

“OnBead” Protocol for Capturing via caproBeads™

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 direct light during the whole experiment.**

1) Mix the sample with all reaction components in a total volume of 100 µl as described below.

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.

For the efficiency of the CCMS approach, place the PCR tube strips in the caproBox for cooling, add all components **exactly** according to the sequence below - mix gently after each step.

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 | | | 49.3 µl | 29.3 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 |
| Total volume | | | 100 µl | 100 µl |

*Performing the first experiments, it is recommended to use the **positive control** (PKA RI) and in addition to the Capture Compound assay “A” and the competition control “C”. **Additional control reactions** are listed under “*checklist for OnBead protocol*”. In addition, keep a 10 µl sample of the prepared Capture Compound assay “A” and competition control “C” solution as a reference for subsequent SDS-PAGE and MS analysis.

2) Preparation of caproBeads™ for each capture reaction: Mix gently 8 µl 100 µM C8-cAMP Capture Compound™ (CC) with 8 µl 100 µM C2-cAMP Capture Compound™, 8 µl 100 µM N⁶-cAMP Capture Compound™, and add 50 µl well resuspended Streptavidin magnetic beads (SA-MB, 10 mg/ml) for each reaction (Capture Compound assay “A”, Control “C”, and any additional control

reactions (cf. step 1 and “Checklist for OnBead protocol” listed below)). Shake reactions vigorously at room temperature for 10 min.

Collect caproBeads as defined in step 7 by using the caproMag™. Wash two times with 200 µl of 1x WB1 as defined in step 8. Discard supernatants after each washing step. After the last washing step keep the freshly prepared caproBeads in the lids of the PCR tube strips at the caproMag.

- 3) Resuspend each single aliquot of the freshly prepared caproBeads with solution “**A**”, “**C**”, and additional control reactions.
- 4) Incubate at least 3 h on a rotator wheel at 4 °C (or gently re-suspend every 5 min).

Note: caproBeads must stay in solution.

- 5) Place solutions “**A**”, “**C**” and any additional control reactions into the caproBox and irradiate for 20 min. Mix solutions at least every 2.5 min by inverting the tubes.

Note: Before irradiation, make sure that no liquid is in the lids of PCR tubes. If necessary shortly centrifuge the suspension. Take care, caproBeads must stay in solution.

- 6) OPTIONAL for displacing non-covalently bound proteins:

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

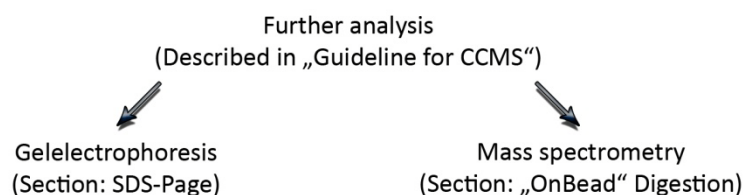
Note: Adding of free competitor after photo cross-linking will displace non-covalently bound proteins. Covalently bound proteins will not be affected by free competitor. This step can be omitted if the Capture Compound experiment is paired with a pull down assay: All affinity driven, non-covalently bound proteins will be isolated in addition to the covalently attached proteins after UV-activation of the reactivity function (cf. “Checklist”).

- 7) Collect beads from “**A**”, “**C**” and any additional control reactions by using the 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 gently resuspend 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 further handling advices, please download “cartoon for using the caproMag” from www.caprotec.com/support/downloads

- 8) Collect the beads with the caproMag and repeat washing step five times without changing the reaction tube by vigorously mixing the reaction suspensions as defined in step 7. Discard supernatants after all wash steps. Wash the beads once with 200 µl ultrapure water.

The collected beads in step 8 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 “OnBead” Protocol and Recommended Additional Control Reactions

| | “ <u>A</u> ” | “ <u>C</u> ” | “PD” | “C-PD” | “ <u>A</u> + PD” | “C1-MB” |
|-------------------------|--------------|--------------|------|--------|------------------|---------|
| H ₂ O | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| Competitor (prior irr.) | - | ✓ | - | ✓ | - | - |
| 5x CB | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| PKA RI* | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| Cell lysate | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| caproBeads | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Incubation | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| Irradiation | ✓ | ✓ | - | - | ✓ | - |
| Competitor (after irr.) | ✓ | - | - | - | - | - |
| Wash | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

CB = capture buffer; caproBeads = (SA-MB + C8-cAMP-CC, C2-cAMP-CC, N⁶-cAMP-CC freshly pre-incubated and washed);

*For the initial experiments, it is recommended to use the 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 target 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 target 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. |

