UPEL with 1.8 ml purified water).

Note: Addition of protease/phosphatase inhibitors (final concentration according to the manufacturer) to 1 x UPEL buffer is strongly recommended

- Thoroughly wash cells of interest twice with PBS
- Adherent cells:
  - Lyse 10 mg cells (~10^7 cells) by adding ice-cold 200 µl 1 x UPEL buffer and leave them on ice for 15 minutes
  - Scrape the cells using a rubber policeman
  - Vortex the lysates
  - Centrifuge lysates 30 minutes 10,000 g at 4°C, the supernatant is the cell lysate
- Non-adherent cells:
  - Centrifuge cells to a pellet and remove washing buffer
  - Lyse 10 mg cells (~10^7 cells) with 200 µl ice-cold 1 x UPEL buffer
  - Vortex the lysates and incubate 15 minutes on ice, vortex again
  - Centrifuge the lysate for 30 minutes, 10,000 g at 4°C, the supernatant is the cell lysate
- Carefully isolate supernatants (cell lysates)

2. Determination of protein concentration

- Determine protein concentration with BCA Protein Assay Kit at absorbance of 562 nm according to manufacturers instruction (Pierce)
  - Make a standard curve measuring concentrations of BSA standards between 0 and 20 µg
  - Measure 1, 5, and 10 µl of cell lysate
  - Determine protein concentration of the cell lysate
- Adjust protein concentration to 2 mg/ml with 1 x UPEL buffer

Note: If protein fractionation is performed before array analysis, the protein concentration of the 'enriched' fraction should also be determined first, and 60 µg should be used for ULS™ labeling.

3. ULS labeling of proteins

- Prepare four tubes for a comparison of lysates from e.g. PERTURBED and CONTROL cells: Mark two PERTURBED and the other two CONTROL. Mark one PERTURBED with FLU and the other with BIO. Repeat for CONTROL. For each labeling reaction add the reagents as shown in Table 1 and according to the tube marks.

Important: FLU-ULS comes as a suspension. Prior to use, thoroughly resuspend the FLU-ULS by pipetting

- Add the reagents in the order of appearance as is shown in Table 1
- Thoroughly mix the samples by pipetting prior to incubation at 37°C
- Incubate the four samples for 3-4 hrs at 37°C using a water bath or incubator.

	volumes
10 x UPEL buffer	10 µl
Purified water	57.5 µl
PERTURBED or CONTROL cell lysate (2 mg/ml)	30 µl
BIO-ULS or FLU-ULS	2.5 µl
Total volume	**100 µl**

Table 1. Cell lysate labeling with BIO-ULS or FLU-ULS

4. Removal of free label using ULS-Trap columns

Preparing the ULS-Trap column

Prepare four ULS-Trap columns:

- Invert the columns several times to resuspend the contents
- Snap off the bottom of the ULS-Trap column and remove the cap
- Put the ULS-Trap column in a 2 ml polypropylene sample collection tube
- Spin column dry: 1 minute maximum speed (20,000 x g) in a tabletop microcentrifuge
- Discard the 2 ml tube together with its content

Note: The content contains 0.02% sodium azide. Therefore, it should be handled as chemical waste

- Put the ULS-Trap column in a new 2 ml polypropylene sample collection tube

Removal of free label

- Prepare 1 ml fresh 10 x Prot*block* solution by dissolving 100 mg PB agent in 1 ml 10 x PB agent solvent (this solvent contains salts which may precipitate upon storage at 4C; ensure complete dissolving before use). The powder does not dissolve completely. Spin the solution for 5 minutes at maximum speed (20,000 x g) in a table top centrifuge. Only the supernatant can be used for blocking purposes which is to avoid aggregates in the incubation mixture.
- Add 10 µl 10 x Prot*block* (freshly prepared, see above) to each sample and mix thoroughly by pipetting.

Important: Immediately proceed with the next step.

- Load each 110 µl sample (containing labeled protein and free ULS label) onto the center of one ULS-Trap column.
- Centrifuge column with sample for 1 minute at maximum speed (20,000 x g) in a tabletop microcentrifuge.
  The flow-through contains the labeled proteins; free ULS is retained in the column.

- Mix 100 µl of the purified BIO-labeled PERTURBED sample with 100 µl of the purified FLU-labeled CONTROL sample (Creating sample A). Accordingly, and to normalize for label performance, mix 100 µl BIO-labeled CONTROL sample with 100 µl FLU-labeled PERTURBED sample (Creating sample B). Mix thoroughly by pipetting.
- Prepare 1 ml 1 x Prot*block*: dilute 100 µl 10 x Prot*block* (as prepared above) in 900 µl purified water.
- Adjust the volume of the 200 µl mixed samples to 400 µl with 200 µl 1 x Prot*block*.

