SERVA Electrophoresis

Application Note:

ICPL™- Coupled Immunoprecipitation (ICPL-IP)

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ImmunoPrecipitation (IP) is widely used as a method to selectively isolate protein complexes from primary tissues. However, due to lack of specificity and selectivity of most antibodies and unspecific binding of the carrier beads, the method can produce false positives. A differential multiplex approach, employing stable isotope labelling and mass spectrometry, can discriminate true positive from false positive IP complexes. Isotope Coded Protein Labelling (ICPL) occurs by labelling of free amino groups of intact proteins with amine specific reagents, and has the advantage, that it can be applied on any species, cell lysates, or tissue types [1]. This method therefore allows a quantitative analysis of any primary tissue including human specimens, without the need of prior metabolic labeling. Stable isotope labelling of proteins prior to separation and digestion allows identical treatment of the multiplexed sample, in this way abolishing experimental variations.

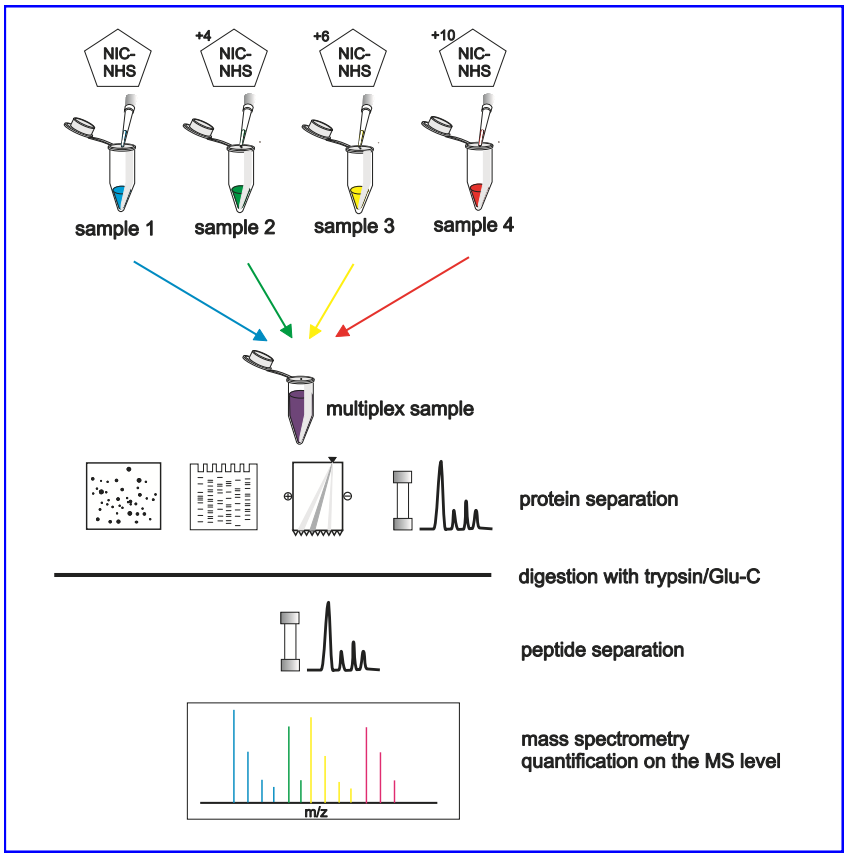

Fig.1: Cartoon of the principle of Quadruplex ICPL (NIC-NHS = *N*-nicotinoyloxy-succinimide).

ICPL labelling is based on a well-established chemistry: the reaction of *N*-nicotinoyloxy-succinimide with the ε-amino group of lysine. The ICPL™-technology is available with up to four labels: as Duplex, Triplex or Quadruplex Kit. The mass differences of 4, 6, and 10 Dalton of the *N*-nicotinoyloxy-succinimide labels are introduced by the different compositions of $^{12}C/^{13}C$ and hydrogen/deuterium isotopes. These kits allow for the relative quantification of up to four different protein samples in one experiment and the efficient, accurate and reproducible quantification of proteins with high sequence coverage. All four labels can be freely combined with each other. In figure 1 the basic concept of ICPL with four different labels is displayed.

As introduced by Vogt *et al.* [2], the tissue extract is split into equal amounts for a control and an IP. The target specific IP is performed with the specific antibody, the control is precipitated with species-specific immunoglobulins (IgG) or an unspecific control antibody. Thereafter, both samples are labelled with different ICPL reagents. The samples are then combined, enzymatically digested to peptides, and analyzed by LC-MS. Peptide ratios of the different samples are defined by isotope mass specific shifts within the spectrogram , and can be quantitatively compared using appropriate data analysis software. Non-specific binder peptides are detected in both samples featuring comparative quantitative levels, whereas the specific binders to a protein of interest are identified by their significant enrichment as a consequence of selective binding to the immunoprecipitated target protein.

Selectivity and specificity of analysis of ICPL-IP has been described for immuno-isolating protein complexes from bovine retinal tissue. In this case the SERVA ICPL™ Triplet Kit is employed. Here, known as well as new protein interactions were identified targeting the small GTPase RhoA, a low abundance protein within the retina. As shown here, even transient changes of protein binding patterns can be studied. More detailed results can be seen in reference 2.

