

“OnBead” Protocol for Capturing via caproBeads™

NOTE: Before proceeding this protocol, please read carefully the instruction in the handbook “Guideline for CCMS”! This is only a brief description of the procedure!

Perform steps 1 - 8 at 0 - 4 °C using the caproBox™ for cooling. Avoid bubbles during handling which may cause denaturation of proteins. We recommend 2 - 5 mg/ml total protein concentration for cell lysates. The assay is designed to use both single 200 µl PCR tubes and 200 µl PCR tube strips.

- 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 your own experiments, please use the provided pipetting scheme (see below) or the reaction volume calculator (see at: www.caprotec.com/support/downloads).



NOTE: For the efficiency of the CCMS approach, place the PCR tube strips in the caproBox for cooling, add all components exactly following the sequence below - mix gently after each addition.

Example to prepare 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			49.3 µl	24.3 µl
cAMP competitor	4 mM	1 mM	--	25 µl
5x CB	5 x	1 x	20 µl	20 µl
PKA RI*	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 Control “C”. **Additional control reaction samples** listed under “*checklist for OnBead protocol*”. Furthermore, take a 1 µl sample of the prepared Capture Compound assay “A” solution as a reference for subsequent SDS-PAGE and MS analysis.

- 2) Preparation of caproBeads for each capture reaction: Mix 25 µl 100 µM Capture Compounds™ (CC) with 50 µl well resuspended Streptavidin magnetic beads (SA-MB, 10 mg/ml) for each approach (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 2 min.

Collect caproBeads as defined in step 7 by using the caproMag™. Wash two times with 200 µl of 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) Resolve 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 30 min. Mix solutions at least every 2.5 min by inverting the tubes.



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

- 6) Add 25 µl cAMP competitor solution to “**A**”, gently mix and incubate for 10 min.



Note: Adding of free competitor after photo cross-linking will displace all 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”).

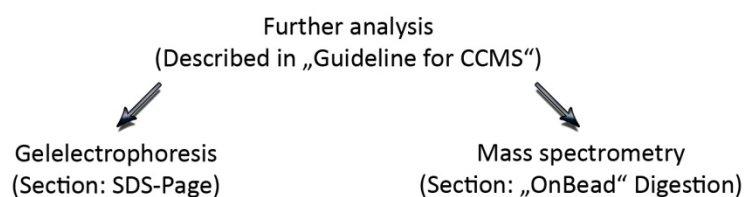
- 7) Collect beads from “**A**”, “**C**” and any additional control reactions by using the caproMag. Allow collection process to proceed for 2 min, until supernatant appears clear. Discard tubes containing supernatant. Add 200 µl WB1 in new PCR tubes and gently resuspend the beads.



