IEO
Istituto Europeo di Oncologia

Research Hospital and Treatment Centre (D. M. 18/1/96)
Via G. Ripamonti 435, 20141 Milan
Phone 02 57489.871 Fax 02 57489.851

Department of Experimental Oncology

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To the Editors of *Journal of Visualized Experiments*

Dear Editors,
I would like to thank you and the reviewers for the accurate assessment of our manuscript and the useful comments received. We have addressed all the criticisms raised and are hereby resubmitting a revised version of the protocol, which we hope you will find ready for publication in your journal.
Point-to-point answers to each Editorial comment and from reviewers' criticisms can be found below, highlighted in red.
Thank you very much for your continuous support.
Best regards

Tiziana Bonaldi, PhD

Head of the Systems Proteomics and Functional Genomics Group
Dept. of Experimental Oncology
European Institute of Oncology
Via Adamello 16- Milano
T: +39 02 94375123 F: +39 02 94375990 E: tiziana.bonaldi@ieo.eu
W: http://www.ieo.it/en/RESEARCH/People/Researchers/Bonaldi-Tiziana/

Point-to-point answers to editorial and reviewers' comments

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
R: Upon reviewing the manuscriprt extensively, we also carried out an extensive proofreading.

2. Please provide the complete addresses for all of the affiliations.
R: We have provided the addresses for all affiliations

3. Please provide an institutional email address for each author.
R: We have provided the email addresses of each author

4. Please include an Abstract (150-300 words). Ensure that the Abstract clearly states the goal of the protocol.
R: We have added an Abstract that includes a clear statement on the goal of the protocol

5. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
R: We have revised the text, avoiding the use of personal pronouns

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Falcon, Sep-Pak, Ultimate 3000-HPLC system, Dionex, Phenomenex Jupiter C12-RP HPLC column, Cell Signaling Technology, Empore, a Q Exactive, Hybrid Quadrupole-Orbitrap Mass 296 Spectrometer, EASY-nano LC, Thermo Fisher Scientific, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.
R: We acknowledge the criticism and we have removed commercial language and reference to commercial products

7. Line 80: Please use standard abbreviations for time units preceded by a numeral. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks
R: We have modified as suggested

8. Line 90-93: Please elaborate on protein quantification steps. Including a citation would suffice.
R: We have included a reference for protein quantification of protein content in total extract.

9. Line 117: Please convert speeds to centrifugal force (x g) instead of revolutions per minute (rpm).
R: We modified as requested, except when the thermomixer was used, for which we cannot convert rmp value into g (see point 2.4)

10. Please include a one-line space between each protocol step and highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
R: We have included the spacing and highlighted the parts thast should be included in the video protocol: when checked, their approximate length covered about 3 pages, as requested.

11. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
R: Done

Reviewer #2:

Manuscript Summary:
A useful, step by step procedure for the isolation of Arg-methylated peptides and their mass spectrometry analysis.

Major Concerns:
All my concerns are minor - see below.

Minor Concerns:
Line 21 - "Protein Arginine Methyltransferases (PRMTs) are the enzymes catalysing 22 R-methylation reaction by the deposition of one or two methyl groups to the nitrogen (N) atoms of 23 the guanidino group of the side chain of Arginine." It might be useful to say here that the dimethylation can be symmetrical or asymmetrical.
R: The reviewer is right and we have added this information in the text (line 47 of the revised manuscript)

Line 32 - "Mass Spectrometry (MS) is recognised as the elective technology.." Use of 'elective' here is a bit strange. Suggest to replace with 'best'. Same for the Abstract.
R: Done

Line 78 - "… addition of protease inhibitors is important.". It would be handy for table 1 to say which protease inhibitor and phosphatase inhibitor should be used. The details are in the reagents table but it would make sense for the reagent to be stated here too.
R: Indeed, we initially thought sufficient adding these details in the Reagents Table; however, we acknowledge the request of reviewer #2 and have included the names of the protease inhibitors in Table 1.

Step 1.3 - presumably the sample is to be put on ice between sonication steps?
R: Indeed not, because the sample is in highly concentrated Urea, which precipitates at ice-temperature. We realised that this was not clear and specifically added a note about this aspect

Step 1.4. What temperature for the spin?
R: The temperature information (RT) was already included at step 1.4, we have double check it.

