



cAMP caproKit™ complete covers a broad range of cAMP-binding proteins

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 (e.g. cAMP binding proteins) within a variety of biological samples.

Introduction

Cyclic adenosine monophosphate (cAMP) is an important second messenger in a plethora of different organisms regulating chemotactic behavior of cells and organisms, the cellular response to hormone stimuli and many more biological processes (1). Many cellular targets of cAMP are defined by the presence of a cyclic nucleotide binding domain (CNBD) in the protein structure. Known proteins of this type are regulatory subunits of the cAMP-dependent protein kinases or cyclic nucleotide-gated ion channels (2). Other signatory protein domains include GAF domains, that occur, e.g., in several eukaryotic phosphodiesterases (3). On the other hand, not all cAMP-binding proteins are known; and also, promiscuity in nucleotide binding was observed in several chemical proteomics studies that used cAMP as the affinity component either with affinity-bead-based experiments (4, 5), or in Capture Compound Mass Spectrometry (CCMS) experiments (6). The biological relevance of the latter still awaits verification.

One crucial aspects in the use of chemical proteomics probes is the attachment orientation of, e.g., cAMP, to the other entities of the probe, in the case of Capture Compounds™, the Capture Compound™ scaffold. Therefore, in search of potential new cAMP binders, differently oriented probes should be used to assess a protein's capability of binding – and likely responding – to cAMP. Also, if the whole subproteome of cAMP-binding proteins is to be probed in a scarce biological sample, the use of a mixture of differently attached cAMP probes may be advisable. Among chemical proteomics probes, the cAMP Capture Compounds™ achieve unparalleled sensitivity, being the only cAMP-probe-based approach that enables the identification of HCN ion channels from complex biological samples (6). In order to enable the profiling of the entire subproteome of cAMP-binding proteins, caprotec bioanalytics offers a range of cAMP Capture Compounds™ with different attachment orientations of cAMP to the Capture Compound™ scaffold (C8-, C2-, and N6- positions of the adenine moiety as attachment points). The distinct capture profiles of these cAMP-CCs have

been described in previous application notes available at www.caprotec.com/support/downloads. A new caproKit™ named cAMP caproKit™ complete is now available that includes all three Capture Compounds™, for the use either in separate capture experiments, or as a mixture. In this application note, the use of the mixture of the three Capture Compounds™ in capture experiments is described. The Capture Compound™ mixture can be efficiently used to isolate a broad range of cAMP-binding proteins from complex biological samples.

Materials

The cAMP caproKit™ complete contains the C8-, C2-, and the N6-cAMP Capture Compound™ (C8-, C2-, and N6-cAMP-CC, Figure 1) at a stock concentration of 100 μM each, cAMP as the competitor solution at a stock concentration of 20 mM, Streptavidin magnetic beads, 5x concentrated capture buffer (5x CB1), and 5x concentrated wash buffer (5x WB1) as well as a preparation of purified regulatory subunit 1 of cAMP-dependent protein kinase (KAPO, at a stock concentration of 1 mg/ml).

We recommend PCR Tube strips for volumes up to 200 μl (Thermo Fisher, cat. No. AB-1114) as reaction vessels to prepare the samples, conduct the capture 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™, and the beads were isolated using the caproMag™. As the protein sample, two rat brains were homogenized by 12 strokes at 900 rpm in a motor-driven glass-teflon homogenizer in a homogenisation buffer containing 0.32 M sucrose, 5 mM HEPES/NaOH pH 7.4, supplemented with protease inhibitors. 10 volumes of homogenization buffer per gram of tissue wet weight were used. The homogenate was filtered through nylon gauze to remove debris, and the filtrate was centrifuged for 10 min at 1,000 xg to remove nuclei. The supernatant was centrifuged for 15 min at 4 °C at 12,000 xg to pellet organelles. The crude organelle pellet was resuspended in homogenization buffer, re-homogenized with 6 strokes at 900 rpm in a motor-driven glass-teflon homogenizer, and centrifuged for 20 min at 4 °C at 12,000 xg.

