

Capturing protein kinases from human HepG2 cells using the Stauro caproKit™

Capture Compound Mass Spectrometry (CCMS) is an innovative technology to reduce the complexity of biological samples through selective isolation of targeted protein or enzyme families. Small synthetic molecules (Capture Compounds™) interrogate native proteins, even lipophilic membrane proteins. CCMS technology enables an efficient complexity reduction of the proteome and allows discovering, isolating and profiling members of functional protein families within a variety of biological samples.

Introduction

The investigation of kinases is of outstanding interest as they perform major roles in development, signaling, and metabolism. Mutations and deregulation of kinases are causative for many human disease and thus placed kinases in the focus for biomarker discovery and drug discovery (1). The selectivity profiling of different kinase inhibitors against large panels of kinases has provided a broad data basis for assessing the utility of certain inhibitors to address subsets of the kinome. As an example, staurosporine is a prototypical ATP-competitive kinase inhibitor which interacts with up to 253 human protein kinases (2). Its broadband affinity makes staurosporine an attractive candidate to probe large sets of kinases within biological samples. Therefore, staurosporine is an ideal selectivity function for a kinase-specific Capture Compound™. Here, we report the application of the Stauro caproKit™ for the isolation and identification of kinases from a lysate of human hepatoma-derived HepG2 cells.

Materials

The Stauro caproKit™ contains the Stauro Capture Compound™ (Stauro-CC) with staurosporine as the selectivity group at a stock concentration of 50 μ M, free staurosporine in a water-soluble form as the competitor solution at a stock concentration of 1 mM, Streptavidin magnetic beads, 5x concentrated capture buffer, and 5x concentrated wash buffer as well as a preparation of recombinant purified catalytic subunit α of cAMP-dependent protein kinase as a positive control (at a stock concentration of 0.37 mg/ml). We further used PCR tube strips for volumes up to 200 μ l (Thermo Fisher, cat. No. AB-1114) as reaction vessels to prepare the samples, conduct the capturing experiments, wash and isolate the magnetic beads. Note that at some stages of the experiment centrifugation of the tube strips in a simple tabletop centrifuge with an appropriate butterfly rotor is desirable.

Irradiation of the samples for photo cross-linking was performed with the caproBox™ equipped with a glass plate covered with a UV-protective film, and the beads were isolated using the caproMag™. As

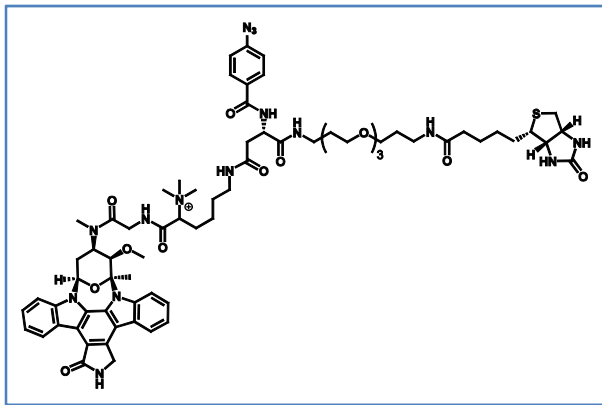


Figure 1 Stauro Capture Compound for selective isolation of protein kinases using staurosporine as selectivity function.

the protein sample, HepG2 lysate at a stock concentration of 7 mg protein/ml was used. HepG2 cells were lysed by French Press in lysis buffer (6.7 mM MES, 6.7 mM NaOAc, 6.7 mM HEPES, pH 7.5, supplemented to 200 mM NaCl, 10 mM β -mercaptoethanol, 1 mM EDTA, and protease inhibitor cocktail). Debris was removed by centrifugation at 30,000 x g for 60 min at 4 °C. The supernatant filtrated through a 0.2 μ m filter and was then dialysed against lysis buffer for the removal of small molecules (optional). After dialysis, centrifugation at 4000 x g was carried out to remove protein precipitated during dialysis.