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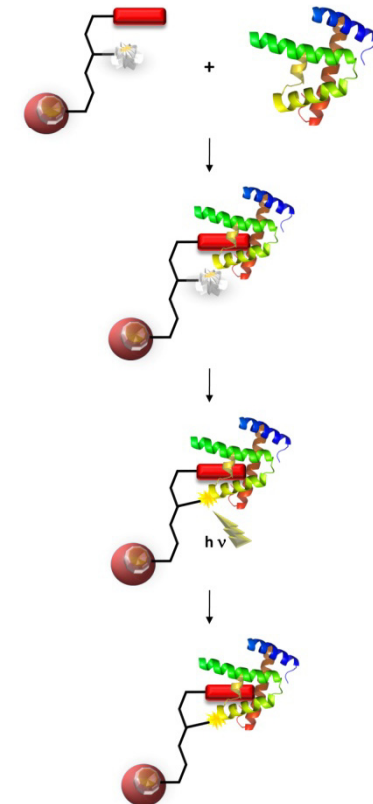
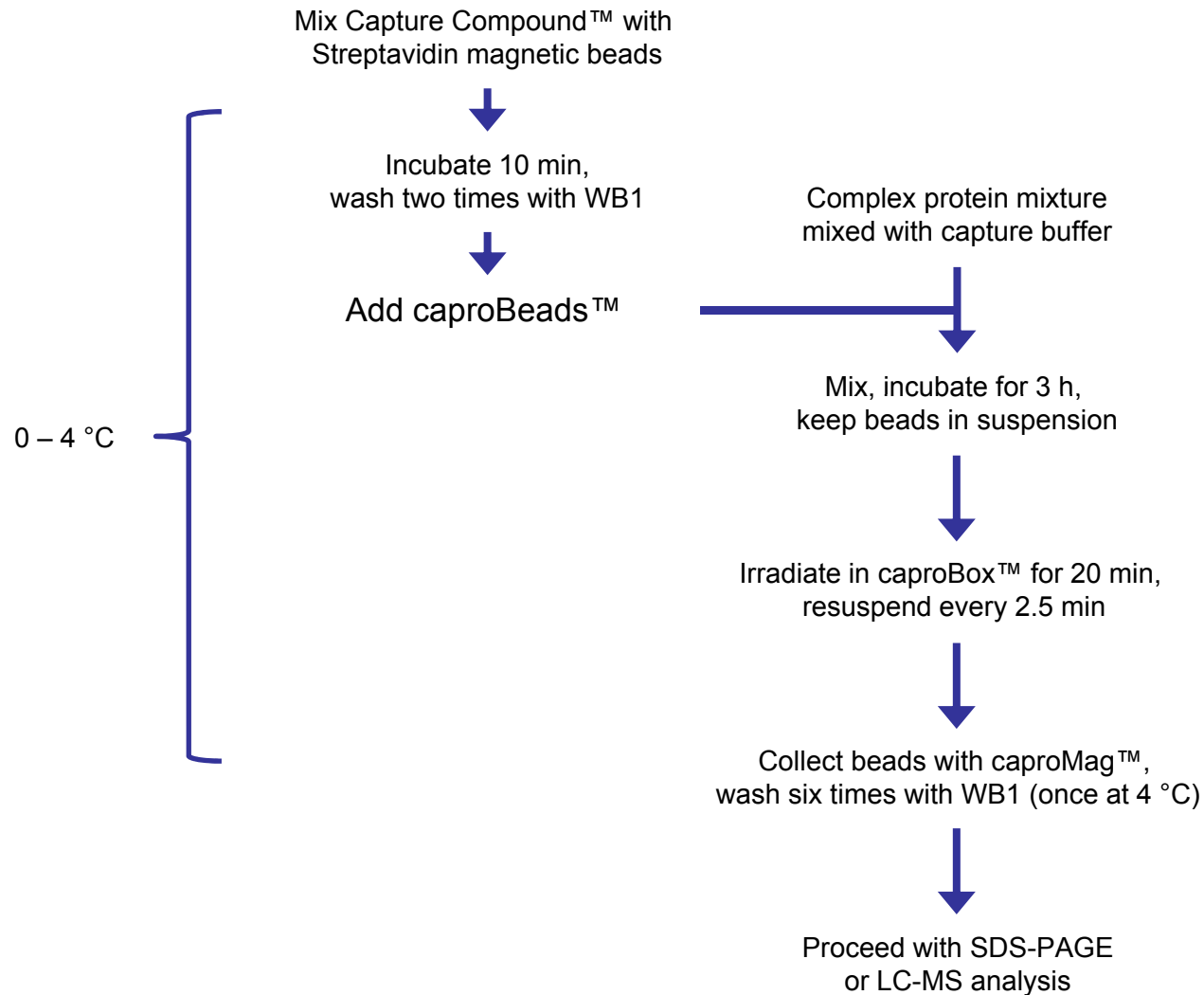
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“OnBead” Protocol for Capturing via caproBeads™



Pipetting Scheme - OnBead

Preparing Capture Compound assay ("A") and Competition control ("C")

| Component | Stock conc. | Assay conc. | Capture Compound assay " <u>A</u> " | Competition control " <u>C</u> " |
|---------------------|-------------|-------------|-------------------------------------|----------------------------------|
| H ₂ O | | | | |
| 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 | | | | |
| Total volume | | | 100 µl | 100 µl |

*For the initial experiments, it is recommended to use the **positive control** (e.g. PKA RI) in addition to the Capture Compound assay "A" and the competition control "C".