Step 2.3 mentions saving an aliquot for SDS-PAGE. This is a good idea. One other thing which is worth checking before proceeding to subsequent steps is the efficiency of heavy methyl-SILAC labelling and that the 1:1 mixing is correct. I would recommend doing that on a slice of a gel which is known to contain a high abundance and heavily Arg-methylated protein (e.g. fibrillarin). If labelling has been successful and 1:1 mixing has been achieved, there should be a 1:1 ratio of light and heavy versions of Arg-methylated peptides present.
R: We thank the reviewer for the suggestion; we have added information/comments both about the incorporation test and the assessment of proper 1:1 H/L mixing, through ad hoc NOTES (after point 1.1 and 1.2 of the revised version)

Steps 6.5 and 6.6. I might have missed something but is each fraction 1ml? It might be useful to say so, explicitly, if so.
R: We added this information

Step 9 - it might be worth while adding a note to explain why this second digestion step is necessary; it's a bit unusual.
R: We agree with the reviewer. We have added the explanantion for the second tryptic digestion and also included a reference for a study when this additional step was also included (see NOTE after point 9.5)

Step 11 - This looks like a CID-based mass spectrometry approach. ETD has been shown to be more successful that CID, by many groups, for detection of arginine methylation. Could this also be, or alternatively be used here? This might be a useful discussion point.
R: We thank the reviewer for this point. We have added a paragraph in the discussion, where we comment on the possible advantages of alternative peptide fragmentation strategies (such as ETD) for PTM detection and site-specific annotation.

Text in Figure 1 says "Mix 1:1, Protein Extraction, Trypsin+LysargiNase digestion". This implies digestion with both enzymes at once. Should be changed to let the reader know that these are being done in parallel but not as a double digestion in one tube?
R: This is a well-taken point; we have modified the related sentences in order to better deliver the concept of parallel digestions

Line 390 - commentary on Figure 4. It might be useful to comment on the relative peak heights one might

expect in a true positive result. This will of course depend on stoichiometry of the methylation but it might be good to fully explain the result and considerations for interpretation.
R: We discuss more extensively the aspect of the relative intensities of the Light and Heavy methyl-peptide peaks in the representative results section (please, see also the response to the point below). We cannot, however, provide comments about the intensity of the unmodified peak counterpart for each methylated one, because: 1) as anticipated by the reviewer himself, it depends on the stoichiometry of the individual methylation and 2) upon methyl-peptide affinity enirched the unmodified peptides are typically depleted in this workflow, so that they are either undetectable or strongly diminished so that their intensities do not reflect their *true in vivo* abundance: hence a stoichimetry calulation in this setting would be biased/skewed.

Line 441 - "Moreover, the intensity ratio between the putative 442 light and heavy methyl-peaks significantly differing from 1, which is not coherent with a 1:1 mixing of the 443 Met0 and Met4 channels and further confirms that this methyl-peptide is not enzymatically deposed." Yes but the light and heavy peaks in Fig 4 (true positive) are not 1:1 either. So this highlights the issue of (i) whether labelling has been done to 100% efficiency and (ii) whether 1:1 mixing was successfully achieved between the light and heavy samples. How is this controlled?
R: This interesting point is linked to the previous one. hmSEEKER classifies as positives those doublets that disaplay a log2 H/L ratio value within a “HL ratio tolerance interval” which was estimated to be ±1 in *Massignani et al., Proteomics 2019*. This is better explained in the Representative Results section. Also, we have also included a brief description of the strategy for the Met-4 incorporation test in the NOTE linked to Points 1.1 and 1.2 and in Figure 2, where we have included a panel displaying the distribution of the H/L ratios for peptides that contain Methionines, from which we derived the HL ration tolerance that is used as criterion to discern false positive, together with the delta mass tolerance and the RT time window. We hope this is clearer now.

Line 484 - "We applied a double proteolytic digestion of the total protein extract with Trypsin and LysargiNase". This suggests both enzymes were used in the same tube, which was not the strategy. Might be better to say something like "We used two separate digestion approaches, with Trypsin and LysargiNase, to generate peptides from the total protein extract."
R: We have modified the text according to the reviewer’s suggestion.

Line 486 - Discussion
The commentary here is useful but would be improved by a thorough discussion of possible issues that may arise in the protocol. A good discussion of possible false positive methylation assignments, and how they arise, would be useful. At least this should alert the reader to the issue associated with methionine-containing peptides that will also be heavy-labelled in any methyl-SILAC approach.
The discussion would also benefit from a thorough commentary on known or potential limitations of the approach.
R: We have extensively reorganised the Discussion paragraph in order to better examine current limitations and possible improvement of the protocol, which cover challenges and restraints of the biochemical protocol, existing potential biases of the MS analysis and source of false positives annotations.

