

“OffBead” Protocol for Capturing in Solution

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

The positive control enzyme (MMP-2) is provided as pro-enzyme and has to be activated prior to use. For activation, add 1 µl 10 mM APMA (4-aminophenylmercuric acetate; FW 351.75 g/mol) in DMSO to 10 µl MMP-2, mix, and incubate 90 min at 37 °C.



NOTE: APMA is very toxic. Carefully read safety instructions provided by the supplier. APMA is not provided with the kit and may be purchased from Sigma-Aldrich (Cat-No A9563-5G).

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 either single 200 µl PCR tubes or 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 “A”	Competition control “C”
H ₂ O			40 µl	35 µl
Marimastat competitor	10 mM	0.5 mM	--	5 µl
5x capture buffer 3 (CB3)	5 x	1 x	20 µl	20 µl
MMP-2*	2.8 µM	0.14 µM	5 µl	5 µl
Cell lysate	10 mg/ml	3 mg/ml	30 µl	30 µl
10 min pre-incubation Proceed to add:				
Marimastat-CC	20 µM	1 µM	5 µl	5 µl
Total volume			100 µl	100 µl

*For the initial experiments, it is recommended to use the **positive control** (MMP-2) added to the Capture Compound™ assay “A” and the control “C”. **Additional control reactions** are listed under “*checklist for OffBead protocol*”. In addition, we suggest to keep a 10 µl sample of


the prepared Capture Compound™ assay “A” solution as a reference for subsequent SDS-PAGE and MS analysis.

- 2) Incubate solutions “**A**”, “**C**” and any additional control reactions (cf. “Checklist”) at 4 °C for 10 min.
- 3) Open tubes and irradiate for 4 min using the caproBox.
- 4) Add 5 µl Marimastat competitor solution to “**A**”, gently mix and incubate for 10 min.




Note: Adding free competitor after photo cross-linking will displace all proteins non-covalently bound to the Marimastat-CC. 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”).

- 5) Add 25 µl 5x WB1 to “**A**”, “**C**” and additional control reactions and gently homogenize.
- 6) Add 20 µl well resuspended Streptavidin magnetic beads (SA-MB) to “**A**”, “**C**” and any additional control reactions. Gently mix the resulting suspensions.
- 7) Incubate for 30 min (at most 3 h) on a rotating wheel at 4 °C (or gently re-suspend every 5 min).



Note: SA-MB must stay in suspension.

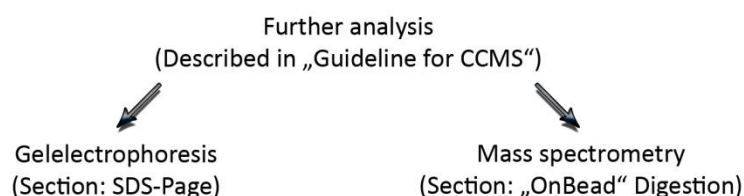
- 8) Collect SA-MB protein complexes (beads) from the reactions by using the caproMag™. Allow collection process to proceed for 2 min, until supernatant appears clear. Discard tubes containing supernatant. Add 200 µl WB1 in new PCR tubes and gently resuspend the beads.



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

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

The collected beads in step 9 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 “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 to irr.)	-	✓	-	✓	-	-	-	-	-	-
5x CB3	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
MMP-2*	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
Cell lysate	✓	✓	✓	✓	✓	-	✓	-	✓	-
Marimastat-CC	✓	✓	✓	✓	✓	-	-	-	-	-
Biotin	-	-	-	-	-	-	-	✓	-	✓
Irradiation	✓	✓	-	-	✓	-	-	-	✓	✓
Competitor (after irr.)	✓	-	-	-	-	-	-	-	-	-
5x WB1	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
SA-MB	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Incubation	✓	✓	✓	✓	✓	-	✓	✓	✓	✓
Wash	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

