**Reviewers' comments:**

**Reviewer #1:**

This JoVE manuscript from Urs Jenal's group is a timely description of a Capture Compound Mass Spectrometry (CCMS) method to identify proteins that bind to c-di-GMP. This is a very useful method for the field in helping to better understand these complex systems. I also think this method would be ideal for JoVE as it seems to involve a lot of steps, and it will be beneficial to actually see these performed. My only comment is that

1. The authors set up the rationale for this method as an approach to identify novel c-di-GMP binding domains. I was thus disappointed when it appears most (all?) of the domains belong to the **PilZ and DGC/PDE** **classes** that are easy to identify.
   * In the representative results described in this paper, only the known components of the c-di-GMP network are shown, as it indicates that the approach works. Apart from FleQ, all the known c-di-GMP related proteins of *Pseudomonas aeruginosa* contain GGDEF, EAL, HD-GYP or PilZ domains. All the novel putative effectors fished don’t harbor one of these domains and belong to new c-di-GMP effectors classes. They first have to be validated for binding and then will be published in a separate research article. Even though the names of the putative novel effectors are not mentioned in this article they are shown in figure 3 as un-circled dots.
2. For example, **FleQ** was not identified using this approach. This is not to say that I do not think the method is valuable, but I think the authors need to address this point. As c-di-GMP has been shown to bind as dimers and in both an extended and closed structure to proteins, I wonder if this CCMS method excludes or inhibits the formation of some of these forms. A discussion of how the tagging impacts c-di-GMP structure would be warranted.
   * Actually, we fished FleQ, but it was not specifically enriched in the capture experiment compared to the c-di-GMP competition experiment (64 spectral count versus 49 respectively), as there was an excess of ATP present. Recently it was shown that c-di-GMP competes with ATP to bind to FleQ (Baraquet and Harwood 2013: “Cyclic diguanosine monophosphate represses bacterial flagella synthesis by interacting with the Walker A motif of the enhancer-binding protein FleQ”; PNAS 110:18478). In the initially performed CCMS experiments, when no ATP was present, FleQ was fished specifically (28 spectral count for the capture experiment, 0 for the competition experiment; Nesper *et al* 2012). The disadvantage of this experimental setup was that we fished many unspecific ATP binders. This point is now addressed in the discussion part (p.10, line 412). We agree with the reviewer that this should be mentioned in the article and included the following text (page 10, line 419): “Although the addition of nucleotides improved the specificity, it may at the same time prevent the capture of proteins that bind different nucleotides at the same site. For example the effector FleQ, which was recently shown to bind ATP and c-di-GMP18 was fished specifically in the absence of ATP in our previous experiment1, but not anymore in the here presented data in the presence of an excess of ATP.”

**Reviewer #2:**

The manuscript by Laventie *et al*. presents a detailed description of the CCMS pulldown procedure applied for identification of c-di-GMP-binding proteins from cell extracts. The authors provide representative data for *Pseudomonas aeruginosa* showing that 74% of the predictable components of the c-di-GMP network in this bacterial species were captured using this technique.

Altogether, this manuscript describes a useful tool for enrichment of putative c-di-GMP binding proteins. The visualization of the CCMS-approach will be certainly very helpful for successful application of this method. I have only a few questions/suggestions as detailed below.

In agreement with our currently limited knowledge about the nature of c-di-GMP-sensors, the authors emphasize the need for an approach allowing the identification of in silico non-predictable c-di-GMP-effectors.

1. However, all c-di-GMP binding proteins enriched using the CCMS-method and shown in table 2 contain the well-defined **GGDEF-, EAL-, or PilZ-domains**. The authors do not mention whether they were indeed able to identify and verify a non-predictable novel c-di-GMP-binding protein using this method.
   * Compare also comment to reviewer 1, point 1. Only the known components of the c-di-GMP network are shown, as the new putative effectors will be published elsewhere.
   * To show that we indeed can identify and verify novel effectors, we included in the manuscript (new paragraph added in the representative results) 3 examples of CCMS hits with non-predictable novel c-di-GMP-binding domains. We included now the following text in the representative result part (page 9, line 355): “The other 63 soluble and 112 membrane proteins are new putative c-di-GMP binding proteins which do not contain one of the known c-di-GMP binding domains. These hits have now to be validated by testing their specific binding to c-di-GMP.