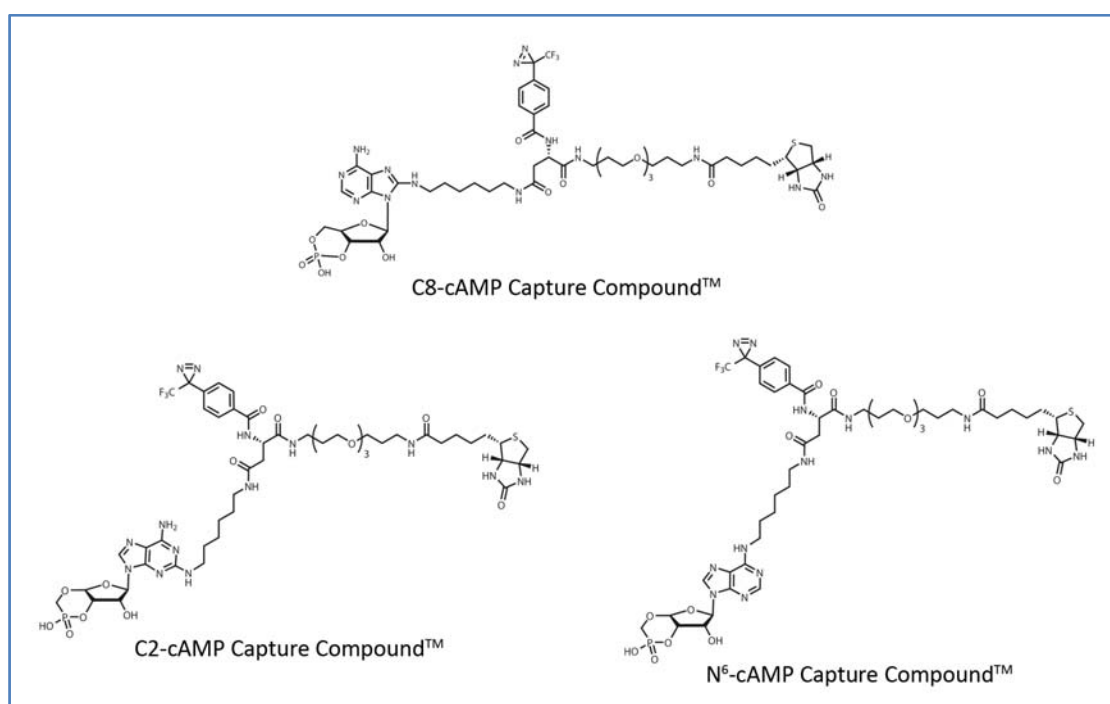


Figure 1 C8-cAMP, C2-cAMP, and N6-cAMP Capture Compound™.

The organelle pellet was then resuspended 1.5 ml/gram of tissue wet weight buffer B (0.32 M sucrose, 5 mM Tris/HCl pH 8.1) with a plastic Pasteur pipette (pastette) and layered on top of a sucrose step gradient consisting of equal volumes of sucrose solution layers of 0.85 M sucrose, 1 M sucrose, and 1.2 M sucrose in an ultracentrifugation tube. Ultracentrifugation was carried out for 2 h at 85,000 \times g at 4 °C. The synaptosome fraction was recovered from the phase border between 1.0 M and 1.2 M sucrose, diluted with \geq 4 volumes of PBS, and pelleted by centrifugation for 30 min at 40,000 \times g. The synaptosome pellet was resuspended in homogenisation buffer, aliquoted, snap-frozen, and stored at -80 °C until further use. The synaptosomes were solubilized using cell opening buffer (6.7 mM MES, 6.7 mM NaOAc, 6.7 mM HEPES pH 7.6, 200 mM NaCl, 10 mM β -mercaptoethanol) with protease inhibitor (Roche, Germany) and 0.5 % DDM. The suspension was rotated at 900 rpm for 1 h at room temperature, centrifuged at 10,000 \times g for 15 min at 4 °C. The supernatant was used for capturing experiments.