Including sample preparation, the whole work-flow can be successfully performed in less than two days.

In the following the standard operation procedure for this application is described in detail. For the application of ICPL-IP it is highly recommended to exactly follow the optimized protocol in the ICPL instruction manual; it is particularly important to employ guanidine elution instead of the alternatives.

Figure 2 shows the workflow of the application of an ICPL triple labelling approach on light-induced alterations within the RhoA-complex (from A. Vogt *et al.* reference 2)

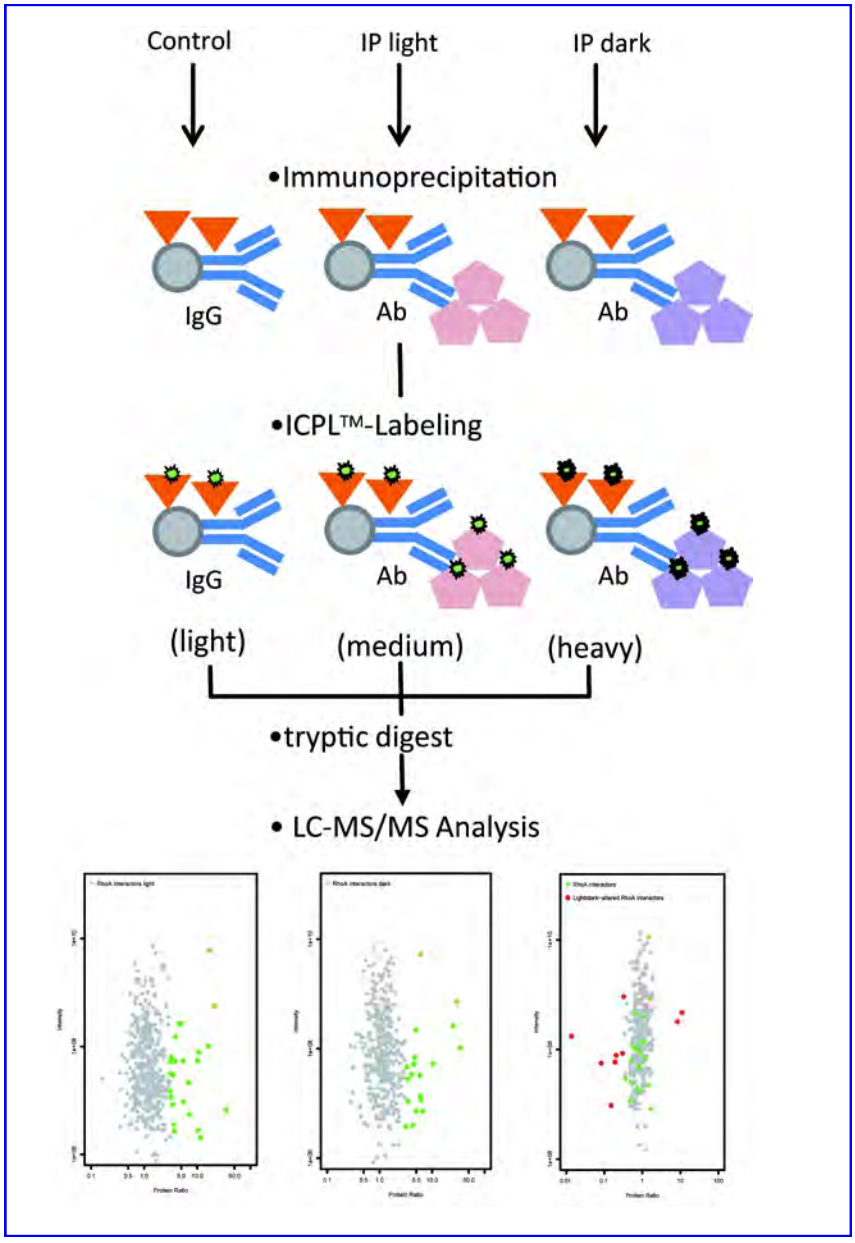

Fig. 2: Light-induced alterations within the RhoA-complex. Applying ICPL-IP with a triple labelling approach (for details see reference 2)

Standard Operation Procedure for ICPL-IP of ROS (rod outer segments)

1. Immunoprecipitation

ROS isolation medium:

5mM HEPES, 2mM $MgCl_2 \cdot 6H_2O$, 130mM NaCl, 20% sucrose, pH 7.2 with HCl

IP buffer:

20mM Hepes pH 7.8, 150 mM NaCl, 1mM EDTA

- Bring 1mg ROS for each condition (control, IP light, IP dark) with ROS Isolation medium to the same volume
- Add Protease Inhibitor Cocktail (1:50) to the samples
- 3x freeze and thaw (with liquid nitrogen)
- Shortly before use prepare 5 mL IP-buffer "master mix": 4350µL IP buffer + 500µL Dodecylmaltoside (from a 10 %w/v stock solution) (Sigma Aldrich) + 100 µL Protease Inhibitor Cocktail (PIC, Roche #11849300) + 50 µL Phosphatase Inhibitor (PI, Sigma #P5726)
- Bring volume of samples to 500 µL with IP-buffer master mix
- Homogenize 3x with G-21 gauge needle (avoid foaming!)
- Incubate lysates for 15 min with overhead rotation (Neolab, Intelli Mixer, Prg. F2 Speed 12) at 4 °C
- 10 min centrifugation (16,000 x g, 4 °C)
- Transfer supernatant to a new Eppendorf reaction tube
- Add antibody-specific IgG (Control) or antibody (IP)
- Incubate 2 h with overhead rotation (Neolab, Intelli Mixer, Prg. F2 Speed 12) at 4 °C
- Add 80 µL Protein G Plus Agarose (Santa Cruz, #sc-2002),
- Incubate overnight with overhead rotation (Neolab, Intelli Mixer, Prg. F2 Speed 12) at 4 °C
- Wash 3x with 500 µL IP-buffer master mix (without detergent)
- Elution: 2 x with 200 µL Guanidine-HCl pH 8.5 for 15 min each at room temperature
- Centrifuge eluates with Micro-Spin Columns (GE Healthcare #27-3565-01) to get rid of residual beads
- (Optional: add internal standard (Ovalbumin, 1ug/µL) to the eluted sample(s))
- Centrifugation with 10 kDa Cut-off column (Sartorius #VS0102) to reduce the samples to about 20uL volume
- Connect the column with an 1.5mL Eppendorf tube by their both openings and reversely centrifuge the sample into the 1.5mL Eppendorf tube

2. Labelling with SERVA ICPL™ Triplex –Kit (#003923101)

- Add 0.5 µL Reduction Solution (yellow)…30 min at 60 °C
- Add 0.5 µL Alkylation Solution (blue)…30 min RT, in the dark
- Add 0.5 µL Stop Solution (green) 15 min RT
- Add 3 µL of the respective ICPL label (0, 4, 6, 10) to each sample
- Overlay with protective gas (argon)
- Vortex for 10 sec
- 1 min ultrasonic bath
- Incubate for 2 h at RT (23°C)
- Add 2 µL Stop Solution (red)
- Incubate for 20 min at RT
- Combine samples
- Add 2µL 2N NaOH,
- Incubate for 20min at RT
- Add 2 µL HCl

3. In-Solution Digest, Mass Spectrometry, and Data Analysis

Because lysines are blocked by ICPL labeling, in-solution cleavage of the sample is ideally performed by double digest with trypsin and endoproteinase GluC. With this measure an improved sequence coverage is achieved.

The method is compatible with both, MALDI and electrospray ionization mass spectrometry, for quantification with ICPL MS/MS is not required.

For the analysis of the mass spectrometry results the following software can be employed:

ICPL Quant can evaluate all ICPL data up to ICPL Quadruplex; it can be downloaded from the following website free of charge: http://www.biochem.mpg.de/en/rg/lottspeich/technologies/ICPLQuant/

The following software packages can also be used:

- Thermo Scientific Proteome Discoverer (v1.4; up to ICPL quadruplex)
- MaxQuant (v1.4.1.2; up to ICPL triplex)

We quote the summary of the original paper by A. Vogt et al [2]: "The results of these experiments demonstrate that the ICPL-IP allows sensitive detection of quantitative changes that are due to altered physiological states. Taken together, the ICPL-IP proves as a highly selective and confident method to determine interactions of proteins at their endogenous cellular levels in primary tissue, devoid of any limitation of species or tissue type.
ICPL-IP also allows the analysis of human biopsy material and opens the door to correlate and validate work performed in human cell lines with primary biopsy material, generating new opportunities especially for medical research."

In short, ICPL-IP has the following features:

- Identification of native protein complexes
- Multiplexing of up to 4 samples
- Specific binders can clearly be recognized
- Unbiased approach

References

[1] Schmidt A, Kellermann J, Lottspeich F. A novel strategy for quantitative proteomics using isotope-coded protein labels. Proteomics 5 (2005) 4-15.

[2] Vogt A, Fuerholzner B, Kinkl N, Boldt K, Ueffing M. Isotope Coded Protein Labeling Coupled Immunoprecipitation (ICPL-IP): A Novel Approach for Quantitative Protein Complex Analysis From Native Tissue. Mol Cell Prot. 12 (2013) 1395–1406.

Ordering Information

	SERVA Cat. No.	
SERVA ICPL™ Kit	**39230.01**	**2 x 6 Reactions**
SERVA ICPL™ Triplex Kit	**39231.01**	**3 x 6 Reactions**
SERVA ICPL™ Quadruplex Kit	**39232.01**	**4 x 6 Reactions**
SERVA ICPL™ Quadruplex Plus Kit	**39233.01**	**4 x 6 Reactions**
Trypsin NB Premium Grade, MS approved from porcine pancreas	37284.01	4 x 25 µg
Endoproteinase Glu-C (V8 proteinase), MS approved from *Staphylococcus aureus*	20986.01	4 x 25 µg

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