CB3 = capture buffer 3; Marimastat-CC = Marimastat Capture Compound™; WB1 = wash buffer 1; SA-MB = Streptavidin magnetic beads; *For the initial experiments, it is recommended to use a positive control (MMP-2) added 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 the Marimastat-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.
C2-MB	Control, examining CC-independent binding to the SA-MB	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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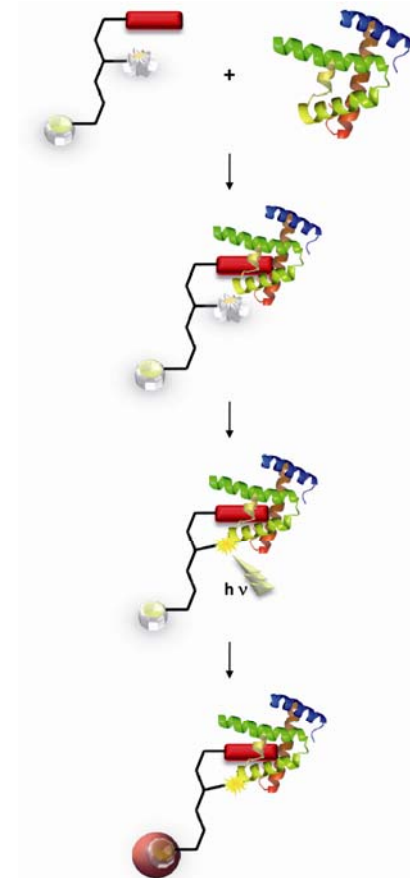
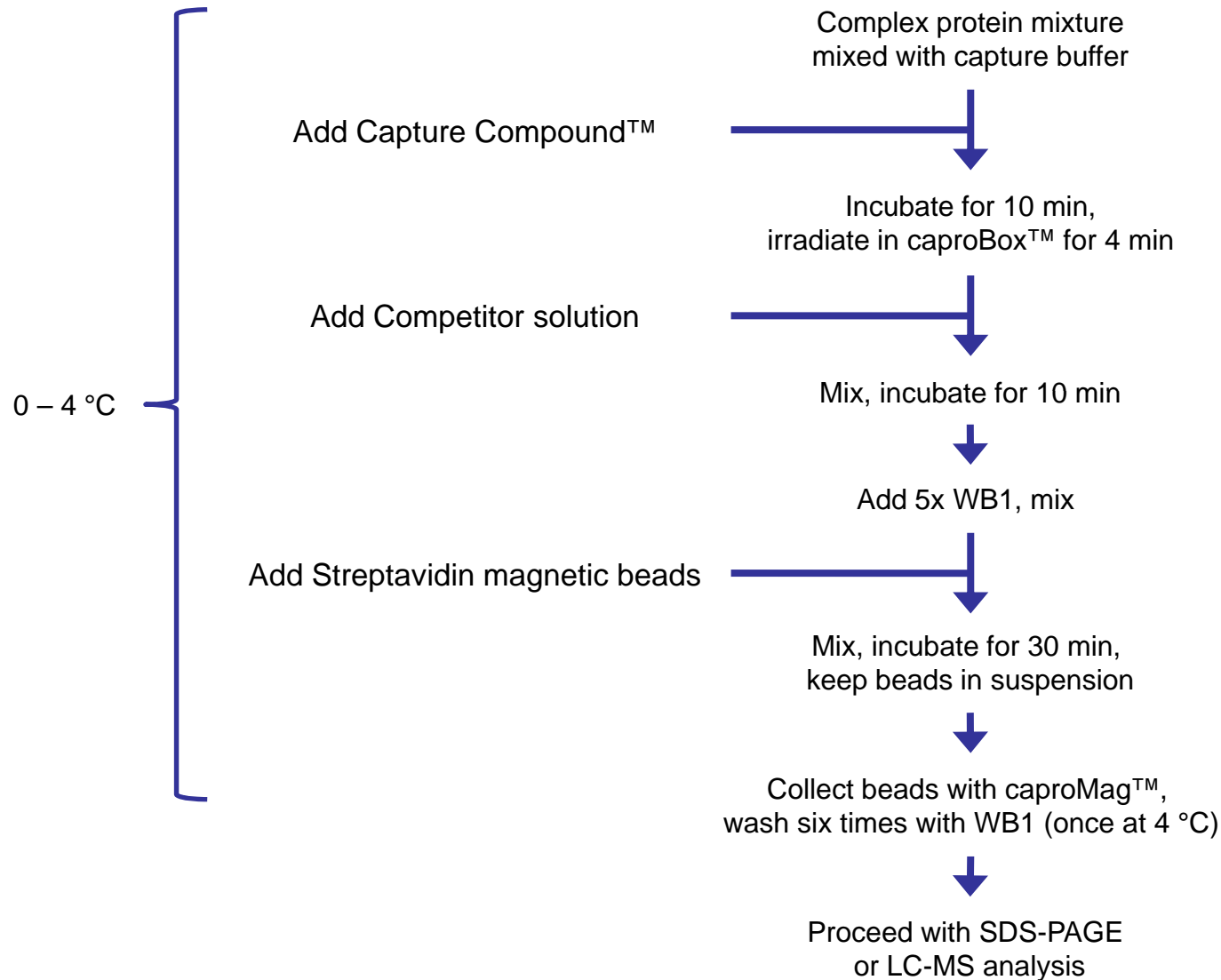
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“OffBead” Protocol for Capturing in Solution



Pipetting Scheme - OffBead

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				
Marimastat competitor	10 mM	0.5 mM	--	5 µl
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Marimastat-CC	20 µM	1 µM	5 µl	5 µl
Total volume			100 µl	100 µl

*For the initial experiments, it is recommended to use the **positive control** (e.g. MMP-2) in addition to the Capture Compound assay "A" and the Control "C".