Capture experiment

The capture experiments were conducted OnBead and OffBead. In each case, equal amounts of each Capture Compound™ were used in the mixture (3.3 μ l of each CC solution for OffBead, 8 μ l of each Capture compound for the preparation of the caproBeads™). For the competition experiments, cAMP was used as the competitor at a final concentration of 4 mM. The experiments were conducted according to the guidelines for OffBead capturing or OnBead capturing, respectively, available at www.caprotec.com/support/downloads. Captured proteins were subjected to OnBead tryptic digestion (www.caprotec.com/support/downloads).

Mass spectrometry and database research

Tryptic digests were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) on an EASY-nLC™ system coupled to a

LTQ-Orbitrap Velos instrument (Thermo Scientific, Bremen, Germany) through a Proxeon nano electrospray ion source. For chromatographic separation samples were first loaded on a reversed phase (RP) precolumn (5 cm, 100 μ m, 100 Å) and separated on a RP analytical column (15 cm, 75 μ m, 100 Å, NanoSeparations, Netherland) performing a 90 min linear gradient (5-35 % acetonitrile, 0.1 % formic acid).

Mass spectrometric detection was performed in the data-dependent mode allowing to automatically switch between Orbitrap-MS and LTQ-MS/MS (MS²) acquisition in a top 20 configuration at 60 K resolution for a full scan with subsequent collisionally induced dissociation (CID) fragmentation. Full scan MS spectra (from m/z 300-2000) were acquired in the Orbitrap analyzer after accumulation to a target value of 5e5 in the linear ion trap. The most intense ions (up to twenty, depending on signal intensity) with charge state \geq 2 were sequentially isolated at a target value of 10,000 and fragmented in the linear ion trap using low energy CID with normalized collision energy of 35 %. Target ions already mass selected for CID were dynamically excluded for the duration of 63 s. The minimal signal required for MS² was 3000 counts. An activation q of 0.25 and an activation time of 10 ms were applied for MS² acquisitions.

All MS/MS data were analyzed using SEQUEST implemented in Proteome Discoverer 1.2 (Thermo Fisher Scientific) and X!Tandem (www.thegpm.org; version 2007.01.01.1, Proteome Software, Portland, OR, USA). Automated database searching against the human UniProtKB/Swiss-Prot database (release 2010_08 contains 518,350 sequence entries and 8994 rat entries) was performed with 10 ppm precursor tolerance, 1 Da fragment ions tolerance, and full trypsin specificity allowing for up to 2 missed cleavages. Methionine oxidation was used as fixed modification in the database search.

Spectra were annotated via human UniProtKB/Swiss-Prot database and analyzed using the software Scaffold (version Q+ 3_00_06, Proteome Software, Portland, OR, USA). First Proteins were selected if the peptide probability was \geq 95 % assigned by the Peptide Prophet algorithm [7] and the protein was identified by at least 1 unique peptide. The minimum protein probability was set to 80 %. The estimated false discovery rate

of peptide identifications was determined using the reversed protein database approach and was $\leq 1\%$.

Results

The results of the capture experiments were best in the OnBead experiments. The exemplary result of a triplicate experiment is shown in

Table 1. Regulatory subunits of the cAMP-dependent protein kinase (KAPs 0-3) were robustly isolated, along with some accessory proteins such as AKAPs 5 and 6. The characteristic features of the distinct capture profiles of the different attachment

positions of cAMP to the Capture Compound™ scaffold were visible in the protein list of the specifically captured proteins: Very robustly, HCN ion channels were identified, that are predominantly addressed by the C8-cAMP-CC. On the other hand, two phosphodiesterases (PDE2A, PDE10A) that are primarily addressed by the C2-cAMP-CC and the N6-cAMP-CC, were also robustly and specifically identified, along with the putative accessory protein myomegalin. Among the other specifically captured proteins, the C2-cAMP-CC-specific creatine kinase KCRU was robustly captured, as well as the N6-cAMP-CC specific lactate dehydrogenases.

