Antibody array-based analysis of protein mixtures extracted from formalin-fixed paraffin-embedded (FFPE) tissue using ULS™ labeling

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ULS™ principle

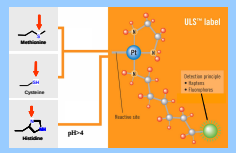
- The Universal Linkage System (ULS™) is a platinum-based technology that allows direct, chemical labeling of proteins, DNA and RNA with any label of choice, including many fluorophores, biotin, and dinitrophenol.
- In proteins, the reaction produces a stable coordinative bond with the sulfur atom in Methionine and Cysteine, and with a nitrogen in Histidine (see Figure).
- ULS™ provides high coverage of the proteome and is suitable in protein microarray applications for the following target samples:

* purified proteins

* recombinant proteins (no expression tag needed)

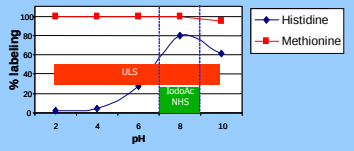
* complex mixtures like:
  - plasma/serum
  - other body fluids
  - cell lysate

* peptides



ULS™ robustness in labeling conditions

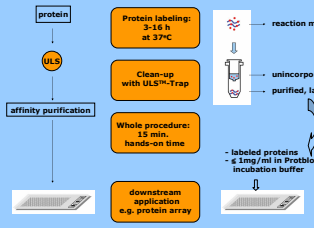
Broad pH range of labeling



- ULS™ is stable in aqueous solution
- ULS™ is stored at 4°C
- ULS™ is compatible with high temperatures (≤ 85°C)
- ULS™ labeling is compatible with generally used reagents:
  - buffers: Tris/glycine, PBS, carbonate etc.
  - detergents: NP40, TritonX-100, Tween-20/80 etc.
  - cations/anions: Mg^{2+}, Na^{+}, K^{+}, Ca^{2+}, EDTA, EGTA, Cl^{-}, SO_4^{2-}, PO_4^{3-} etc.

ULS™ labeling of proteins

An easy protocol giving reproducible results



ULS™-Trap columns

affinity purification of labeled proteins

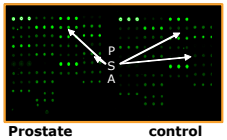
ULS™-Trap columns ensure low backgrounds and high signals in downstream protein applications by combining optimal removal of unincorporated label with recovery of nearly all proteins without molecular size exclusion, in contrast to gel filtration methods (see Table).

	ULS™-Trap	Gel-filtration
Label removal	> 99.6%	94-98%
Principle	affinity purification => No cut-off	size exclusion => cut-off: 7 kDa
Protein loss	< 1%*	10-30%

* for complex mixtures when protblock is used before clean-up

ULS™ serum/cell lysate labeling for antibody arrays

Two-color serum profiling (Whatman)

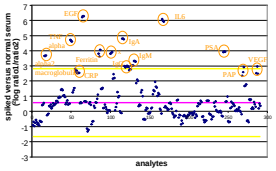


Prostate Cancer Patient

control

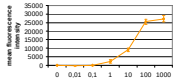
Serum from a prostate cancer patient with high levels of PSA detected by two different antibodies and compared to a pool of sera from healthy individuals

Relative abundance of spiked serum versus unspiked serum on Serum Biomarker Chip (SBC ~120 analytes)
Purple line represents mean of all abundance ratios. Yellow lines represent 2*SD away from mean abundance ratio. Spiked analytes are highlighted in orange. Note that one antibody showed cross-reactivity (haptoglobin= x)

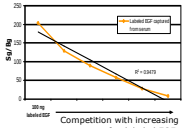


Sensitivity and specificity using ULS™ labeling

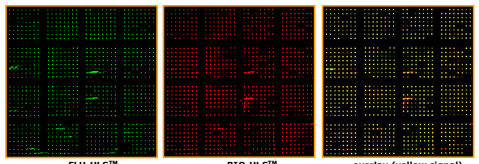
Upper figure: Indicated amounts of EGF were spiked into serum and labeled with BIO-ULS™. Each spiked sample was incubated on a FAST® array containing a monoclonal anti-EGF antibody. Of note is the dynamic range of 3-4 log, and the **sensitivity** of low ng/ml.



Lower figure: 100 ng EGF was spiked into serum and labeled with BIO-ULS™. Before array incubation, increasing amounts of "cold" non-labeled EGF was spiked into each one of the samples. Data points show EGF signal obtained for each competition. Of note is the complete competition towards zero indicative for EGF signals only (**specificity**).