In a previous screen we fished GlyA2 (PA2444), GlyA3 (PA4602) and Gsp69 (PA1127)1. These 3 proteins were cloned, overexpressed, and purified from *E. coli* and could be validated to bind c-di-GMP in UV-cross linking experiments using 33P labelled c-di-GMP15. The *K*ds were determined to 1.0, 2.0 and 6.9 µM respectively, indicating that indeed novel effectors can be identified by using CCMS.”

1. Moreover, *Pseudomonas aeruginosa* encodes for a known c-di-GMP-binding transcription factor **FleQ**, belonging to the non-predictable class of c-di-GMP-responsive proteins (Hickman JW, Harwood CS, 2008). FleQ was successfully captured in the initial analysis using the CCMS-technique (Nesper *et al.,* 2012) but is not listed in the representative results in the manuscript by Laventie *et al*. Could the authors comment on that? Is that possible that changes in the protocol were rather disadvantageous?
   * Compare also comment to reviewer 1, point 2.With the protocol that we describe here, we actually fished FleQ, but it was never specifically enriched in the capture experiment compared to the c-di-GMP competition experiment, and therefore was below our threshold of selection. The recently published paper Baraquet and Harwood (2013) “Cyclic diguanosine monophosphate represses bacterial flagella synthesis by interacting with the Walker A motif of the enhancer-binding protein FleQ” in PNAS, the authors showed that c-di-GMP probably competes with ATP to bind FleQ.
   * In case of FleQ the addition of ATP was therefore rather disadvantageous, on the other hand less ATP binding proteins were fished, decreasing the list of proteins to test for specific binding.
   * We included the following text in this article (page 10, line 419): “Although the addition of nucleotides improved the specificity, it may at the same time prevent the capture of proteins that bind different nucleotides at the same site. For example the effector FleQ, which was recently shown to bind ATP and c-di-GMP18 was fished specifically in the absence of ATP in our previous experiment1, but not anymore in the here presented data in the presence of an excess of ATP.”
2. The protocol is written in a quite clear and understandable way but a **table** containing the required materials (including source for reproducibility) and buffer compositions would be very helpful for the user. It would be also useful to mention, that the capture buffer, the washing buffer and the magnetic beads are included in the c-di-GMP caproKit.
   * The table containing the required materials has been updated, and a new table 1 with the buffers compositions and the chemicals sources has been added. The “JoVE\_Materials” materials tables provide a list of specific materials required.
3. It might be more user-friendly to describe the treatment of the **soluble fraction and membrane fraction** **in** **two separate sections** since it is confusing to follow the different handling of the two samples.
   * The steps 2 and 6.1 are soluble fraction specific, and the steps 3 and 6.2 are membrane fraction specific. The intermediate steps 4 and 5 are common, and the step 7 almost identical. We chose not to separate the soluble fraction and membrane fraction in two sections in order to save space as the manuscript gets to long otherwise.
4. The **logic of highlighting of critical steps** is not very clear and not consistent e.g. headlines 1-7 are highlighted whereas 8-10 are not. Some notes are also highlighted as being critical, although they are not (e.g. note following 7.7 "the samples can be stored at -20°C at this stage").
   * Highlighted text corresponds to the sections that will be featured in the video, and is limited by JoVE to 2.75 pages of protocol text. As this protocol is 5.5 pages long, we chose to focus on the wet lab part that is more specific to this method and will probably benefit more from a video compared to the *in silico* analysis, although they have equal importance. Highlighting has now been slightly adjusted to re-focus on critical steps.
5. The authors discuss the major drawbacks of the CCMS method but do not mention whether pronounced **losses of the compound** are expected in the sample due to 1) the c-di-GMP degradation activity of EAL-domain containing phosphodiesterases in the cell lysate and 2) the hydrophobic nature (with affinity towards the cell membrane itself) of the scaffold tail of the capture compound.
   * The degradation of the c-di-GMP moiety of a fraction of the capture compound by phosphodiersterases may occur. This is one of the reasons why the procedure has to be carried out at 4°C and the buffer does not contain Mg, which PDEs need for activity, therefore limiting the phosphodiersterases activity. After cross linking, the captured protein cannot be released from the capture compound even if the c-di-GMP moiety is degraded (as they are covalently bound to the reactive moiety).
   * This point is now integrated in the discussion section (page 11, line 445) and reads: “This could be due to a poor access or a low affinity of the cdG-CC to the binding site, or to the degradation of the cdG-CC by EAL proteins.”
   * Regarding the hydrophobic nature of the scaffold tail of the capture compound, we do not expect problems for the soluble fraction as all the membranes and membrane associated components are removed by centrifugation at 100 000 *g*. For the membrane fraction there might be a loss, as it could in principle integrate into the mixed detergent/membrane micelles. On the other hand this seems not to happen under the experimental setup we are using, relatively high amounts of Capture compound are added, as we fish almost all of the known proteins.
6. In addition, it might be noteworthy that after the on-beads digest, Strep**tavidin-derived peptides** are present in a very high abundancy, thus possibly masking some potential peptide-spectra of desired proteins. For completion, the workflow **Figure 2** should also include the highly pronounced presence of Streptavidin peptides.
   * Indeed Streptavidin-derived peptides are highly abundance. We included that now in Figure 2. It might also mask some peptide-spectra of fished proteins. However we don’t think that this is a problem, as there are always several experiments performed and analyzed together and it is unlikely that always the same peptides are masked. But this may explain why some hits appear only in 2 or 3 of the performed experiments and not in all 4.