Capture experiment

The capture experiments were carried out in the OffBead configuration as described in the Guideline for the Stauro caproKit™ (www.caprotec.com/support/downloads). In brief, the assays were prepared in the order of pipetting water and 5x capture buffer into PCR tubes and the assays were vortexed thoroughly. Cell lysate was added to a final concentration of 5 mg/ml and vortexed thoroughly. The Stauro Capture

Compound™ needs to be protected from bright light, such as sunlight or neon light in the laboratory. Prior to the handling of the Stauro Capture Compound™, the laboratory light was therefore switched off. The Capture Compound™ or in addition, the Stauro competitor for the competition experiment, was added to the sample and incubated for 30 min at 4 °C under rotation in the dark. The lids were removed from the tubes and the samples were placed in a pre-cooled caproBox™. Before irradiation of the samples, a UV filter frame is placed on the top of the sample tubes. The filter frame ensures that upon irradiation the reactivity function undergoes a photo cross-link to the target proteins while the integrity of the selectivity function is preserved. The samples were irradiated for 10 min at 2-4 °C. Following irradiation, competitor solution as described in the Guideline was added to the Capture assay samples and the lids are re-fitted onto the PCR tubes. After 10 min rotation of the samples at 4 °C, wash buffer 1 was added, and the samples were vortexed thoroughly. Finally, Streptavidin magnetic beads were added and the samples rotated for 30 min at 4 °C. Capture Compound-protein conjugates were washed and isolated using the caproMag™ as described in the Guideline (www.caprotec.com/support/downloads).

The capture samples were then either incubated with 2x concentrated sample buffer for SDS-PAGE and boiled for 5 min or subjected to OnBead tryptic digestion. For this purpose, the beads were washed twice with HPLC grade water and incubated with 10 μ l per assay of 50 mM NH_4HCO_3 , 5 mM CaCl_2 , and supplemented with 0.5 μ g trypsin. The bead suspension was transferred into a new tube. Tryptic digest was allowed to proceed over night at 37 °C under vigorous shaking. Beads were then fixed at the tube walls by using the caproMag™ and the supernatants with the peptides recovered, desalted via Stage Tips™ (Proxeon, Odense, Denmark), and then directly subjected to LC-MS/MS analysis.

Mass spectrometry

LC-MS/MS experiments were performed using a nanoflow HPLC system (Proxeon) coupled on-line to an ESI-LTQ-Orbitrap system. From the LC column, peptides were eluted during an 80-min linear gradient from 5 % ACN/0.1 % FA to 40 % ACN/0.1 % FA followed by additional 2 min to 100 % ACN/0.1 % FA and remaining at 100 % for another 8 min with a controlled flow rate of 300 nl/min. MS/MS fragmentation was performed in a data-dependent mode using one survey MS scan followed by four MS/MS scans per second. Proteins were identified by automated database searching against the human UniProtKB/Swiss-Prot database using SEQUEST implemented in BioworksBrowser 3.3.1 SP1 (Thermo Fisher Scientific). Specific search parameters used in the SEQUEST analyses were 5 ppm precursor tolerance, 1 amu fragment ions tolerance, and full trypsin specificity allowing for up to two missed cleavages. Phosphorylation at serine, threonine, and tyrosine, oxidation of methionines, deamination at asparagines and glutamine, acetylation at lysine and serine, formylation at lysine, and methylation at arginine, lysine, serine, threonine and asparagine were allowed as variable modifications. No fixed modifications were used in database search. The SEQUEST peptide identifications were required to satisfy minimum XCorr values of 2, 2.5, and 3 for singly, doubly, and triply charged peptides, a minimum ΔC_n of 0.1, and a P (pep) of ≤ 0.001 . Peptides with a better score than this were accepted for analysis without further validation. The estimated false discovery rate (FDR) of peptide identifications was determined using the reversed protein database approach and was $< 0.5\%$.