Note: these steps yield a 400 µl mixed sample each, with a cell lysate protein end-concentration of 275 µg/ml, which is sufficient for 2-pad arrays. For multiple pad arrays: lower incubation volumes can be used down to 100 µl. If higher volumes are needed for single pad arrays, see Appendix I, Changing the array format.

5. Incubation of ULS labeled proteins on the antibody array

Preparation of the antibody array slide

- Mount an antibody microarray slide into a FAST® Frame Multi-Slide Plate with a FAST® 2-well Incubation Chamber

Note: When home-made slides are used and/or incubation chambers are not available, mark a chamber using a hydrophobic pen (e.g. PAP pen)

- Block the arrays on the slide with 500 µl of PAB buffer for at least 15 minutes at RT while gently rocking

Incubation of ULS labeled and purified proteins on the antibody array

- Remove the PAB buffer from the arrays. Discard the buffer by inverting the slide, but don't let the surfaces dry out!
- Add 400 µl sample A to one array pad of the slide. Accordingly do the same with sample B on the other array pad.

- Incubate the arrays for at least 1.5 hours at RT while gently rocking.

6. Washing to remove unbound proteins

- Wash the slide thoroughly 6 x 1 minute with 500 µl PAW buffer. Discard the PAW buffer after each wash step by inverting the slide, but don't let the surfaces dry out.

7. Detection of FLU-ULS and BIO-ULS label with anti-Flu and Streptavidin fluorescent conjugates.

- Prepare the Detection Solution during the washing steps: For one 2-pad slide, add 100 µl of 10 x Prot*block* to 900 µl of purified water. Add 10 µl (1:100) anti-Flu-DY547 and 1 µl (1:1000) Streptavidin-DY647. Vortex the solution prior to use!
- Add 500 µl of Detection Solution to each array pad and incubate the slide for 45 minutes at RT while gently rocking. Protect the slides from light during the incubation step!
- Wash the slide thoroughly 4 x 1 minute with 500 µl PAW buffer. Discard the PAW buffer after each wash step by inverting the slide.
- Remove the slide from the incubation chamber
- Wash the slide 2 x 1 minute with 40 ml PBS in a 50 ml tube
- Wash the slide 1 x 1 minute with 40 ml of purified water in a 50 ml tube
- Carefully dry the slide with compressed nitrogen gas. Alternatively, spin dry (5 minutes, at 1,500 g in a slide holder) or air dry for at least 20 minutes

8. Scanning and analyzing the microarray slides to measure bound antigen

- Scan the slide within 24 hours of performing the experiment using an array scanner. For GenePix™ (Axon Instruments) optimal PMT gain values for the DY547 conjugate (green channel, 532 nm) and for the DY647 conjugate (red channel, 635 nm) should be determined depending on the array surface and/or scanner used.
- Determine net signals (signal minus background) for each specific spotted antibody and normalize. The normalized ratio's can be calculated as follows:

First calculate Ratio A = IO-Perturbed / LU-control

Then calculate Ratio B= IO-Control / LU-Perturbed

Normalized Ratio = ^{2}Log (Ratio A / Ratio B)

This ratio represents the relative abundance of an antigen in the Perturbed sample over the Control sample.

D. Trouble Shooting

Problem	Possible Causes	Suggestions
Weak signal	Incorrect scanner settings	• Adjust PMT gain settings • Adjust laser power • Run calibration of scanner
	Incomplete detection	• Increase concentration of detection conjugate
	Poor labeling	• Check L/P ratios, see appendix. When needed increase degree of labeling by increasing labeling time or decreasing protein concentration during labeling. • Increase concentration of labeled protein during incubation on array • Make sure the used extraction buffer is ULS-compatible. If not: change buffer
High background	Sticking of labeled protein to array surface	• Prolong washing times • Increase Prot*block* concentration • Shake slides while washing • Incompatible array background: try another array blocking buffer or another array surface
	Excess of detection conjugate used	• Further dilute detection conjugate
Protein precipitation after labeling	Protein degradation	• Use freshly made samples • Add (more) protease inhibitors
	Labeling density too high	• Check L/P ratios, see appendix. When needed decrease degree of labeling by decreasing labeling time or decreasing protein concentration during labeling
Non-homogenous background	Protein precipitation after labeling	• Centrifuge sample after labeling to check for the presence of precipitates. If precipitates are present: see above
	Washing/ drying artifact	• Increase time or number of PBS washes after detection step. Traces of wash buffer may result in "stripes" on the array

E. Appendix

I. Changing the array format

This manual has been written for 2-pad antibody arrays, but can be easily adjusted for use on single pad or multiple pad slides. Incubation volumes can be adjusted from 400 µl down to 100 µl. Use table 2 and take this protocol for two-pad arrays with an incubation volume of 400 µl as a reference. When using 8-, 12-, or 16-pad arrays, additional Two-color ULS Cell Lysate Protein Labeling and Detection Kits are required.

