Physical interactions between proteins are at the basis of building cellular structures and executing physiological cellular processes. To identify the constituents of a protein complex, immunoprecipitation (IP)-based approaches are widely applied. In this workflow, the affinity isolation of protein complex is often executed in Eppendorf tube by pulling down the bait protein with a specific antibody. Next, proteins are separated on SDS-PAGE, digested by trypsin and the resulting peptides are characterized by LC-MS/MS.

We applied the protocol to reveal synaptic protein–protein interactions. Frozen mouse brain cortex was extracted in 0.5% n-dodecyl β-D-maltoside extraction buffer as previously described (one cortex in 3 mL extraction buffer), and centrifuged twice at 16 000 × g for 20 min to remove insoluble materials. We recovered about 2 mg protein per mL of buffer. Four hundred and fifty µL of supernatant was dispensed to each well of the 96-well plate (Deepwell plate 96/500 µL from Eppendorf). Ten µg of antibody (rabbit polyclonal antibodies against DmXL2, Gpr158, Gabra4, and Mpp2, custom-made by Genscript) and 60 µL of Protein A Magbeads (Genscript) were added to each well. This standard protocol works well for most IPs on typical synaptic proteins. We expect that 5–10-fold less input material and antibody should be sufficient in case a highly sensitive LC-MS/MS platform is employed, such as the Orbitrap HF-X or the ion mobility QTOF mass spectrometer (TimsTOF pro) equipped with PASEF technology.

The plate was covered with a water-tight silicone sealing mat (Eppendorf) and incubated at 4 °C for 3 h in rotation at 20 rpm on an ELMJ Intelli-Mixer. The plate was then placed on a 24-pins magnetic plate (MagnaBot 96 Magetic separation device from Promega), in which the beads were pulled rapidly to the side of the well closest to the magnetic bar. This facilitates the aspiration of solution from the bottom of the well with no interference from on-bead by trypsin in an aqueous buffer. The absence of organic solvent concomitantly releases proteins/peptides from the beads into the digestion buffer. The consecutive IP followed by SP3 can be completed in 1 day, which is substantially faster than the classical approach that requires ≥3 days.

A simple and fast immunoprecipitation (IP) protocol is designed with the sample preparation incorporated, applicable to both low and high throughput. This new protocol combines two procedures based on magnetic beads in 96-well plate format. Protein complexes are captured by antibodies and magnetic beads conjugated with protein A. Proteins are washed and on-bead digested by using Single-Pot solid-phase sample preparation (SP3). The whole IP-SP3 approach can be completed in one day, which is considerably faster compared to the classical approach. No major quantitative differences are found between SP3 and FASP (filter-aided sample preparation) or a longer incubation protocol. Taken together, the IP-SP3 protocol is a fast and economical approach easily applicable for large-scale protein interactome analysis.
Figure 1. A) Boxplot showing the coefficient of variation of all identified proteins (LFQ, label-free quantitation) in the different experiments (Dmxl2, Gpr158, Gabra4, and Mpp2 IPs using SP3 or FASP). Each experiment was performed in triplicate. The median and number of proteins is indicated in each box. B) Overlap of proteins identified between SP3 and FASP protocol with each antibody and all combined. Only proteins identified by at least two peptides were included (N). C) LFQ intensity comparison between SP3 and FASP protocol for the bait protein of each antibody and two well-known interactors. D) LFQ intensity comparison for Dmxl2 and two well-known interactors between overnight and 3 h incubation for antibody-antigen interaction using IP-SP3 protocol. E) Potential interactors most enriched in each IP-SP3 experiment. Each node represents a protein, color-coded by iBAQ intensity (log10 scale).

The solution was discarded. The washing procedure was repeated four times with 350 µL 0.1% Triton-X 100 in 25 mM HEPES buffer (pH 7.4) at room temperature. Next, 50 µL 2% SDS in 75 mM Tris buffer (pH 7.5) containing 1 µL Tris(2-carboxyethyl)phosphine hydrochloride was added to each well and shaken in a Thermoshaker (Eppendorf) at 800 rpm, 50 °C, for 45 min. Afterward, 0.5 µL S-Methyl methanethiosulfonate was then added and shaken for 5 min at room temperature.

The eluted proteins from Magbeads were transferred to a 96-well microplate with conical bottom (Microplate 96/V-pp from Eppendorf). Ten µg of Sera-Mag Speed beads[6] (Thermo Scientific, combination of GE Healthcare catalogue number 2004310281k) was added and shaken for 5 min at room temperature.

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In short, we demonstrated that SP3 is a viable alternative to FASP for IP-based interaction proteomics. Significantly, the cost of Sera-Mag Speed bead (~0.1 euros per experiment) is substantially lower than the Microcon-30 filter (about 4.5 euros per experiment), making the SP3 protocol more economical. With state-of-the-art mass spectrometers, such as the Orbitrap HF-X or the ion mobility QTOF mass spectrometer (TimeTOF pro) equipped with PASEF technology, it is expected that an IP sample could be analyzed in <15 minutes. The IPs and MS analyses are processed independently, each of which can be completed in a single day. Thus, the IPs and MS analysis of 96 samples from one plate could be synchronized in consecutive days. Taken together, this protocol opens up an avenue for a high-throughput, fast, and economical approach to large-scale protein interactome analysis (see ref. 13).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

96-well plate, immunoprecipitation, magnetic beads, proteomics, sample preparation

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