Specific comments on writing:

Introduction (end of 2nd sentence): it is more appropriate to **cite** ?Hengge, R. 2009. Principles of cyclic-di-GMP signaling. Nature Rev. Microbiol. 7:263-273" instead of Hengge, R. Cyclic-di-GMP reaches out into bacterial RNA world, see Reference no 2" in this context

* We replaced the citation.

Protocol 1.1: Could the authors mention which **culture volume** they used for e.g. log-phase and stat. phase culture to get the desired pellet size/total protein amount.

* Culture volume now integrated in the protocol section (1.1)

Protocol 1.4: Could the authors specify which **French press** cell they used to be able to French press the volume as little as 1 ml without losing the material.

* The French press cell model is now mentioned in the protocol section 1.4 (page3, line 96) (Miniature French Pressure Cell, suitable for volumes from 1 to 4 mL, Cat. N° FA-003, Thermo Electron Corporation)

Protocol 1.9: refer to step 3 at the end of the sentence

* We changed it.

Protocol 2: It is confusing that the authors first remove nucleotides using a PD10 column and then add nucleotides in step 5. Could the authors explain why the **removal of nucleotides** is necessary at all?

* The PD10 desalting column allows us to remove first all small molecules of the cell lysate including free c-di-GMP, which could compete with binding to the c-di-GMP capture compound. It is especially required when users are using this method with cells containing elevated c-di-GMP concentrations (*e.g.* engineered strains overexpression a diguanylate cyclase, or *Pseudomonas aeruginosa* small colony variants). By adding a selection of nucleotide the overall specificity of the capture reaction can then be improved.
* We changed the text in the protocol section 2 (page 3, line 110) to: “Removal of free c-di-GMP and other nucleotides (soluble fraction only)”

Protocol 3.1: the **composition of the capture buffer** should be mentioned here (or in separate table as suggested above) and not in the later step 5.1

* A table (Table 1) with the buffers compositions and the chemicals providers has been added.

Protocol 5.1: refer to table 1 at the end of 1st note and to step 7.1 at the end of 3rd note

* done

Protocol 5.3: refer to table 1 at the end of 1st note

* done

References: **References** no 2, 3, 4, 5, 9, and 10 do not specify the journal in which the cited work has been published.

* References were adapted.

**Reviewer #3:**

In this manuscript, the authors describes how to use a capture compound technology to identify candidate proteins that bind c-di-GMP. The manuscript is well-written and presented and the procedures easy to follow.

Comment

1. This type of technology could potentially be used with other selectivity determinants. Are there examples in the literature for other usages of the a capture compound technology?
   * Indeed there are examples of other applications of similar capture compounds. Caprotec, the company selling the c-di-GMP capture compound, also sells capture compound with other selectivity groups, such as GDP, S-adenosyl-(L)-homocysteine (SAH) or staurosporine. These past years, several studies using the CCMS technology were published; here is a selection:
     1. Luo, Y., Fischer, J.J., Baessler, O.Y., Schrey, A.K., Ungewiss, J., Glinski, M., Sefkow, M., Dreger, M., and Koester, H. (2010). **GDP-capture compound**--a novel tool for the profiling of GTPases in pro- and eukaryotes by capture compound mass spectrometry (CCMS). Journal of Proteomics 73, 815-819.
     2. Lenz, T., Poot, P., Weinhold, E., and Dreger, M. (2012). Profiling of methyltransferases and other **S-Adenosyl-L-homocysteine**-binding proteins by Capture Compound mass spectrometry. Methods Mol Biol 803, 97-125.
     3. Fischer, J.J., Graebner Baessler, O.Y., Dalhoff, C., Michaelis, S., Schrey, A.K., Ungewiss, J., Andrich, K., Jeske, D., Kroll, F., Glinski, M., et al. (2010). Comprehensive identification of **staurosporine**-binding kinases in the hepatocyte cell line HepG2 using Capture Compound Mass Spectrometry (CCMS). Journal of proteome research 9, 806-817.

