

N⁶-cAMP caproKit™ extends the functional protein isolation of cAMP-binders in addition to C2-cAMP and C8-cAMP 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 (e.g. cAMP binding proteins) within a variety of biological samples.

Introduction

3',5'-cyclic adenosine monophosphate (cAMP) is a key second messenger molecule in signal transduction processes from bacteria to mammals (1). This second messenger is generated from ATP by adenylyl cyclases and catabolized by phosphodiesterases. Most known mammalian proteins that are directly regulated through cAMP binding contain one or more cyclic nucleotide binding domain (CNBD). Among these proteins are regulatory subunits of cAMP-dependent protein kinases, cyclic-nucleotide-gated ion channels, cAMP-dependent rap guanine nucleotide exchange factors, and some phosphodiesterases. The latter can bind cAMP or its guanosine-derived relative, 3',5'-cyclic guanosine monophosphate (cGMP) through a second class of regulatory protein domains, the GAF domains. However, results obtained in-house at caprotec and by others suggest that there is some additional promiscuity in the binding of nucleotide cofactors to a range of proteins (2, 3). The physiological relevance of these observations still remains to be clarified. However, the concept of

localized formation of up to millimolar concentrations of cAMP suggests that these proteins deserve attention in the study of cAMP-dependent signal transduction, and may well be regulated through direct cAMP binding under physiological conditions. Cyclic nucleotide monophosphates may bind to target proteins in different orientations (e.g., binding to CNBDs versus GAF domains). In the broader context of promiscuous nucleotide binders, different binding orientations may prevail among different types of proteins. This is of relevance for the capturing of cAMP-binding proteins with tri-functional Capture Compounds™, because the attachment site of cAMP to the Capture Compound scaffold is lost for interaction with the target proteins. Therefore, caprotec has synthesized a range of Capture Compounds with cAMP (or cGMP) as selectivity functions with different attachment orientations to the Capture Compound scaffold: a Capture Compound with the cAMP attached via the C8-position, a Capture Compound with cAMP attached via the C2-position, and now newly available, a Capture Compound with cAMP attached via the N6-position of the adenine moiety. This

application note is focused on the use of the N⁶-cAMP Capture Compound to the capturing of cAMP-binding proteins from rat synaptosomes.

Materials

The N⁶-cAMP caproKit™ (1-1032-010, 1-1032-050) contains the N⁶-cAMP Capture Compound (N⁶-cAMP-CC) at a stock concentration of 100 μM, 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). The structure of the N⁶-cAMP Capture Compound is depicted in Figure 1.

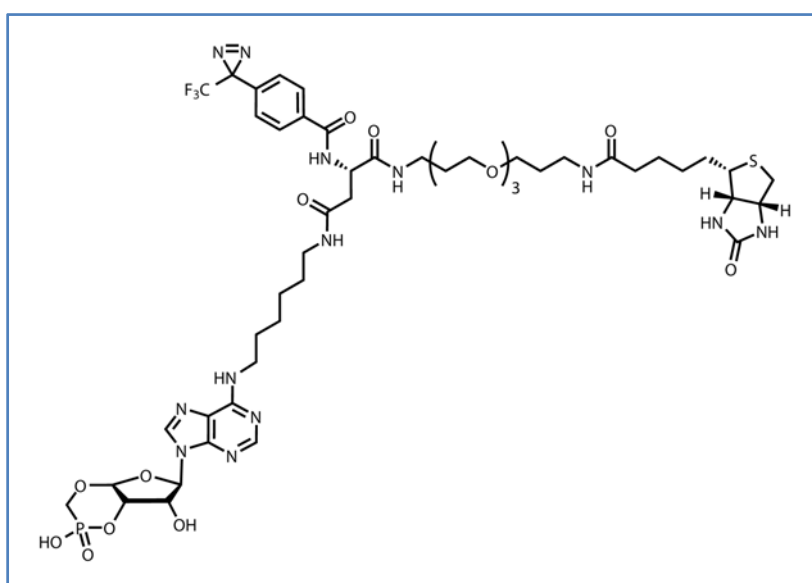


Figure 1 Structure of N⁶-cAMP Capture Compound™.

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 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™, 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 a nylon gauze to remove debris, and the filtrate was centrifuged for 10 min at 1000 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. 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 hours at 85,000 xg at 4 °C. The synaptosome fractions was recovered from the phase border between 1.0 M and 1.2 M sucrose, diluted with ≥ 4 volumes of PBS, and pelleted by centrifugation for 30 min at 40,000 xg. The synaptosome pellet was then 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 respectively. The suspension was rotated at 900 rpm for 1 h at room temperature, centrifuged at 10000 \times g for 15 min at 4 °C. The supernatant was used for capturing experiments.

