

## Improved functional protein enrichment using the Biotin Capping Kit

The Biotin Capping Kit allows the masking of endogenously biotinylated proteins (e.g. carboxylases). Using the Biotin Capping Kit before capturing prevents endogenously biotinylated proteins that naturally occur in complex samples, to bind on Streptavidin magnetic beads. As the result, the entire bead capacity is available for binding of Capture Compound protein conjugates. Consequently, LC-MS/MS analysis is improved by the reduction of the background proteins.

### Introduction

Capture Compound mass Spectrometry (CCMS) is a uniquely effective approach to address and isolate functional sub-proteomes from complex biological samples on the basis of small molecule-protein interactions (1, 2). Two possible workflows can be pursued using the tri-functional Capture Compounds™ (CCs): either the “OnBead” workflow, where the CCs are pre-bound to Streptavidin beads prior to incubation with the sample or the “OffBead” workflow, where reversible binding of the selectivity function of the CC target proteins and photo cross-linking take place in the sample prior to addition of the Streptavidin beads. While some of the caproKit™ CCs can be used in either way, compounds such as the Stauro-CC should be used in the “OffBead” workflow. In addition, the new S-adenosyl-L-homocysteine (SAH)-CC with a tetrafluoro phenylazide reactivity function was designed to particularly enhance “OffBead” capture results (see respective application note). One potential drawback of the “OffBead” configuration is the co-purification of proteins that are endogenously biotinylated, such as the five carboxylases/ carboxylase subunits from mammalian samples, or the biotin carboxyl carrier protein of acetyl-CoA carboxylase (BCCP) from *E. coli* (Table 1).

Co-purification of these proteins by Streptavidin magnetic beads consumes binding capacity, as well as measurement capacity in the LC-MS/MS analysis. It is therefore desirable to prevent endogenously biotinylated proteins from binding to the Streptavidin magnetic beads, while retaining the binding capacity for CC-protein-conjugates from the capture reaction.

Here, a solution for this problem is presented which is now available as Biotin Capping Kit. This kit allows masking endogenously biotinylated proteins without interfering with the binding of CC-protein-conjugates. Endogenously biotinylated proteins are no longer co-purified with the CC-protein-conjugates, and more measurement capacity can be allocated to truly captured proteins in LC-MS/MS experiments. Capping of endogenously biotinylated proteins is depicted in Figure 1.