Reviewer #3:
Manuscript Summary:
While a lot is known about the role of other PTMs, in particular phosphorylation but also lysine methylation, the study of arginine methylation has been lagging behind due to the lack of tools. In this manuscript, the authors describe a full experimental pipeline to identify bona fide arginine methylation sites with peptide mass spectrometry, from cell culture to sample prep to data analysis. The provided protocol is detailed and including safe stopping points given and useful experimental notes. It should be easy to follow, making it potentially very useful for the researchers in the field.

Major Concerns:
The authors provide a good general introduction to the field, however some of the details could be explained in more depth, either in the introduction or in the description of the experimental procedures.

Some questions to consider here are:

Why double digestion?
R: This is an interesting point also asked by Reviewer #2; we have better argued the choice of the double parallel Trypsin and LysArgiNase digestion

Why concatenating fractions?
R: In order to address this point, which was requested by Reviews #2, we have explained better the strategy of non-contiguous fraction concatenation upon high pH RP chromatography

What is the purpose second digestion step?
R: As also replied to reviewer #2, we have introduced in a NOTE (at point 9.5) where be better explain the rationale of the second Tryptic digestion step

A concise introduction to hmSEEKER, what is the purpose of it and brief explanation of the algorithm should be moved to introduction.
R: We have included a brief introduction to hmSEEKER at the end of the Introduction paragraph. We thank the reviewer for this suggestion.

Whilst the aim of this manuscript is clearly to provide a step-by-step tutorial, adding some theoretical background and the broad rationales behind the protocol's steps is necessary for the potential users to be able to decide whether this pipeline or some parts of it are suitable for their particular applications. Such material is currently mostly missing from the introduction and experimental procedures. The authors do provide some explanations (e.g., the of the two enzymes for digestion or the rationale for pooling fractions after the HpH-RP) towards the end of the manuscript in the Discussion. This is rather unusual and makes it harder to follow the procedure steps described before. Providing at least cursory explanation, either in the introduction or within the relevant steps, would be very helpful in making the protocol more accessible.
R: We agree with the reviewer and we have added a more extended explanation and critical discussion of strategic points/steps within the protocol text, as NOTES; correspondingly, we have reduced the corresponding part in the Discussion.

In addition, it would be useful to know what level of enrichment of methylated peptides is expected after the preparation and how does it compare to other methods, such as widely used IP with pan-methyl antibodies?
R: We have included a table in Figure 3B which summarises the numbers of mono- and di-methylated peptides identified in the input and in corresponding anti-pan-R-methy- IPs, from one exemplificative experiment carried out in the lab, which allow extrapolating information about enrichment and efficiency of the methyl-peptide affinity kits. We have also added a comment in the Discussion (around line 605) on the different efficiency of the anti-MMA, -ADMA and -SDMA antibodies with the consequent bias in the global MS analysis of protein-R-methylation that is linked to this.

Specific comments:

Generally, it was rather hard to figure out what fractions are pooled together and when. Since this is a critical issue, the authors should strive to make it clearer throughout the description of the experimental procedure.
R: We agree and we have added in the protocol a description on the strategy for non-contiguous concatenation, that- together with the schematic outline of the fractions pooled, should make easier to understand the rationale and logic behind this (please, see point 6.12 and lines 592-599 in the Discussion)

Adding time estimates for the experimental steps would be very useful for the readers to plan the experiments
R: Good point: we have added time estimation to complete crucial parts of the experiments.

Minor Concerns:
HpH-RP should be explained when it is used for the first time: High pH Reverse Phase separation technique (HpHRP)
R: Correct. Done

The required starting material in terms of the cell numbers is well described. However, it would have been useful to know on which cell line are these calculations based, as depending on the cell type the total protein and the arginine methylation levels can differ by an order of magnitude.

R: We have added the specification on the cell line used as reference in this protocol.

Are other reducing (TCEP) and alkylating agents (chloroacetamide) compatible?
R: We have added specific comments on these two aspects, with linked references, in ad hoc NOTE, after point 2.1 (for TCEP) and after point 2.2 (for Chloroacetamide). We thank the reviewer for this suggestion.

Points 6.5-6.10: What is the volume of the fractions?
R: We have added the missing information.

Point 7.9: Is the antibody resin added to the peptides in this step? The way it is written is ambiguous and suggest that the beads suspension is simply divided into 16 parts. Something like (…) and finally divide them equally between the 16 fractions would be clearer. Also, are the ADMA and SDMA beads pooled together at this stage? Not clear. And if so, what would be the rationale behind this step?
R: Indeed, not: the two anti-ADMA and anti-SDMA IPs are carried out in parallel, as explained at point 7.7. of the revised draft and further explained in the Discussion, when we debate the different efficiency of the antibodies/kits. We hope that this is not better explained and clearer.

Point 7.11: Probably "after the incubation" would be clearer.
R: Done