**Reviewer #4:**

*Major Concerns:*

1. I am concerned about a relatively sloppy statistics pertaining to the assessment of performance of the c-di-GMP CCMS method. This information is necessary to researchers who want to choose a method of detecting c-di-GMP-binding proteins.
   * One on the major challenge with the CCMS data is to define a threshold to decide which candidates are real c-di-GMP receptors. In our experience, the selection of the best candidates depends on the specificity of the capture that can be expressed by the ratio of detected spectral counts between the capture and competition experiments. Known c-di-GMP binding proteins are always within this category and putative novel once that are already validated also belong to this class. We therefore think that this threshold should be adjusted according to the specificity and significance of the known c-di-GMP related proteins for each experiments and each species investigated
   * In addition, we performed a modified t-test (empirical Bayes method, reference 17) on the spectral counts to provide p-values that represents the enrichment significance; that we believe is a statistical strategy widely used in proteomic studies.
2. What exactly does "74% of the known or predicted c-di-GMP-binding proteins" mean?
   * In *Pseudomonas aeruginosa* PAO1 there are 51 proteins related to c-di-GMP. They are predicted diguanylate cycles (DGCs), phosphodiesterases (PDEs), PilZ proteins, plus FleQ that doesn’t belong to the previous categories. 38 of them were captured by CCMS under the different conditions tested. In principle it could be that the 13 missing ones are not expressed under the tested conditions or that they do not bind c-di-GMP at all (see also point 3). Meaning that **at least** 74 % of the proteins can be identified.
3. How many of these bind c-di-GMP (as verified by other techniques), and what is the basis of predictions for unverified proteins?
   * See also point 2. At least the 38 identified are expected to bind c-di-GMP.
   * The prediction was originally based on bioinformatics (apart for FleQ). Regarding the DGCs and PDEs, they can harbor conserved or degenerated motifs (GGDEF, EAL, HD-GYP), and can be enzymatically active or not. Some may only synthetize or degrade c-di-GMP without being c-di-GMP receptors, but this information is not available for most of them. At least 16 DGCs and PDEs were shown to be active enzymes *in vitro* or *in vivo*, without information about their capacity to bind c-di-GMP. The DGCs harboring an “I-site” (site that down regulates the DGC activity upon c-di-GMP binding) are likely to bind c-di-GMP (as a negative feedback control). Affinity values are only available for 5 of the 8 PilZ proteins (summarized in Christen 2010, Science) and FleQ (Baraquet 2013, PNAS).
4. Is the percentage of false positives detected by the c-di-GMP CCMS known?
   * We don’t know what the fraction of false positive is. We will learn it over time, but it is first necessary to validate several CCMS hits to be able to state if they are real c-di-GMP receptors or false positives.
5. Is the percentage of false negatives known, i.e., how many of the known c-di-GMP-binding proteins have been detected by MS but not detected by c-di-GMP CCMS?
   * To be able to calculate this percentage, it could be considered to create a proteome atlas of *Pseudomonas aeruginosa* prepared in the same conditions that the CCMS samples. We did not create such an atlas, and are thus unable to calculate the percentage of false negatives.

*Minor Concerns:*

Page 2 "Most of these compounds?": The accuracy of this sentence is questionable. Many c-di-GMP-binding proteins have emerged from genetic analysis. Delete.

* Sentence deleted.

Page 3; 2.1: What is PD10 column? Manufacturer?

* PD10 columns are desalting columns. We changed the text (page 3, line 112) to: “Wash a PD10 desalting column (GE Healthcare) with 10mL of cold lysis buffer”

Page 4, 5.3: Do you mean add cdG-CC to 10 µM, final concentration?

* To clarify this step, it is now mentioned that it correspond to 10µM final concentration (page 4, line 157), and we added a reference to the Table 2 which details the capture reaction mix.

Page 4, 5.5: Need details about UV light: flux (intensity); wavelength; distance from the source.