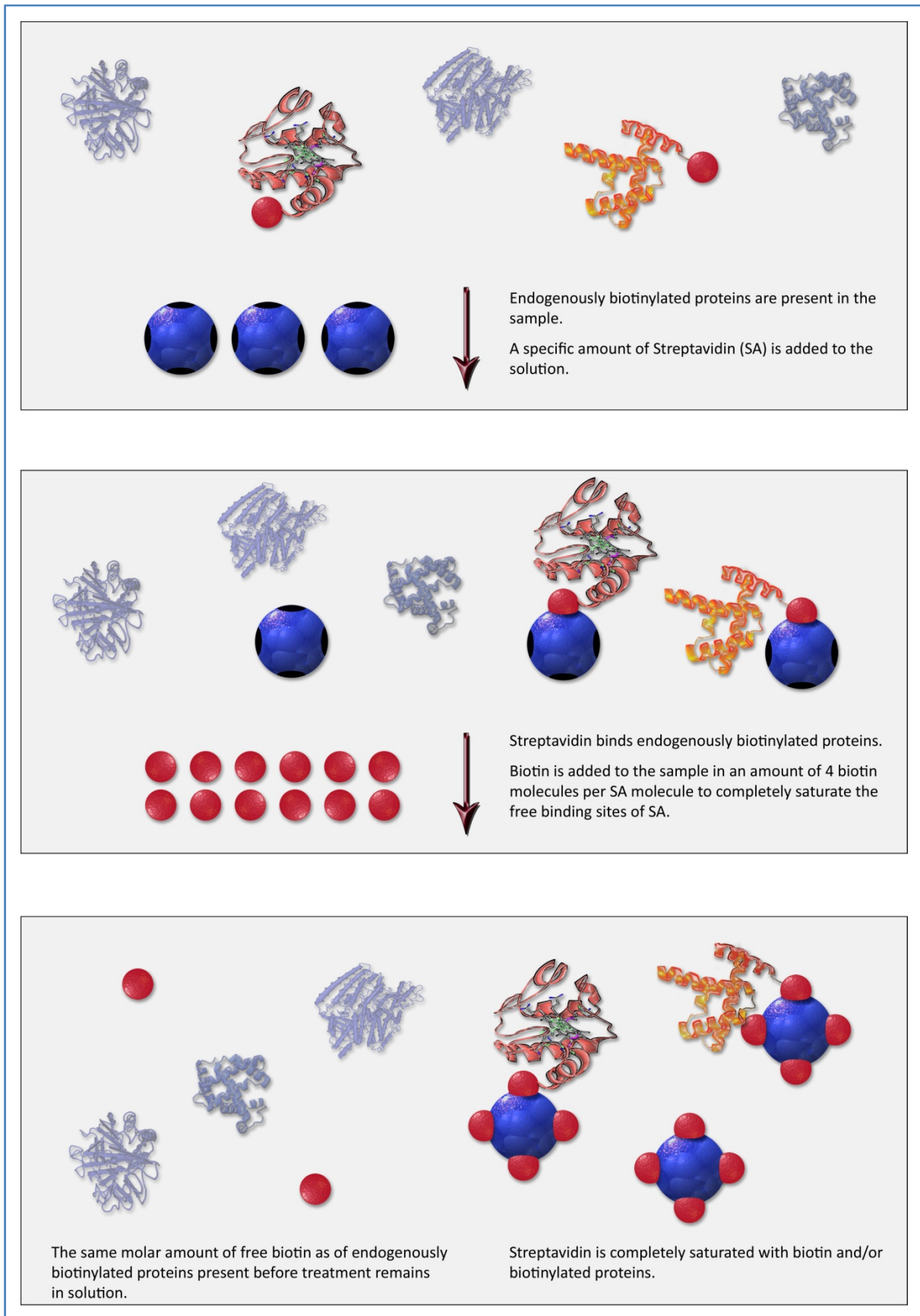


Figure 1 Workflow for application of the Biotin Capping Kit.

**Table 1** Examples of endogenously biotinylated proteins from human and *E. coli* cell lysate.

Protein name	Gene names	UniProtKB accession number	UniProtKB entry name	Molecular weight / kDa
<b>Human:</b>				
Acetyl-CoA carboxylase 2	ACACB	O00763	ACACB_HUMAN	276.6
Acetyl-CoA carboxylase 1	ACACA	Q13085	ACACA_HUMAN	265.6
Pyruvate carboxalase, mitochondrial	PC	P11498	PYC_HUMAN	129.6
Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial	MCCC1	Q96RQ3	MCCA_HUMAN	80.5
Propionyl-CoA carboxylase alpha chain, mitochondrial	PCCA	P05165	PCCA_HUMAN	77.4
<b><i>E. coli</i>:</b>				
Biotin carboxyl carrier protein of acetyl-CoA carboxylase (BCCP)	accB	POABD8	BCCP_ECOLI	16.7

## Materials

The Biotin Capping Kit consists of Streptavidin solution and biotin solution. To conduct the experiments for this application note, the SAH caproKit was used for capturing methyltransferases from human and *E. coli* cell lysates, either with or without prior application of the Biotin Capping Kit. *E. coli* DH5 $\alpha$  lysate was prepared from cells grown to the stationary phase, and, subsequent to harvesting by centrifugation, resuspension in lysis buffer (6.7 mM MES, 6.7 mM NaOAc, 6.7 mM Hepes, 1 mM EDTA, 10 mM 2-mercaptoethanol, 200 mM NaCl, 0.2 mM PMSF, 10 % glycerol, pH 7.5), and sonication, the lysate was cleared of debris by centrifugation and concentrated to a final concentration of 12 mg/ml. For capture experiments, 280  $\mu$ g of total *E. coli* lysate protein were used per assay.

HepG2 cell lysate was prepared as follows: 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 2-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  $\mu$ 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. The total protein concentration was 7 mg/ml. For capture experiments, 350  $\mu$ g of total HepG2 lysate protein were used per assay.