Table 2. Conversion table for using multiple array formats

Array formats	FLU-ULS or BIO-ULS to use per array pad (µl)	Amount of protein to be labeled per array pad (µg)	Minimal incubation volume per array pad (µl)	Amount of ULS labeling kits required	Protocol action required
1-pad incubation chamber external dimension: 57 x 26 mm well dimension: 53 x 22 x 3 mm (LxWxD)	5	120	800	1	Perform standard labeling reactions in duplicate by using 2 x 2.5 µl ULS to label 2 x 60 µg. Combine duplicates and create sample A and B by diluting the 400 µl mixed samples to 800 µl with 1x protblock. Adjust array blocking and wash step volumes to 800 µl.
2-pad incubation chamber external dimension: 51 x 25 mm well dimension: 21 x 21 x 3 mm (LxWxD)	2.5	60	400	1	Follow the standard protocol.
8-, 12-, or 16-pad incubation chamber external dimension: 75 x 25 mm well dimension: 7 x 7 x 3 mm (LxWxD)	2.5	60	100	8-pad: 2 12-pad: 3 16-pad: 4	Use only 100 µl of the 200 µl mixed samples A and B (see page 20) for incubation on the array. Duplicate incubation on arrays is possible using the remainder of mixed samples, adjust array blocking and wash steps volumes to 100 µl.

II. Determination of the labeling density

Although the ULS labeling method is robust, there may be occasions that one wishes to check the degree of labeling of the proteins. This is done by measuring the fluorescence of Fluorescein and calculating the amount of molecules using the molar extinction coefficient. For Biotin this is not possible. Instead, biotin can be determined using the HABA/avidin method.
Essential to both methods is that after the labeling reaction the excess of free label is removed. Choose one of the following protocols.

Determination of FLU-ULS to protein ratio:

- Label the cell lysates as described
- Remove excess FLU-ULS using ULS-Trap column
- Perform a Protein assay on a fraction of the labeled and purified material. In contrast to the protein determination before labeling, we recommend to use BioRad Rc/Dc protein assay (Bio-Rad # 500-0113, 500-0114, 500-0115, 500-0119), since the BCA assay is not compatible when proteins are purified over the ULS-Trap column.
- Measure FLU absorbance in a spectrophotometer at 495 nm
- Calculate the label / Protein ratio using the following formula. The FLU-ULS / protein ratio should be > 2.

$$\frac{\text{Absorbance at } \lambda \text{ max} * \text{Mol weight protein (g/mol)}}{\text{Molar Extinction coefficient (mol}^{-1}* \text{l} *\text{cm}^{-1}) * \text{cuvette path length (cm)} *\text{concentration of protein (g/l)}}$$

Take as average molecular weight for cell lysate proteins 60,000 Da.
The molar extinction coefficient for FLU at λ max (495 nm) is 83,000 $mol^{-1}* l *cm^{-1}$.

Determination of Biotin-ULS to Protein ratio:

- Label the cell lysates as described
- Remove excess BIO-ULS using ULS-Trap column
- Perform a Protein assay on a fraction of the labeled and purified material. In contrast to the protein determination before labeling, we recommend to use BioRad Rc/Dc protein assay, since the BCA assay is not compatible when proteins are purified over ULS-Trap column
- Determine the molar ratio of biotin incorporated into a protein using the HABA/avidin method. The HABA dye (2-Hydroxyazobenzen-4'-carboxylic acid) binds to avidin to produce a yellow-orange colored complex which absorbs at 500 nm. Free biotin will displace the HABA dye and cause the absorbance to decrease. A standard curve can be established using the free biotin to estimate the number of moles of biotin incorporated after biotinylation of a protein. We recommend to use HABA from Pierce (catalogue #28010) using the manufacturers instructions
- Take as average molecular weight for cell lysate proteins 60,000 Da
- Determine the molar amount of biotin per mole of protein
- The BIO-ULS/Protein ratio should be > 2

F. References:

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