PROTEIN LABELING KIT

ULS™

Two-color ULS™ Serum Protein Labeling and Fluorescent Detection Kit

4 dual Flu/Bio labeling and detection reactions for expression profiling on antibody arrays

Product code
PLK-001

For research use only
Not for diagnostic purposes

KREATECH BIOTECHNOLOGY

Instruction manual

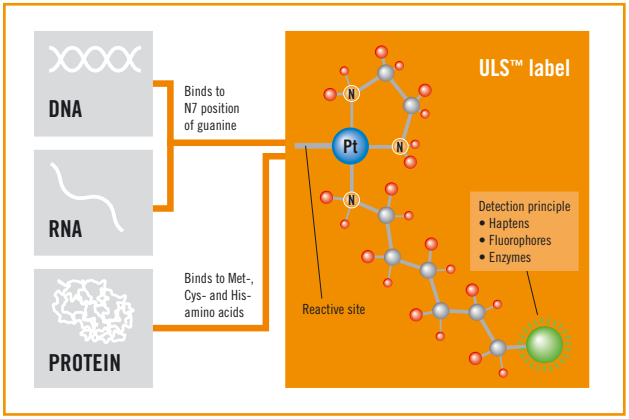

Figure 1. ULS™, the Universal Linkage System that labels your DNA, RNA and proteins.

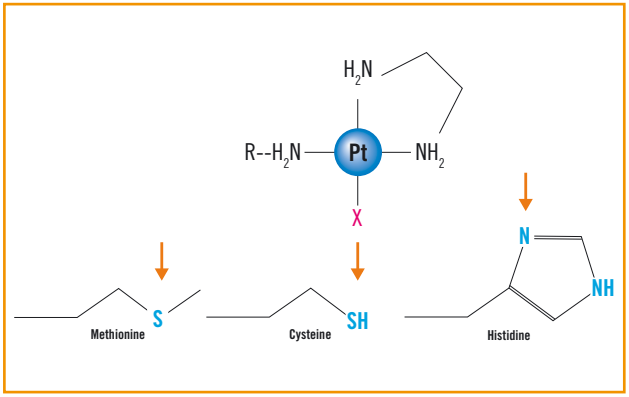

Figure 2. ULS™ labels proteins by forming a coordinative bond on:

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

KREATECH's Two-color ULS™ Serum Protein Labeling and Detection Kit

For serum 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.

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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 UPL buffer	10 x ULS™ Protein Labeling buffer	80 µl	Transparent
FLU-ULS™	Fluorescein-ULS™ label	20 µl	Green
BIO-ULS™	Biotin-ULS™ label	20 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 columns	16	-
PAB buffer	Protein Array Blocking buffer	2 ml	Black
PAW buffer	Protein Array Washing buffer	20 ml	Transparent
Rabbit anti-Flu-DY™547	Fluorescein detection conjugate	20 µl	Green (brown vial)
Streptavidin-DY™647	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)
- 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)
- 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™ Serum 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 kind 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.

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), Tributylphosphine (TBP).

This kit has especially been designed for serum protein labeling and detection for profiling protein expression on antibody arrays. In the Two-color ULS™ Serum 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-DY™547 conjugate to detect FLU-ULS™ and a Streptavidin-DY™647 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™ Serum 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.

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 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™ Serum 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 4-5 hours hands-on time to complete including an overnight labeling step.

The protocol contains the following steps:

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

1. Sample preparation

This kit has been designed for serum protein expression profiling and as such can be used to compare the expression of serum protein from patients versus a reference. As reference 'normal' serum can be used. When one has multiple patient samples to compare with each other, one can make a mixture of all the samples and use that as reference and test this against each individual sample. Samples should be stored frozen after blood sampling and coagulation since the composition of serum proteins may change in time.

It is strongly recommended to use fresh samples. Using old samples may give inadequate results.

The ratio of protein to ULS label is important for successful labeling. Human serum has an average protein concentration of 50- 60 µg/µl. In this protocol a standard volume of 2 µl of serum is used for labeling with a fixed amount of ULS™, and this has been proven to give reliable and reproducible results. In case a substantial higher or lower amount of protein is expected or in case high abundant proteins are removed from serum prior to labeling, an adequate determination of the protein concentration is important since the correct ratio of protein to label is important to the success of the experiment. In that case an equivalent amount of protein should be used in the labeling reaction (100-120 µg). We recommend using the BCA protein assay reagent since the recommended amounts of ULS™ for labeling are based on the determination of protein with the BCA assay.

2. 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 has been optimized to overnight (16 hours) at 37°C. This may be shortened to a minimum of four hours at 37°C, but this may yield lower labeling densities and thus lower signals. If preferred, one may decrease the labeling time by performing the labeling reaction at 65°C for four hours instead of overnight at 37°C. By doing so, the labeling, incubation and scanning steps can all be carried out within one day.

Generally, with this protocol good results have been obtained without the use of protease inhibitors. If one wants to add them we recommend Roche Complete™, which is compatible with the labeling procedure.
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.

3. 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 highly 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.

4. 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 Test sample and Reference 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 array 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. 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.

5. 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 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 serums, 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.

