Reviewer #1:

Manuscript Summary:

In this manuscript, the authors reported a detailed protocol for using protoplasts to study protein import into chloroplasts, which is very helpful for investigating the protein import process in vivo.

The manuscript is suitable for publication in JoVE, and some points I am concerned are listed below.

Major Concerns:

To better adapt to the title "A simple and efficient method for studying protein import into chloroplasts using protoplasts", the protocol of purifying the chloroplasts from protoplasts could be included in this manuscript, which looks like a protocol of how to make protoplasts and gene expression in protoplasts right now.

A : We modified the title.

Minor Concerns:

1. In 1.2, what is the expiration date of the medium plates?

A : Medium plates are usually used up within one month. We recommend to use medium plates within 2 months before they dry out.

2. In 3.1.2, the volume of the deionized water should be pointed, although everybody knows, because 21 g of sucrose has been pointed clearly here.

A : Corrected.

3. In 3.2, some people would like cutting the leaf tissues into very small pieces. Is that required and helpful for the digestion.

A : It would be helpful for the digestion by expanding contact with enzyme solutions but at the same time can have negative effects for the protoplast condition due to more damage and stress

4. In 3.3, the centrifuge time is required.

A : Corrected

5. In 3.4, again, the centrifuge time is required.

A : Corrected

I had a hard time to understand the sentence "carefully transfer protoplasts in the top fraction (enzyme solution) and at the interface between the enzyme solution and sucrose solution to a 50 mL conical tube ".

A : We modified the expression (the top fraction 🡪 the most upper layer)

6. In 4, "Do not invert the tube". Why?

A : The sentence is unnecessary. It is removed

7. In 4.3, please check the dimension of 300 mL.

A : Corrected (300 ml 🡪 300 µl)

Instead of the proper amount of plasmid, could you show the range or an example?

A : Corrected

8. In 4.4, similarly, please check the dimension of 300 mL.

A : Corrected (300 ml 🡪 300 µl)