

“OffBead” Protocol for Capturing in Solution

Note: Before performing this protocol, please carefully read the instructions in the “Guideline for CCMS”. This is only a brief outline of the procedure.

We recommend the “OnBead” configuration when the cAMP Capture Compounds™ are used in a mixture.



Perform steps 1 - 8 at 0 - 4 °C using the caproBox™ for cooling. Avoid formation of bubbles during handling as this may accelerate denaturation of proteins. We recommend 2 - 5 mg/ml total protein concentration for cell lysates. The assay is designed to use either single 200 µl PCR tubes or 200 µl PCR tube strips. **Protect the Capture Compounds™ from sunlight or other highly UV-emitting light sources during the whole experiment. Usual ceiling lighting in the lab is tolerable.**

- 1) Place the PCR tubes or tube strips into the caproBox sample holder for cooling and add all components according to the pipetting scheme shown below – follow the given sequence (first H₂O, then competitor etc.) and mix thoroughly after each step (but without producing bubbles if proteins are involved).

Note: The schemes below are examples to prepare the reactions. For planning your own experiments, please use the provided pipetting scheme below or the reaction volume calculator at: www.caprotec.com/support/downloads.

Example to prepare Capture Compound assay (“A”) and competition control (“C”)

| Component | Stock conc. | Assay conc. | Capture Compound assay “A” | Competition control “C” |
|--|-------------|-------------|----------------------------|-------------------------|
| H ₂ O | | | 39.4 µl | 19.4 µl |
| cAMP competitor | 20 mM | 4 mM | -- | 20 µl |
| 5x CB1 | 5 x | 1 x | 20 µl | 20 µl |
| PKA RI* (43 kDa) | 16 µM | 0.11 µM | 0.7 µl | 0.7 µl |
| Cell lysate | 10 mg/ml | 3 mg/ml | 30 µl | 30 µl |
| 10 min pre-incubation Proceed to add: | | | | |
| C8-cAMP-CC | 100 µM | 3.3 µM | 3.3 µl | 3.3 µl |
| C2-cAMP-CC | 100 µM | 3.3 µM | 3.3 µl | 3.3 µl |
| N ⁶ -cAMP-CC | 100 µM | 3.3 µM | 3.3 µl | 3.3 µl |
| Total volume | | | 100 µl | 100 µl |

*For the initial experiments, it is recommended to use the **positive control protein** (PKA RI) in addition to the Capture Compound assay “A” and the competition control “C”. **Additional control reactions** are listed under “*checklist for OffBead protocol*”. In addition, keep a 1 µl sample (or 10 µl in case no lysate and only the positive control protein is involved) of the

prepared Capture Compound assay “A” and competition control “C” solution as a reference for subsequent SDS-PAGE or MS analysis.

- 2) Incubate solutions “A”, “C” and if applicable any additional control reactions (cf. “Checklist”) on a rotating wheel at 4 °C for 30 min (or gently re-suspend every 5 min).

- 3) Irradiate for 10 min using the caproBox.

Note: Before irradiation, make sure that all the liquid is spun down into the bottom of the PCR tubes and that the lids are removed from the tubes.

- 4) OPTIONAL for displacing proteins non-covalently bound to the Capture Compound:

Add 20 µl cAMP competitor solution to “A”, mix and incubate for 10 min.

Note: Adding competitor after photo cross-linking will displace proteins non-covalently bound to the Capture Compound, i.e. proteins that are not photo cross-linked to the Capture Compound but that may still non-covalently interact with the selectivity functions of the Capture Compounds. Proteins covalently photo cross-linked to the Capture Compound will not be affected by the competitor. This step can be omitted if the Capture Compound experiment is paired with a pull down assay: Affinity driven, non-covalently bound proteins can be isolated in addition to the Capture Compound-Protein-conjugate (cf. “Checklist”).

- 5) Add 25 µl 5x WB1 to “A”, “C” and additional control reactions and gently homogenize.

- 6) Add 50 µl well re-suspended Streptavidin magnetic beads (SA-MB) to “A”, “C” and any additional control reactions. Gently mix the resulting suspensions.

- 7) Incubate for 30 min on a rotating wheel at 4 °C (or gently re-suspend every 5 min).

Note: SA-MB must stay in suspension.

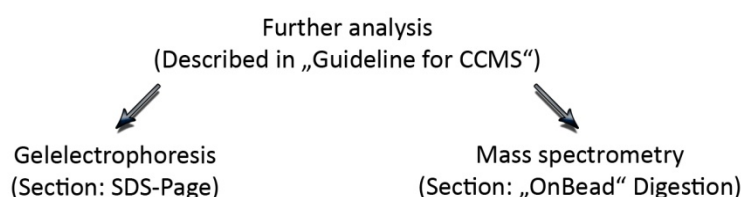
- 8) Collect SA-MB protein complexes (beads) from the reactions by using caproMag™. Allow collection process to proceed for 2 min, until supernatant appears clear. Discard tubes containing supernatant. Add 200 µl 1x WB1 in new PCR tubes and re-suspend the beads.