For the preparation of the samples, 12 PCR tube strips 0.2 ml (Thermo Scientific cat no AB-1114) were used. Irradiation of the samples in the capture experiment was carried out using the caproBox™. The caproMag™ was used for handling Streptavidin coated magnetic beads (SA-MB). Further materials are required for SDS-PAGE analysis in the minigel format as well as trypsin and 50 mM ammonium bicarbonate buffer for proteolytic protein digest.

## Method

**Note that the capping kit is applied to the samples prior to any addition of CC or competitor.**

*Capping of endogenously biotinylated proteins in cell lysates*

The volumes of the Biotin Capping Kit solutions used for the experiments described in this application note can be used as starting point also for other samples from different cell lines or organisms. If the samples contain highly abundant endogenously biotinylated proteins, higher volumes of the Biotin Capping Kit solutions need to be applied. Note that the ratio of volumes of the Biotin Capping Kit Streptavidin solution and biotin solution must be kept constant. **Any deviation will lead to sensitivity loss in a subsequent capture reaction.**

Since the ratio between Streptavidin and biotin is essential for the functionality of the kit, the

following precautions and recommendations should be considered.

- Before usage, the Biotin Capping Kit Streptavidin solution and Biotin Capping Kit biotin solution must be at room temperature. It may take up to 45 min for the solutions to thaw and come to room temperature. Only open vials when they have reached room temperature to avoid condensation and, thus, change of concentration of the solutions. Homogenize by inversion and briefly centrifuge.
- Use the same pipette for either solution to avoid volume errors caused by differences in calibration between different pipettes.
- Pay attention on accurate pipetting and ensure complete transfer of both solutions, i.e. Biotin Capping Kit Streptavidin solution and Biotin Capping Kit biotin solution, into the lysate.

The actual protocol is simple and only consists of two pipetting steps performed in a 200 µl PCR tube of a PCR tube strip (Thermo Scientific cat no AB-1114):

1. 5 µl of Biotin Capping Kit Streptavidin solution were transferred to the respective lysate (23.5 µl *E. coli* lysate containing 280 µg total protein for *E. coli* or 50 µl HepG2 lysate containing 350 µg total protein), mixed gently but thoroughly by inversion, and incubated for 20 min at 0-4 °C.
2. 6.5 µl of Biotin Capping Kit biotin solution were transferred to the lysate, mixed gently but thoroughly by inversion, and incubated for 20 min at 0-4 °C.

For the *E. coli* lysate, additional controls and a concentration series were prepared in parallel (Table 2).

For the HepG2 lysate, samples were prepared analogously (Table 3)

## Capture Experiment

All solutions for capture experiments were used from the SAH caproKit™. The capturing procedure was carried out as described in detail in the guideline for the application of the SAH caproKit™ in the “OffBead” configuration (available at [www.caprotec.com/support/downloads](http://www.caprotec.com/support/downloads)) for the functional isolation of SAH-binding proteins, among them methyltransferases, from complex biological mixtures.

Briefly, to each of the above described samples the other necessary components of the Capture Compound assays or competition control were added. Addition of the components was performed in the following sequence at 0-4 °C with mixing in between: 20 µl of 5x concentrated capture buffer, 10 µl 10 mM SAH competitor to sample 2/*E. coli*, sample 2/HepG2, and sample 4/HepG2 (competition controls) or 10 µl Milli-Q water to the other samples followed by 10 min incubation, Milli-Q water to reach a sample volume of 95 µl, and 5 µl 100 µM SAH-CC. All samples were UV irradiated for 4 min within the caproBox™. To each of the samples, 25 µl of 5x concentrated wash buffer 1 were added, followed by 50 µl of Streptavidin magnetic beads. The samples were allowed to incubate for 1 h on a rotation wheel at 4 °C. Afterwards, Streptavidin magnetic beads were isolated using the caproMag™ and washed 6 times with 200 µl of 1x concentrated wash buffer 1 and once with 200 µl Milli-Q water as described in the guideline for using the caproMag™ available at [www.caprotec.com/support/downloads](http://www.caprotec.com/support/downloads). Subsequent to the final isolation step, 8 µl of 2x concentrated Laemmli sample buffer for SDS-PAGE was added to the beads of *E. coli* samples and heated to 95 °C for 5 minutes. These samples were subjected to SDS-PAGE and the proteins stained using a commercially available silver staining kit (ProteoSilver, Sigma-Aldrich). HepG2 samples were washed additionally 6 times with 200 µl 80 % acetonitrile and once with 200 µl Milli-Q water and analyzed by tryptic digestion of the captured proteins using sequencing grade trypsin (Roche) followed by LC-MS/MS analysis (see [www.caprotec.com/support/downloads](http://www.caprotec.com/support/downloads) for a recommended protocol for digestion of captured proteins on Streptavidin magnetic beads).