Two-color cell lysate profiling (Clontech)



Same versus same data (~512 analytes)

	Assay performance	
	Assay 1	Assay 2 (reproduction)
INR* (non-differentials)	0.77-1.3	0.8-1.27
% spots fitting the criteria**	87.5%	91.5%

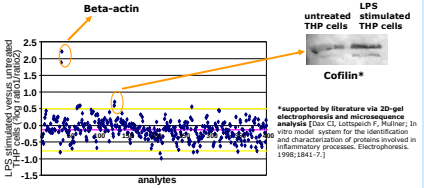
*INR= internalized normalization ratio

**CRITERIA FOLLOWED 1: intra-slide spot variation < 20%
2: inter-slide spot variation < 30%
3: sig/bg > 1.5
4: normalization via mean log2 ratio/ratio of all spots

Assay performance	Mean values of all spots	
	BIO-ULS (PMT gain 600)	FLU-ULS (PMT gain 500)
CV (%)	21	21
signal/ background	13.9	16.3
netsignals	5600	9200
background	450	600

Specifications of BIO-ULS™ and FLU-ULS™ labeling and detection on Clontech arrays

Western blot validation (Sigma)



*supported by literature via 2D-gel electrophoresis and microsequence analysis [Dax CS, Lottspeich F, Hulmar; In vitro model system for the identification and characterization of proteins involved in inflammatory processes. Electrophoresis. 1998;1841-7.]

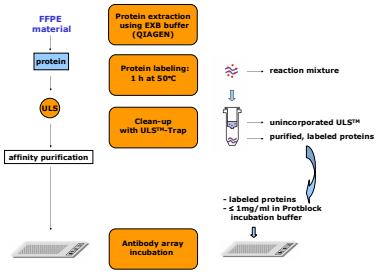
Relative abundance of analytes from LPS stimulated THP cells over untreated cells. Panorama™ arrays (~200 analytes) were analyzed. Purple line represents mean of all abundance ratios. Yellow lines represent 2*SD away from mean ratio. All cell lysate experiments were performed in collaboration with NV Organon (A. van Gool and S. Aarle-van der Locht, Section Genomics & Proteomics, Target Discovery Unit).

ULS™ labeling of protein extracted from FFPE material for antibody arrays

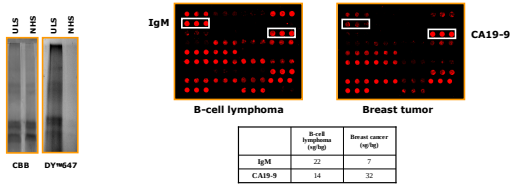
Introduction to experiments: protein extracted from FFPE material

Just recently, several protocols and commercial buffers have become available to extract complex protein mixtures from formalin-fixed paraffin-embedded tissue (FFPE). This opened the opportunity to analyze archival material by Western blotting, lysate arrays and by other types of assays. Rather than analyzing one particular analyte from these extracts, multiplex analysis of expression levels from one sample enables the discovery of biomarkers/protein profiles for specific diseases.
Antibody arrays are ideal for quick screening in multiplexed format. For this, direct chemical labeling of the complex protein mixture is required. During formalin fixation cross-linking occurs via primary amines; decross-linking during protein extraction does not yield genuine primary amines, but modified ones. Since ULS™ labeling targets other amino acid residues, it can easily be applied for labeling of FFPE extracted protein. In addition to the labeling itself, we have developed an optimal protocol which, for the first time, allows screening of multiple analytes from FFPE material on antibody arrays.

Antibody array protocol for FFPE material



Differential expression of analytes between two distinct cancer types

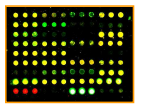


	B-cell lymphoma (sg/bg)	Breast cancer (sg/bg)
IgM	22	7
CA19-9	14	32

Left part: protein extracted from FFPE material with Qiagen's EXB buffer has been directly labeled with Dy647-ULS or Dy647-NHS. Labeled proteins were separated on SDS-PAGE. Shown are Coomassie Brilliant Blue staining (left image) and the fluorescence image (right image) as obtained using a KODAK image station. ULS efficiently labeled de-cross-linked proteins. Note that CBB intensities are somewhat skewed by Dy647 labeling.

Right part: Protein was extracted from 7-year old B cell lymphoma (left image) and breast tumor (right image) 10µm FFPE slices. Extracted protein mixtures were labeled with ULS, purified, and incubated on a home-brew PATH slide containing 36 antibodies spotted in triplicate. IgM and CA19-9 were the analytes showing most predominant differences in expression levels between both samples (table).

Two color assay



IN CONCLUSION

- Protein extracted from FFPE material can be directly labeled with ULS
- For the first time this allows analyses of FFPE material on antibody arrays
- Two-color assays are possible using differentially labeled samples
- Protocol is compatible with commercially available high-density arrays

ULS™-labeling is the method of choice for biomarker discovery using FFPE material on high-density antibody arrays