Note: Take care not to pinch your fingers between the Neodymium magnet and the steel plate. Keep away from pace makers or other metallic objects. For handling advices, please download “cartoon for using the caproMag” from www.caprotec.com/support/downloads

- 9) Collect the beads with the caproMag and repeat the washing step five times without changing the reaction tube. Discard supernatants after all wash steps. Wash the beads once with 200 µl ultrapure water.

Note: Aggregated beads can be suspended by using an ultrasound bath.

The collected beads in step 9 are now ready for further analysis. For sample storage add 100 µl ultrapure water and keep at 4 °C for up to one week.



Checklist for “OffBead” Protocol and Recommended Additional Control Reactions

| | <u>A</u> | <u>C</u> | PD | C-PD | <u>A</u> + PD | C1-MB | C2-MB | C3-MB | C4-MB | C5-MB |
|-------------------------|----------|----------|----|------|---------------|-------|-------|-------|-------|-------|
| H ₂ O | ✓ | ✓ | ✓ | ✓ | ✓ | - | ✓ | ✓ | ✓ | ✓ |
| Competitor (prior irr.) | - | ✓ | - | ✓ | - | - | - | - | - | - |
| 5x CB | ✓ | ✓ | ✓ | ✓ | ✓ | - | ✓ | ✓ | ✓ | ✓ |
| PKA RI* | ✓ | ✓ | ✓ | ✓ | ✓ | - | ✓ | ✓ | ✓ | ✓ |
| Cell lysate | ✓ | ✓ | ✓ | ✓ | ✓ | - | ✓ | - | ✓ | - |
| C8-cAMP-CC | ✓ | ✓ | ✓ | ✓ | ✓ | - | - | - | - | - |
| C2-cAMP-CC | ✓ | ✓ | ✓ | ✓ | ✓ | - | - | - | - | - |
| N ⁶ -cAMP-CC | ✓ | ✓ | ✓ | ✓ | ✓ | - | - | - | - | - |
| Biotin | - | - | - | - | - | - | - | ✓ | - | ✓ |
| Irradiation | ✓ | ✓ | - | - | ✓ | - | - | - | ✓ | ✓ |
| Competitor (after irr.) | ✓ | - | - | - | - | - | - | - | - | - |
| 5x WB1 | ✓ | ✓ | ✓ | ✓ | ✓ | - | ✓ | ✓ | ✓ | ✓ |
| SA-MB | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Incubation | ✓ | ✓ | ✓ | ✓ | ✓ | - | ✓ | ✓ | ✓ | ✓ |
| Wash | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

CB = capture buffer; C8-cAMP-CC = C8-cAMP Capture Compound; C2-cAMP-CC = C2-cAMP Capture Compound; N⁶-cAMP-CC = N⁶-cAMP Capture Compound; WB1 = wash buffer 1; SA-MB = Streptavidin magnetic beads; *For the initial experiments, it is recommended to use a positive control (PKA RI) in addition to the Capture Compound assay “A” and the Control “C”.

| Synonym | Description | Comments |
|---------------|--|--|
| <u>A</u> | Capture Compound assay | |
| <u>C</u> | Control of Capture Compound assay | Proteins only detected in <u>A</u> or detected in a much lower extent in <u>C</u> are cAMP binders. The proteins are covalently cross-linked to CC. The cross-link position within the protein sequence may be determined by MS. |
| PD | Pull down assay | |
| C-PD | Control of pull down assay | Proteins only detected in PD in comparison to C-PD are strong or highly abundant cAMP binders. They are not removed from the SA-MB by washing steps. No covalent cross-link between CC and proteins occurred. |
| <u>A</u> + PD | Combined Capture Compound and pull down assay | Use <u>C</u> as control. |
| C1-MB | Control, examining Streptavidin magnetic beads (SA-MB) only. | Only Streptavidin should be detected. |
| C2-MB | Control, examining CC-independent binding to the SA-MB | Endogenously biotinylated proteins are detected if not removed by pre-treatment of the lysate. Additionally, proteins with unspecific binding to the SA-MB may be detected (compare C3-MB). |
| C3-MB | | Control, examining CC-independent binding to the SA-MB excluding biotinylated proteins |
| C4-MB | | Control, examining CC-independent binding to the SA-MB including irradiation induced unspecific binding |
| C5-MB | | Control, examining CC-independent binding to the SA-MB including irradiation induced unspecific binding and excluding biotinylated proteins |

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