Table 1 Triplicate capture assays and competitions using a mixture of C2-cAMP-CC, C8-cAMP-CC, and N6-cAMP for on-bead capture experiments in solubilised rat brain synaptosomes. Free cAMP was used as the competitor. Numbers of unique peptides identified per protein in each run are given.

Protein name	Accession number	A1	A2	A3	C1	C2	C3
Regulatory subunits of cAMP-dependent protein kinases (KAPs) and accessory proteins							
KAP0	P09456	7	8	8	0	0	0
KAP1	P81377	5	4	4	0	0	0
KAP2	P12368	10	12	15	0	0	0
KAP3	P12369	16	14	19	0	0	1
A-kinase anchoring protein 5	P24587	11	9	10	1	0	1
A-kinase anchoring protein 6	Q9WVC7	2	0	1	0	1	1
cAMP-dependent protein kinase catalytic subunit beta	P68182	1	0	0	0	0	0
Hyperpolarization and cyclic nucleotide-gated (HCN) ion channels							
HCN1	Q9JKB0	9	9	10	1	0	0
HCN2	Q9JKA9	11	15	15	0	0	0
HCN3	Q9JKA8	2	2	2	0	0	0

Phosphodiesterases (PDEs) and accessory proteins							
PDE2A	Q01062	19	19	18	0	0	0
PDE10	Q9QYJ6-2	8	8	10	0	0	0
Myomegalin	Q9WUJ3	1	1	2	1	0	1
Other specifically captured proteins							
Phosphatidylinositol-5-phosphate 4-kinase 2 beta	O88377	4	4	6	2	0	0
Creatine kinase U-type, mitochondrial	P25809	3	2	4	1	0	0
L-lactate dehydrogenase A chain	P04642	11	9	9	0	1	1
L-lactate dehydrogenase B chain	P42123	1	2	4	0	0	0
P2X purinoreceptor 6	P51579	2	3	0	1	0	1
5'-AMP-activated protein kinase catalytic subunit alpha-1	P54645	2	1	2	0	0	0
5'-AMP-activated protein kinase subunit gamma-1	P80385	1	1	1	0	0	0
5'-AMP-activated protein kinase catalytic subunit alpha-2	Q09137	1	0	0	0	0	0
1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase epsilon-1	Q99P84	1	0	2	0	0	0

Numbers of unique peptides are given. A = Capture Compound assay, C = Competition control.

The data provided in this application note demonstrate that the three cAMP-CCs contained in the cAMP caproKit™ complete can not only be used individually in separate assays as described in other application notes (please refer to www.caprotec.com/support/downloads), but also in combination as a mixture of the three CCs in one assay.

The use of the three CCs individually in separate assays is recommended if the binding mode of cAMP to a previously not functionally annotated candidate binder is in the focus of interest. For a comprehensive profiling of cAMP-binding proteins in, e.g., functional proteomics experiments to track expression changes between healthy and diseased populations, it may be advisable to use a mixture of the three CCs in a single assay to cover the entire range of cAMP-binding proteins.

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Downloads:

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Item Nr.	Description
1-1030-050	C8-cAMP caproKit™ 50 rxns
1-1030-010	C8-cAMP caproKit™ 10 rxns
1-1031-050	C2-cAMP caproKit™ 50 rxns
1-1031-010	C2-cAMP caproKit™ 10 rxns
1-1032-050	N ⁶ -cAMP caproKit™ 50 rxns
1-1032-010	N ⁶ -cAMP caproKit™ 10 rxns
1-1035-000	cAMP caproKit™ complete 30 rxn

The caproKit includes the respective Capture Compound, all buffers, protein controls, competitor, and Streptavidin magnetic beads. The cAMP caproKit™ complete includes all cAMP Capture Compound variations, all buffers, protein controls, competitor, and Streptavidin magnetic beads.

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