* Details added in the text (page 4, line 166) (λ = 310 nm, Irradiance ≥ 10 mW/cm², distance from the source = 2 cm).

Page 6, 7.19: Sonication parameters? Brief spin parameters?

* The sonication and centrifugation parameters have been added to the text (page 6, line 253) (“Sonicate 20 sec (pulse cycle 0.5, amplitude 100%, UIS250v sonicator with VialTweeter, Hielscher ultrasound technology), and spin down 5 sec, 12 000 rpm (benchtop centrifuge).

Page 8 last paragraph; Page 9 first paragraph; Table 2:

If Table 2 contains "at least four peptides identified in the four individual experiments" and proteins not detected with empty beads (i.e., "n/0" in four replicates), then why proteins having "0/0" are listed?

* We agree with the reviewer that the sentence was misleading. We changed the text in the manuscript (page 9, line 349) to: “Lastly, we considered only robust hits with at least four peptides identified in the four experiments for the 2 capture compound concentrations taken altogether”

Page 9 (last sentence of Representative Results): The statement "this technique is most effective and powerful" needs better statistics (see above) about performance of this method and its comparison to performance of other methods of identifying c-di-GMP-binding proteins.

* With this sentence, we were not intending to compare the efficiency with other techniques.. Beside, only the paper by Düvel *et al*. (2012) provides a comparable method of screening of c-di-GMP effectors. However as this paper only provides a list of selected hits (and not the raw MS data), it was not possible to accurately compare the 2 methods. As far as we can compare, for the same sample (log phase *Pseudomonas aeruginosa* culture, soluble fraction), Düvel *et al* co-immunoprecipitated 14 proteins that are known or predicted to bind c-di-GMP, while we captured 13.
* We corrected this sentence to moderate this statement (page 9, line 370) to: “This together with the observation that most of these components were captured with high specificity (Table 2) strongly argues that this technique is effective and powerful.”

Page 10 Discussion:

- Why "a concentration of 10 mg/mL being difficult to reach in some cases"?

* For liquid cultures it is relatively easy to reach proteins concentration of 10 to 15 mg/mL. But it can be more challenging to obtain enough biomass to reach a suitable amount of protein when growing cells under special conditions or using special strains (*e.g.* biofilms, small colony variants, or cells collected from infected mice).
* The text was adjusted and reads now (page 10, line 413): “The protein concentration is a critical parameter with a concentration of 10 mg/mL being difficult to reach when cells are grown under specific growth conditions (*e.g.* biofilms or small colony variants)”

- Why "various nucleotides [were added] to the capture reaction to minimize non-specific capturing of nucleotide binding proteins"? Shouldn't only non-labeled c-di-GMP be added? If this is not so, explain and provide details.

* The nucleotide mix (1mM GDP, GTP, ATP, CTP) were added in both the capture and the competition experiment, while the unmodified c-di-GMP was added only in the competition experiment. In our previous CCMS paper Nesper et al., 2012, we were not using this excess of free nucleotides, and noticed that we fished a large proportion of nucleotide binding proteins. Although some of these hit can be real c-di-GMP effectors, we feared to fish many aspecific proteins. As the c-di-GMP binding validation is a long and tedious procedure, we opted for this additional step that efficiently decrease the amount of (hopefully aspecific) nucleotide binding proteins fished. The downside is to loose c-di-GMP effectors such as FleQ, which possess a unique binding site for both ATP and c-di-GMP.
* The following text was added (page 10, line 419) (compare also Reviewer 1 and 2): “Although the addition of nucleotides improved the specificity, it may at the same time prevent the capture of proteins that bind different nucleotides at the same site. For example the effector FleQ, which was recently shown to bind ATP and c-di-GMP18 was fished specifically in the absence of ATP in our previous experiment1, but not anymore in the here presented data in the presence of an excess of ATP.”

- Provide details regarding light sensitivity: "cdG-CC should be carefully protected from light". What light environment is safe?

* Details provided in the discussion section and reads as follows (page 10, line 425): “Although ambient light contains only a small fraction of UV, it is recommended to keep the capture compound stock wrapped in aluminum foil, as well as the capture mix previous to the activation by UV irradiation.”

- Poor detection of EAL domain proteins is explained by "low affinity of cdG-CC for the active site of these proteins". Poor access to the binding site, or degradation of the cdG-CC by EAL domain phosphodiesterases seem more plausible.

* The text has been adjusted as suggested (page 11, line 445).