One-step multiplex toolkit for efficient generation of conditional gene silencing human cell lines

Tsz Kwan YEUNG, Ho Wai LAU, Hoi Tang Ma, and Randy Poon

Corresponding author(s): Hoi Tang Ma, Hong Kong University of Science and Technology

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|-------------------------------------|------------|
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Editor-in-Chief: Matthew Welch

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
Dear Dr. Ma,

Your manuscript has been reviewed by two experts. They both recommend that the method you developed can be of value to the community, only if the plasmids are readily available and a clear step-by-step protocol is provided. They also raised a number of specific technical concerns that should be addressed. If you are able to satisfy these requests, we would be happy to consider a revised manuscript. Thank you for your interest in publishing with MBoC.

Best regards,
Rong Li
Monitoring Editor
Molecular Biology of the Cell

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Dear Dr. Ma,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor’s decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your
Reviewer #1 (Remarks to the Author):

In this manuscript, Yeung et al. described a series of new plasmids used for a one-step generation of tight conditional cell lines. The auxin-inducible degron (AID) system is a popular protein-knockdown methodology by which AID-fused proteins can be degraded in a short time after the addition of auxin. The authors previously published a methodology paper in which they combined the tetracycline-controlled TRE promoter and AID for tight control of AID-fused proteins (Ng et al. Cell Cycle, 2019). In the previous paper, they introduced a transgene and subsequently inactivated the endogenous gene through multiple processes of transfection and colony isolation. The current manuscript shows a series of new plasmids containing multiple components required for the TRE-AID control of transgenes and developed a protocol for gene knockout and introduction of a transgene by transfecting multiple plasmids at once. The authors also showed that two genes (CDK1 and CDK2) could be inactivated and complemented by the AID-fused transgenes controlled by the TRE-AID system.

The concept of TRE-AID for the tight expression control has been already published by the authors and others (Ng et al. Cell Cycle, 2019; Tanaka et al. Yeast, 2015). It is also true that other groups have also published the concept of one-step gene inactivation and complementation by an AID-fused transgene (Nishimura et al. Chromosome Res, 2017; Nishimura et al. NAR, 2020; Yesbolatova et al. Pharmaceuticals, 2020). Therefore, the described idea itself is not entirely novel.

Even though the concepts are not novel, the plasmids and protocols are potentially useful for the community of cell biology. For the evaluation of this paper, the following points should be clarified and addressed.

1. They showed data using a population of cells after drug selection (Fig 2A, 2B, 3A, 3B, 4A, 5A, S1A, S1B) and an isolated clone (Fig 3E, 4C, 5C). Why did the authors not use data using isolated clones mainly? Taking the fact that the expression level of an AID-fused protein level in the population is variable, it is presumably recommended to isolate a clone showing an appropriate expression level for evaluation and characterization (Fig 2B, 3A, 3B, S1A, and S1B).
2. Fig 2A lane 2: Why you did not detect the endogenous STG1 and saw a high expression level of mAID-STG1 and TIR1 even without any drug selection?

3. Fig 2: It is unclear if the mAID-STG1 protein shown is functional or not because this protein is not essential for cell viability. Can you see any phenotype after depletion of the fusion protein?

4. Fig 2 and 3: An advantage of using AID is rapid depletion. It is crucial to evaluate the depletion kinetics in the presence of Dox, IAA, or DI in a time course assay. For this experiment, it is also essential to use a clone showing a similar expression level of the transgene to that of the endogenous gene. I also suggest that the expression levels should be quantified and presented in a graph with statistical analyses.

5. Fig 3C: The most critical information in this paper is experimental protocol. However, it is unclear how many milligrams of the four plasmids were transfected and how many cells were used. The experimental conditions should be described more in details. It is the same about the selection using multiple drugs. In some cases, the authors used two drugs throughout the selection process. In other cases, they used consecutive selections. Do you have standard protocols of antibiotic selection using multiple drugs? If so, those should be described.

6. Similarly, it is unclear about the availability of the described plasmids. Ideally, all plasmids should be deposited to a bioresource center such as Addgene. The availability of the materials should be addressed.

7. Not only about about the advantages, but limitations of the described methodology should also be addressed.

Reviewer #2 (Remarks to the Author):

This manuscript is more of a methodological paper than a research paper. The authors report a useful technique that could be widely used by everybody who want to study loss-of-function of genes in mammalian cell lines. Therefore, this information deserves to be distributed widely in the community.

The manuscript describes in simple way, how the authors developed the system and evaluates the different steps before ending up with the final "product". The fact that they use a single plasmid is remarkable and there also very useful.

The authors need address the following issues:
1. There are a few references that could be improved...for example on page 6 for CDK1 (Fung and Poon 2005) and on page 7 for CDK2 (Woo and Poon 2003). There are better or additional references that represent these genes more appropriately.
2. The final sentence on page 10 is incomplete "Further improvements of the AID system, such as the use of a TIR1 inhibitor (8) or the use of AID2 (TIR1(F74G) mutant and a ligand 5-Ph-IAA) (18)." Probably the authors should expand this discussion and also add potential limitations of their approach.
3. In figure 3D, in the CDK1 WB panel, there is a band that runs slightly faster than AID-CDK1. Do the authors know where this band is coming from and why it cross reacts with this band? One can note that this band is not visible in the HeLa control lane...
4. In Figure 4C, in the CDK2 WB panel, AID-CDK2 is staining only weakly. Is this specific to this clone (of the cell line) or are there other reasons why the expression of AID-CDK2 is low compared to CDK2?
5. Although the "Materials and Methods" are generally ok, I wonder whether the authors should not include a step-by-step protocol for generating a knockout cell line. This would be very useful for everybody who wants to use this technique.
Dear Editor,

We are submitting the revised version of the manuscript “One-step multiplex toolkit for efficient generation of conditional gene silencing human cell lines” (manuscript# E21-02-0051) for your consideration for publication in *Molecular Biology of the Cell*.