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 10 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; cAMP-CC = cAMP Capture Compound; caproBeads = (SA-MB + 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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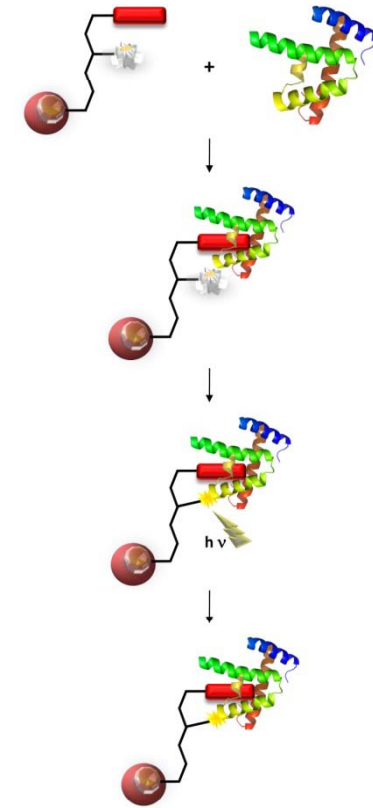
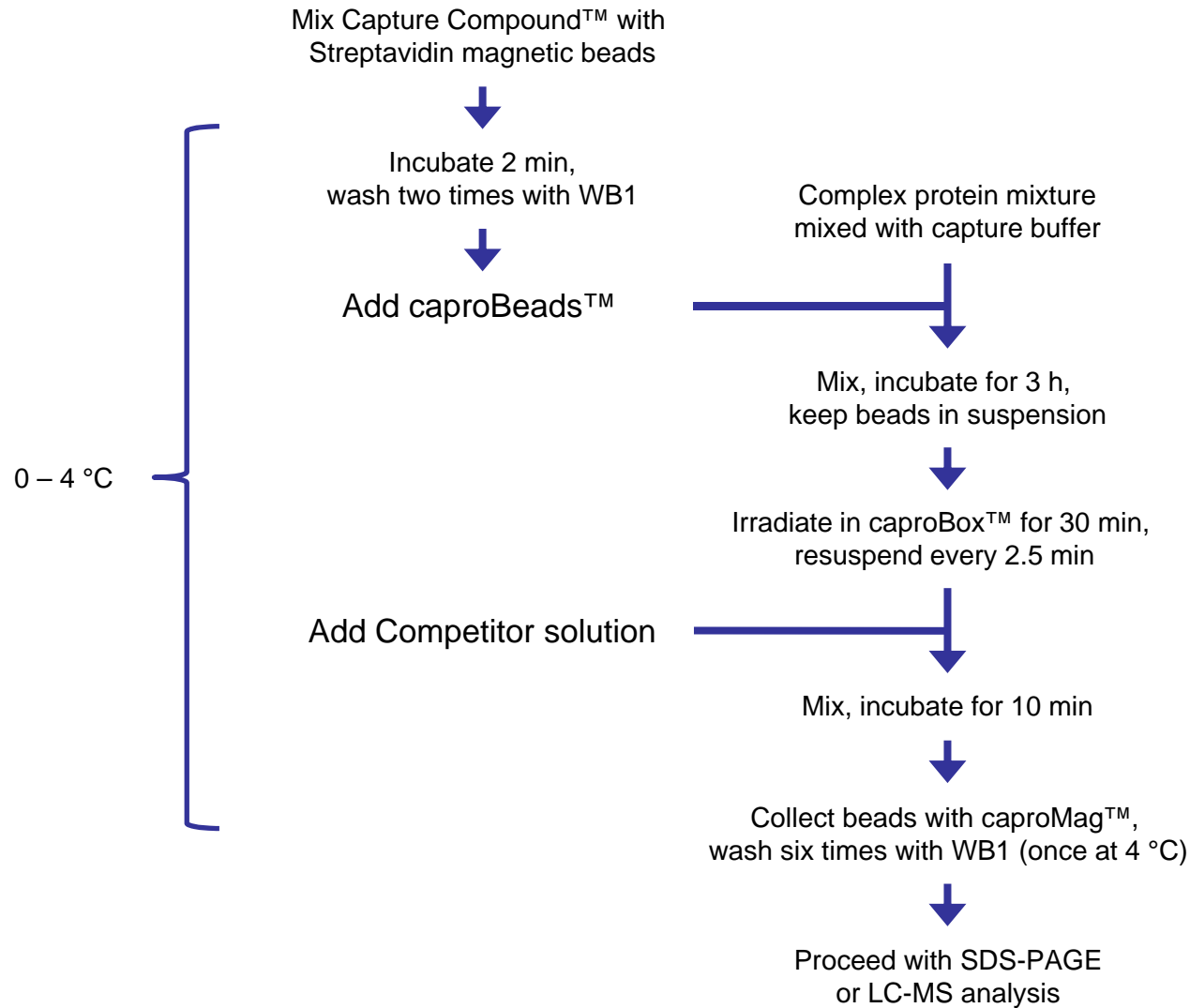
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Pipetting Scheme - OnBead

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	40 mM	4 mM	--	25 µl
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*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 Control "C".