**Table 2 Pipetting scheme used for preparation of *E. coli* lysate samples.**

Sample No.	1	2	3	4	5	6	7	8	9	10	11
Description	w/o	w/o	w	SA	B	w	SA	B	w	SA	B
	A	C	A	A	A	(1:5)	(1:5)	(1:5)	(1:25)	(1:25)	(1:25)
	A	C	A	A	A	A	A	A	A	A	A
E. coli lysate (12 mg/ml) [μl]	23.5	23.5	23.5	23.5	23.5	23.5	23.5	23.5	23.5	23.5	23.5
Streptavidin solution [μl]	-	-	5	5	-	5	5	-	5	5	-
Milli-Q water [μl]	5	5	-	-	5	-	-	5	-	-	5
	Incubate 20 min at 4 °C										
Biotin solution [μl]	-	-	6.5	-	6.5	6.5	-	6.5	6.5	-	6.5
Milli-Q water [μl]	6.5	6.5	-	6.5	-	-	6.5	-	-	6.5	-
	Incubate 20 min at 4 °C										

w/o = without Biotin Capping Kit; w = using Biotin Capping Kit; SA = Streptavidin solution only; B = Biotin solution only; (1:5) = 1:5 dilution of the solution; (1:25) = 1:25 dilution of the solution; A = Capture Compound assay; C = Competition control.

**Table 3 Pipetting scheme used for preparation of HepG2 lysate samples.**

Sample No.	1	2	3	4	5	6	7
Description	w/o	w/o	w/o	w/o	w	w	w
	A	C	A	C	A	(1:5)	(1:25)
	A	C	A	C	A	A	A
E. coli lysate (12 mg/ml) [μl]	50	50	50	50	50	50	50
Streptavidin solution [μl]	-	-	-	-	5	5	5
Milli-Q water [μl]	5	5	5	5	-	-	-
	Incubate 20 min at 4 °C						
Biotin solution [μl]	-	-	-	-	6.5	6.5	6.5
Milli-Q water [μl]	6.5	6.5	6.5	6.5	-	-	-
	Incubate 20 min at 4 °C						

w/o = without Biotin Capping Kit; w = using Biotin Capping Kit; (1:5) = 1:5 dilution of the solution; (1:25) = 1:25 dilution of the solution; A = Capture Compound assay; C = Competition control.

## Mass Spectrometry

Tryptic digests were analyzed by online nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) on an Ultimate 3000 (Dionex, Idstein, Germany) coupled to a LTQ-Orbitrap XL instrument (Thermo Scientific, Bremen, Germany) through a Proxeon nano electrospray ion source. For chromatographic separation samples were loaded on a reversed phase (RP) precolumn (20 mm, 100  $\mu$ m i.D., 120  $\text{\AA}$ ) and separated on a RP analytical column (10,3 cm, 75  $\mu$ m i.D., 120  $\text{\AA}$ , NanoSeparations, Netherland) performing a 90 min linear gradient (5-35 % acetonitrile, 0.1 % formic acid). LTQ Orbitrap XL was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. and in a top 5 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 5 most intense peptide ions with charge states  $\geq 2$  were sequentially isolated to a target value 10,000 and fragmented in the linear ion trap by low energy CID with normalized collision energy of 35 %. Ion selection threshold was 1500 counts for MS/MS, an activation q=0.25 and activation time of 30 ms were used.

All MS/MS data were analyzed using SEQUEST implemented in Bioworks Browser 3.3.1. SPI (Thermo Scientific, Bremen, Germany) and X!Tandem. Scaffold (version Q+, Proteome Software Inc., Portland, USA) against human UniProtKB/Swiss-Prot database (release July 2010). Specific search parameters were 5 ppm precursor tolerance, 1 amu fragment ions tolerance, and full trypsin specificity allowing for up to two missed cleavages. Methionine oxidation was allowed as variable modifications.