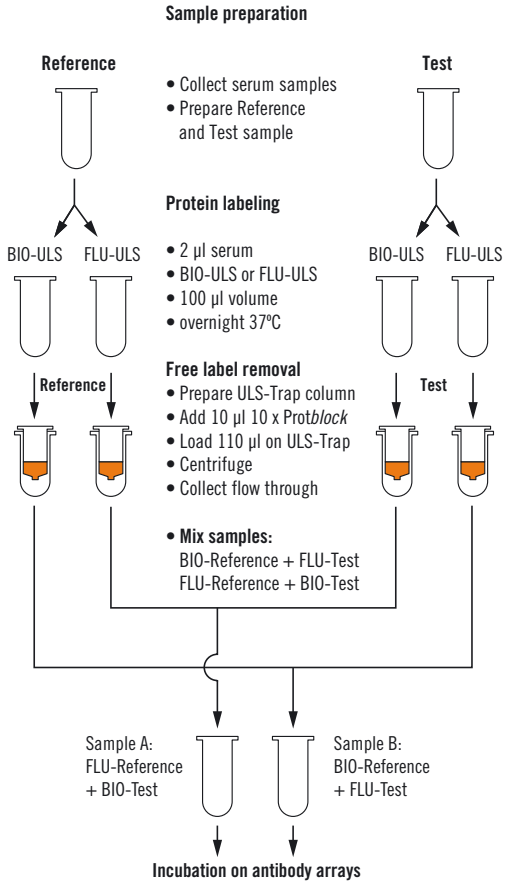
6. Detection of FLU-ULS™ and BIO-ULS™ label with anti-Flu and streptavidin fluorescent conjugates.

The FLU-ULS™ and BIO-ULS™ -labeled proteins are detected by fluorescent conjugates: rabbit anti-Fluorescein-DY™547 for FLU-ULS™ and Strepavidin-DY™647 for BIO-ULS™. DY™547 and DY™647 have the same spectroscopic characteristics as Cy™3 and Cy™5, respectively.
This indirect method of detection has two major advantages 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.

7. 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 completion 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 Test sample over the Reference sample. See also a review article for details on this subject (2).

IV. Schematic overview of ULS™ serum 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™ serum protein labeling and detection process.

C. Protocol

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

1. Sample preparation

- Collect fresh serum samples or thaw frozen serum samples
  - Optional: remove high abundant serum proteins
  - Optional: Determine protein concentration using the BCA Protein Assay Kit at absorbance of 562 nm according to manufacturer's instruction (Pierce):
  - Make a standard curve measuring concentrations of BSA standards between 0 and 20 µg
  - Determine protein concentration of the samples

Note: If high abundant proteins are removed, the protein concentration of the 'enriched' fraction should also be determined first, and 100-120 µg should be used for ULS™ labeling.

2. ULS™ labeling of proteins

- Prepare four tubes for a comparison of serum from e.g. TEST and REFERENCE sample: Mark two TEST and the other two REFERENCE. Mark one TEST with FLU and the other with BIO. Do the same for REFERENCE. 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

	volumes
10 x UPL buffer	10 µl
Purified water	83 µl
Test or Reference serum sample (50-60 µg/µl)	2 µl
BIO-ULS™ or FLU-ULS™	5 µl
Total volume	**100 µl**

Table 1. Serum protein labeling with BIO-ULS™ or FLU-ULS™

- Incubate the four samples for overnight (16 hrs) at 37°C in a water bath or incubator.
  - Optional: the samples may be incubated at 65°C for four hours to reduce labeling time

3. 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 at maximum speed (20,000 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 Protblock solution by mixing 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). Vortex for 1 minute. The powder does not dissolve completely. Spin the solution for 5 minutes at maximum speed (20,000 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 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 TEST sample with 100 µl of the purified FLU-labeled REFERENCE sample (Creating sample A). Accordingly, and to normalize for label performance, mix 100 µl BIO-labeled REFERENCE sample with 100 µl FLU-labeled TEST sample (Creating sample B). Mix thoroughly by pipetting.
- Prepare 1 ml 1 x Prot*block*: dilute 100 µl 10 x Prot*block* 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, 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.

4. Incubation of 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 the 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

5. 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!

6. 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-DY™547 and 1 µl (1:1000) Streptavidin-DY™647. 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 with 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 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.

7. 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 DY™547 conjugate (green channel, 532 nm) and for the DY™647 conjugate (red channel, 635 nm) should be determined depending on the array surface and/or scanner used.

- Determine net signals (signal-background) for each specific spotted antibody and normalize. The normalized ratio's can be calculated as follows:

First calculate Ratio A = Bio-Test / Flu-Reference

Then calculate Ratio B= Bio-Reference / Flu-Test

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

This ratio represents the relative abundance of an antigen in the Test sample over the Reference 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
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 (page 27) 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™ Serum 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 serum to be labeled per array pad (µl)	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)	10	4	800	1	Perform standard labeling reactions in duplicate by using 2 x 5 µl ULS to label 2 x 2 µl serum. 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)	5	2	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)	5	2	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 step 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 serum samples 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.

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

Take as average molecular weight for serum proteins 60,000 Da.
The molar extinction coefficient for FLU at λ max (495nm) is 83.000 $mol^{-1} * l * cm^{-1}$.

Determination of Biotin-ULS™ to Protein ratio:

- Label the serum samples 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 the 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 serum proteins 60,000 Da
- Determine the molar amount of biotin per mole of protein
- The BIO-ULS™/protein ratio should be > 2

F. References:

1. Kusnezow W. and Hoheisel J. (2002) Antibody Microarrays: Promises and Problems. *BioTechniques* 33:S154-S23

2. Quackenbush, J., (2002) Microarray data normalization and transformation. *Nature Genet.* Suppl., 32, 496-501

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