Results

The SDS-PAGE-based analysis (Fig.2) revealed a number of proteins that were specifically captured from HepG2 cell lysate with the Stauro Capture Compound™, while many of these were largely absent from the competition sample.

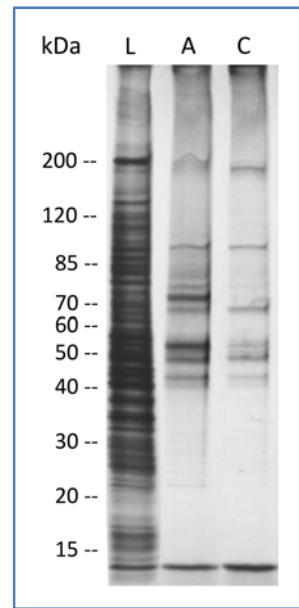


Figure 2 Capturing in HepG2 cell lysate reduces the complexity of biological samples. CCMS yield a higher amount of specifically captured proteins. SDS-Page followed by silver staining. L: HepG2 lysate; CC: Capture Compound assay; C: Control of CC using staurosporine as competitor.

Table 1 Identified protein kinases in HepG2 cells.

Family	Captured kinases
Serine-Threonine kinases	25
Small molecule kinases	7
Tyrosine-protein kinases	5
Dual specificity kinases	3
Unknown	1

The more sensitive direct LC-MS/MS analysis revealed that in a single run, more than 40 different kinases were identified from a typical HepG2 lysate sample size of 500 μg , covering many different functional classes of kinases. Moreover, the comparison of the number of kinases identified from a captured sample as compared to a sample that was not irradiated (and thus resembled a “traditional” small molecule bead affinity pull down experiment) revealed that the capture experiment is clearly superior over the pull down approach (Fig. 3).

Therefore, CCMS using the Stauro caproKit™ enables a particularly easy, sensitive, and effective profiling of kinases from biological samples.

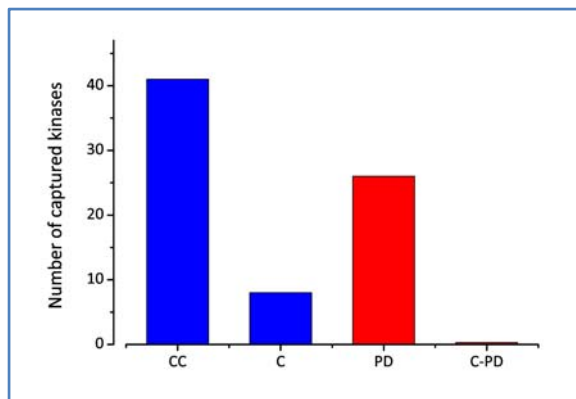


Figure 3 CCMS yield a higher amount of specifically captured protein kinases in HepG2 cell lysate in comparison to pull-down assay. CC: Capture Compound assay; C: Control of CC using staurosporine as competitor; PD: Pull-down assay, no irradiation; C-PD: Control of PD using staurosporine as competitor.

Conclusion

The Stauro caproKit™ is an effective tool for the isolation of staurosporine binding proteins from complex protein mixtures. The selectivity of the analysis is demonstrated by the competition experiments.

CCMS is an outstandingly sensitive method to discover, isolate and profile members of functional protein families within a variety of biological samples.

References

- 1) Cohen, P. (2002) *Nat Rev Drug Discov* **1**, 309-15
- 2) Karaman, M.W. et al. (2008) *Nat Biotechnol* **26**, 127-32.

Downloads:

www.caprotec.com/support/downloads

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Ordering information:

Item Nr.	Description
1-1020-050	Stauro caproKit™ 50 reactions
1-1020-010	Stauro caproKit™ 10 reactions

The caproKit™ includes the Stauro specific Capture Compound™, all buffers, protein controls, Stauro competitor, positive control, and Streptavidin magnetic beads.

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