Scaffold was used to validate MS/MS-based peptide and protein identifications based on probability thresholds. Only peptides with  $\geq 95$  % probability as specified by the Peptide Prophet algorithm were considered. Because non of the single-peptide protein hits reached a protein identification probability higher than 85 % irrespective of the spectrum quality, this value was arbitrarily set to  $\geq 50$  %. Single-peptide protein identifications were

only accepted if the peptide probability was  $\geq 95$  %. Furthermore, these protein hits were manually validated by inspection of the MS/MS spectra.

## Results

The SDS-PAGE analysis of captured *E. coli* proteins (Figure 2) revealed that the untreated samples 1 and 2 contain a strong protein band at  $\sim 17$ -18 kDa corresponding to the biotin carboxyl carrier protein of acetyl-CoA carboxylase (BCCP), which is largely diminished in the sample 3 to which the Biotin Capping Kit was applied. Specific proteins (SAH binding, present in Capture Compound assay sample 1, absent in SAH competition control sample 2) are detected equally well with applying the Biotin Capping Kit (sample 3) than without (sample 1). Using only Streptavidin solution and no biotin solution (sample 4) results in the absence of both, BCCP and captured proteins. BCCP is capped by Streptavidin but also the biotin sorting function of the CC has bound to the excess Streptavidin making it and the CC-protein-conjugates unavailable to the Streptavidin magnetic beads. Using only biotin and no Streptavidin (sample 5) leads to a significant reduction of both, BCCP and captured proteins. In this case, the excess biotin competes with BCCP and the CC-protein-conjugates in binding to the Streptavidin magnetic beads. Lowering the amount of Biotin Capping Kit components (samples 6-11, see Table 2) gives unsatisfactory results in terms of diminishing BCCP.

The LC-MS/MS analyses of peptide mixtures from captured proteins from HepG2 lysate samples with or without applying the Biotin Capping Kit are summarized in Tables 4 and 5.

From these measurements, it becomes apparent that the endogenously biotinylated carboxylases together with their complex partners are abundant in uncapped samples, but nearly absent from capped samples, at all concentrations of the Biotin Capping Kit tested.

In total, 15 established or putative methyltransferases were specifically captured. The yield of methyltransferases was similar in uncapped and capped samples. The only methyltransferase detected in uncapped but not in capped samples was TRM6. However, six methyltransferases were

detected in the capped but not in the uncapped samples (RRMJ3, PIMT, LCMT1, CP013, WDR4, SET1A).

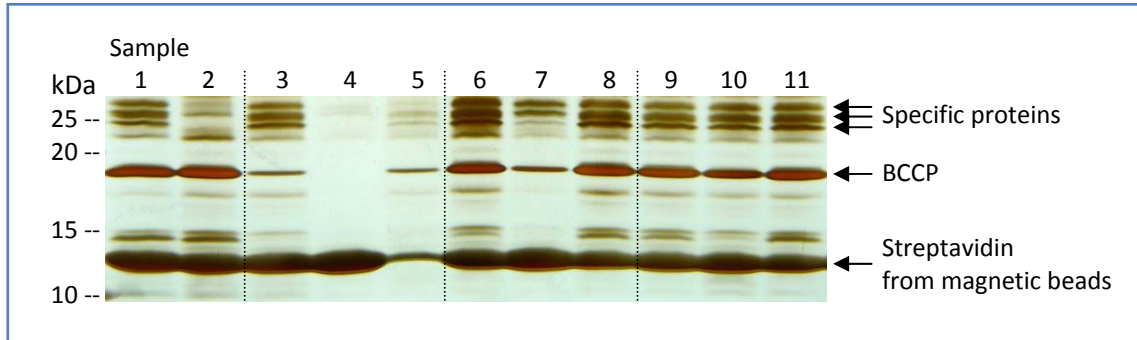


Figure 2 SDS-PAGE/silver stain of captured proteins from *E. coli* lysate samples prepared according to Table 2.

Table 4 Endogenously biotinylated carboxylases and their interaction partners identified by Capture Compound Mass Spectrometry (CCMS) from HepG2 lysate samples with and without applying the Biotin Capping Kit. Numbers denote peptide spectral counts.