Capture experiment

The assays were performed according to the caproKit™ OnBead Guideline (see www.caprotec.com/support/downloads) and prepared as follows: for preparation of the caproBeads™, per assay 50 μ l Streptavidin magnetic beads were mixed with 25 μ l of the N⁶-cAMP-CC solution, respectively and incubated for 2 min at room temperature under vigorous shaking. Afterwards, the tube strip was fitted in the caproMag™, the beads collected in the tube lids, and washed twice with wash buffer and ultimately collected in the tube lids according to the caproMag™ Guideline (see www.caprotec.com/support/downloads). In the meantime, the assays were prepared in the following order: Water, 5x capture buffer, and lysate were mixed by vortexing to achieve a final reaction volume of 100 μ l with a final protein concentration of 3.5 mg/ml. Note: for the competition sample add cAMP competitor prior to the addition of the lysate. The caproBeads™ were thoroughly resuspended in the sample solutions and allowed to incubate for 3 hours at 4 °C under rotation. The samples were very briefly centrifuged in a tabletop centrifuge with a butterfly rotor, in order to remove any liquid from the tube lids, but short enough to avoid pelleting of the caproBeads™. Afterwards, in 12 intervals of 2.5 min the samples were irradiated in the caproBox™ at 2-4 °C and finally washed six times with wash buffer WB1. The capture samples were then washed twice with 200 μ l 80 % acetonitrile and once with 200 μ l MS-grade water, and subjected to OnBead tryptic digestion as described in the respective caproKit™ guideline MassSpec (<http://www.caprotec.com/support/downloads>).

Mass spectrometry and database research

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 90-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 and Tandem X! as implemented in the software scaffold 2.0. Specific search parameters 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.

Results

The N⁶-cAMP-Capture Compound was found to specifically target cyclic nucleotide binding domain containing proteins from rat brain synaptosomes. These are listed in Table 1.

All proteins were consistently competed by free cAMP, thus absent from the competition samples. The predominant proteins specifically captured were regulatory subunits of cAMP-dependent protein kinase (KAPs). Two A-kinase anchoring proteins, AKAP 5 and AKAP 18, were identified as well. In addition, several phosphodiesterases were identified, along with one member of the hyperpolarisation and cyclic nucleotide gated ion channel family, HCN1. Among specifically captured proteins that do not contain a known CNBD, lactate dehydrogenase is most prominent.

Table 1 Proteins specifically captured by N⁶-cAMP Capture Compound from rat brain synaptosomes.

Protein Name	Acc. No.	A	C
KAPs and AKAPs			
cAMP-dependent protein kinase type II-beta regulatory subunit	KAP3_RAT	15	0
cAMP-dependent protein kinase type II-alpha regulatory subunit	KAP2_RAT	21	0
cAMP-dependent protein kinase type I-alpha regulatory subunit	KAP0_RAT	10	0
A-kinase anchor protein 5	AKAP5_RAT	13	0
AKAP18 delta isoform (A kinase (PRKA) anchor protein 7)	Q6JP77_RAT	2	0
PDEs			
cGMP-dependent 3',5'-cyclic phosphodiesterase	PDE2A_RAT	12	0
PDE10A14	Q6S9E6_RAT	7	0
PDE10A12	Q6S9E8_RAT	1	0
PDE10A13	Q6S9E7_RAT	1	0
cGMP-inhibited 3',5'-cyclic phosphodiesterase A	PDE3A_RAT	1	0
Ion channels			
Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1	HCN1_RAT	9	0
Others			
L-lactate dehydrogenase B chain	LDHB_RAT	6	0
L-lactate dehydrogenase A chain	LDHA_RAT	5	0

Numbers of unique peptides are given. A representative capture experiment is shown. A = Capture Compound assay, C = Competition control.

Lactate dehydrogenase is a known binder of NADH, thus specific binding of cAMP points to promiscuous nucleotide binding. However, from a structural point of view, The N⁶-cAMP-CC is the only cAMP Capture Compound with an attachment orientation of cAMP to the Capture Compound scaffold that would permit binding to lactate dehydrogenase similar to the established co-factor NADH (4). This is supported by the observation that lactates dehydrogenase is not captured by the C2- or C8-cAMP Capture Compounds.

The N⁶-cAMP caproKit complements effectively the existing range of cAMP caproKits with cAMP attached to the Capture Compound scaffold in different orientations. Especially with respect to the discovery of potential new cAMP binders, the availability of a range of differentially attached cAMP-CCs is useful, because different proteins may bind cAMP in different orientations.

References:

- 1) Beavo, J. A., and Brunton, L. L. (2002) Cyclic nucleotide research -- still expanding after half acentury. *Nat Rev Mol Cell Biol* **3**, 710-718.
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- 4) Read, J.A., et al (2001) Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase. *Proteins* **43**, 175-185.

Downloads:

www.caprotec.com/support/downloads

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Ordering information:

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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