We thank the positive comments from you and the reviewers, and have revised the manuscript accordingly. Attached below is a point-by-point address of the Editor’s and the reviewers’ comments. We hope that the revisions have addressed the points raised by the reviewers, and the manuscript is now acceptable for publication in *Molecular Biology of the Cell*.

Please do not hesitate to contact me if you require further information or clarification.

Yours sincerely,

Hoi Tang Ma
**Reviewer #1:**

In this manuscript, Yeung et al. described a series of new plasmids used for a one-step generation of tight conditional cell lines. The auxin-inducible degron (AID) system is a popular protein-knockdown methodology by which AID-fused proteins can be degraded in a short time after the addition of auxin. The authors previously published a methodology paper in which they combined the tetracycline-controlled TRE promoter and AID for tight control of AID-fused proteins (Ng et al. Cell Cycle, 2019). In the previous paper, they introduced a transgene and subsequently inactivated the endogenous gene through multiple processes of transfection and colony isolation. The current manuscript shows a series of new plasmids containing multiple components required for the TRE-AID control of transgenes and developed a protocol for gene knockout and introduction of a transgene by transfecting multiple plasmids at once. The authors also showed that two genes (CDK1 and CDK2) could be inactivated and complemented by the AID-fused transgenes controlled by the TRE-AID system.

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As suggested by the reviewer, we have now included a new panel showing degradation of mAID-STG1 in the presence of Dox and/or IAA. The signals on the Western blots were quantified using serially diluted standard curves. As shown before (Ng et al., 2019), this experiment also showed that the depletion of mAID-tagged proteins was more efficient in the presence of both Dox and IAA compare to the individual chemicals alone.
5. Fig 3C: The most critical information in this paper is experimental protocol. However, it is unclear how many milligrams of the four plasmids were transfected and how many cells were used. The experimental conditions should be described more in details. It is the same about the selection using multiple drugs. In some cases, the authors used two drugs throughout the selection process. In other cases, they used consecutive selections. Do you have standard protocols of antibiotic selection using multiple drugs? If so, those should be described.

- This is an excellent suggestion and we have now included a short protocol in the Supplemental information.

6. Similarly, it is unclear about the availability of the described plasmids. Ideally, all plasmids should be deposited to a bioresource center such as Addgene. The availability of the materials should be addressed.

- We have planned to deposit the vectors to Addgene. This is now stated in the text.

7. Not only about bout the advantages, but limitations of the described methodology should also be addressed.

- As suggested by the reviewer, we have now included a discussion on limitations of the system (including the lack of endogenous promoter).

Reviewer #2:
This manuscript is more of a methodological paper than a research paper. The authors report a useful technique that could be widely used by everybody who want to study loss-of-function of genes in mammalian cell lines. Therefore, this information deserves to be distributed widely in the community.

The manuscript describes in simple way, how the authors developed the system and evaluates the different steps before ending up with the final "product". The fact that they use a single plasmid is remarkable and there also very useful.

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The authors need address the following issues:
1. There are a few references that could be improved...for example on page 6 for CDK1 (Fung and Poon 2005) and on page 7 for CDK2 (Woo and Poon 2003). There are better or additional references that represent these genes more appropriately.

- As suggested by the reviewer, we have replaced the review articles with more recent reviews.

2. The final sentence on page 10 is incomplete “Further improvements of the AID system, such as the use of a TIR1 inhibitor (8) or the use of AID2 (TIR1(F74G) mutant and a ligand 5-Ph-IAA) (18).” Probably the authors should expand this discussion and also add potential limitations of their approach.

- We thank the reviewer’s comment and have now amended the sentence. We have also included a discussion on limitations of the system in the Discussion.

3. In figure 3D, in the CDK1 WB panel, there is a band that runs slightly faster than AID-CDK1. Do the authors know where this band is coming from and why it cross reacts with this band? One can note that this band is not visible in the HeLa control lane...

- The band is present in all AID-CDK1 samples (as long as the exposure is strong). Importantly, it also disappeared after the addition of Dox and IAA (e.g., Fig 3A, C), indicating that it is likely to a truncated product (either protein degradation or internal stop).

4. In Figure 4C, in the CDK2 WB panel, AID-CDK2 is staining only weakly. Is this specific to this clone (of the cell line) or are there other reasons why the expression of AID-CDK2 is low compared to CDK2?

- We agree that the AID-CDK2 in this clone is weaker than the endogenous. This is only specific for this clone as other clones do have AID-CDK2 expressed at levels higher than the endogenous CDK2 (see Fig 4B).

5. Although the "Materials and Methods" are generally ok, I wonder whether the authors should not include a step-by-step protocol for generating a knockout cell line. This would be very useful for everybody who wants to use this technique.

- We agree with the reviewer and has now included a short protocol in the supplemental information. In addition, we will deposit the vectors to Addgene once the paper is in press.
Dear Dr. Ma,

Thank you for submitting your revised manuscript "One-step multiplex toolkit for efficient generation of conditional gene silencing human cell lines". I am happy to let you know that the revision is satisfactory and the manuscript is now acceptable for publication. As a suggestion for future submission of revised manuscripts, it is always helpful if you could indicate the figure or main text locations at which you additional data or discussion have been included. You did that for some of the additions but not for all of them.

Congratulations and many thanks for publishing your work with MBoC.

Best regards,
Rong Li, PhD
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Ma:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

Within approximately four weeks you will receive a PDF page proof of your article.

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