Sample No.	1	2	3	4	5	6	7
Description	w/o	w/o	w/o	w/o	w	w (1:5)	w (1:25)
	A	C	A	C	A	A	A
Propionyl-CoA carboxylase alpha chain, mitochondrial (PCCA_HUMAN)	31	35	32	33	-	-	-
Pyruvate carboxylase, mitochondrial (PYC_HUMAN)	25	35	30	31	1	3	2
Acetyl-CoA carboxylase 1 (ACACA_HUMAN)	20	23	14	17	-	-	-
Propionyl-CoA carboxylase beta chain, mitochondrial (PCCB_HUMAN)	17	20	16	17	-	-	-
Methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial (MCCA_HUMAN)	15	16	15	16	-	-	-
Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial (MCCB_HUMAN)	14	16	14	14	-	-	-

w/o = without Biotin Capping Kit; w = using Biotin Capping Kit; (1:5) = 1:5 dilution of the solution; (1:25) = 1:25 dilution of the solution; A = Capture Compound assay; C = Competition control.

**Table 5 Methyltransferases identified by Capture Compound Mass Spectrometry (CCMS) from HepG2 lysate samples with and without applying the Biotin Capping Kit. Numbers denote peptide spectral counts.**

Sample No.	1	2	3	4	5	6	7
Description	w/o	w/o	w/o	w/o	w	w (1:5)	w (1:25)
	A	C	A	C	A	A	A
Putative ribosomal RNA methyltransferase NOP2 (NOP2_HUMAN)	9	-	11	-	9	8	7
Arsenite methyltransferase (AS3MT_HUMAN)	4	-	5	-	6	6	5
tRNA (guanine-N(7)-)-methyltransferase (TRMB_HUMAN)	2	-	1	-	2	1	2
Catechol O-methyltransferase (COMT_HUMAN)	1	-	1	-	1	1	1
tRNA guanosine-2'-O-methyl-transferase TRM11 homolog (TRM11_HUMAN)	1	-	1	-	1	1	1
Histamine N-methyltransferase (HNMT_HUMAN)	2	-	1	-	1	-	-
tRNA (cytosine-5)-methyltransferase NSUN2 (NSUN2_HUMAN)	1	-	1	-	-	-	1
DNA (cytosine-5)-methyltransferase 1 (DNMT1_HUMAN)	-	-	1	-	1	-	-
tRNA (adenine-N(1)-)-methyl-transferase non-catalytic subunit (TRM6_HUMAN)	1	-	1	-	-	-	-
Putative rRNA methyl-transferase 3 (RRMJ3_HUMAN)	-	-	-	-	2	-	-
Protein-L-isoaspartate(D-aspartate) O-methyltransferase (PIMT_HUMAN)	-	-	-	-	1	-	-
Leucine carboxyl methyltransferase 1 (LCMT1_HUMAN)	-	-	-	-	1	-	-
UPF0585 protein C16orf13 (CP013_HUMAN)	-	-	-	-	1	-	-
tRNA (guanine-N(7)-)-methyltransferase subunit WDR4 (WDR4_HUMAN)	-	-	-	-	-	1	-
Histone-lysine N-methyltransferase SETD1A (SET1A_HUMAN)	-	-	-	-	-	-	1

w/o = without Biotin Capping Kit; w = using Biotin Capping Kit; (1:5) = 1:5 dilution of the solution; (1:25) = 1:25 dilution of the solution; A = Capture Compound assay; C = Competition control.

## Summary

The newly introduced Biotin Capping Kit is an effective tool to prevent endogenously biotinylated proteins from complex biological mixtures to interact with Streptavidin magnetic beads. This saves bead capacity for the binding of truly captured proteins and improves the allocation of detection capacity for those proteins in LC-MS/MS runs. Also, background in the form of highly abundant endogenously biotinylated proteins is reduced, especially with beneficial effects on the detection of specifically captured proteins in a Streptavidin-horseradish peroxidase western blot.

### References:

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- 2) Luo, Y., et al. (2009) From PKA to HCN: The cAMP-capture compound mass spectrometry as a novel tool for targeting cAMP binding proteins. *Mol Cell Proteomics* **8**, 2843-56
- 3) Karaman, M.W. et al. (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* **26**, 127-132

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