Peer Review File

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Reviewer comments

Comments 1:

Among answers/response to comments by this referee, the Replies 4 to 6 and 8 to 9 were unreliable and the Reply 6 was definitely fabric.

Original comment was the following;

“In immunoblotting analysis, authors used acrylamide gel of 10%. However, mTOR which is a protein of 290 to 300kd could not be detected in such concentrated gel.”

Then, authors replied as

“Reply 6: In our western blotting analysis, 8 or 15% SDS-PAGE were used to separate proteins according to their molecular weight. Protein with higher molecular weight was separated with lower concentration gel and vice versa. The mTOR was separated by 8% SDS-PAGE in our study.”

However, anyone who experienced immunoblotting for mTOR knows it cannot be resolved by 8% gel, but possible only by 3-5% gel. Indeed, it had been described in the previous literature:

- Cancer 2009;115:107–118
- Mod Pathol. 2009 Oct;22(10):1328-40, etc.

Furthermore, the answer for the request which was “all gels were cropped, but whole gel should be presented in Supplementary Figure” was the following;

“Reply 9: --- Because of the severe epidemic in Shanghai, our laboratory was temporarily closed. I have no access to the relevant original data saved in lab computer, which included the whole gel images. Cropped version of original images has been uploaded on Supplemental Files. And the molecular weight markers were illustrated in the figures.”

This is non-excusable!! Authors apparently tried to hide the fact and attribute it to

Corona!! All images of Gels in Supplemental Files were those of cropped ones. Scientists are supposed to be holding indispensable data with themselves, and the image of whole gel is a typical example.

The manuscript should be re-submitted after the Lock-down in Shanghai.

Reply 1:

When our group tried to complete the western blotting of mTOR at the very beginning, we referred to some literatures (see the detailed information later), which mentioned the use of 7.5% SDS-PAGE gels. Our laboratory had 8% gels available, so we used 8% SDS-Page gels. Our experiment yielded the expected results, indicating the trend of the hypothesis. Then, we wanted to reduce the time of electrophoresis and separate the macromolecule proteins more thoroughly. So in the subsequent formal experiment, we changed to 5% gels for the experiment. The original picture is attached. At the beginning, 8% gels were used in the experiment record, so when we wrote the article and submitted it, we wrote 8% gels. We have revised the manuscript. We also thank the reviewers for their professionalism, rigor and carefulness. We will be more rigorous and objective in future scientific research work.

We uploaded the original pictures of WB when we re-submitted this time. We hope to help reviewers and editors confirm the credibility of our conclusions.

Several previous literatures which described the application of 7.5% gels to complete Western blotting of mTOR are listed below[1-3]

1. Gels with a higher acrylamide concentration (e.g.20%) impede the movement of larger proteins to a greater degree than those of a smaller molecular weight but better resolve those of lower molecular weights (e.g.4EBP1 ~ 20kDa) (Chrambach &Rodbard 1971). Similarly, if the desired target is a large protein (e.g.mTOR ~289 KDa) a lower concentration gel (e.g. 7.5%) may be required for optimal resolution. Alternatively gradient gels (e.g. 4–12%) provide

uniform resolution across the molecular weight spectrum (Rath et al. 2013). Other gel types such as agarose may be used, but are less common as they are predominantly used for very large molecular weight proteins (e.g. titin isoforms 700–4200 kDa) giving superior separation when compared to polyacrylamide gels (Warren et al.2003).

2. Cultured cells were harvested and lysed in lysis buffer (25 mM TrisHCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, leupeptin, 1 mM Na3VO4, and 1 mM PMSF) for 30 min. Lysates were centrifuged at 10 000 rpm for 5 min at 4 1C. Each protein sample (10 mg) was mixed with 5 sample buffer containing 10% mercaptoethanol and boiled for 5 min. The total cellular protein extracts were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) on 7.5% gels for the examination of mTOR and p-mTOR, and on 12.5% gels for the examination of p70S6K, p-p70S6K, 4E-BP1, p-4E-BP1, and b-actin.

3. Briefly, 20-µg of protein was loaded into each lane, separated electrophoretically by SDS–PAGE using 7.5% Tris–HCl gels, and electroblotted onto nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked with 5% non-fat dry milk in TBS-T buffer (0.1% Tween-20 in Tris-buffered saline) for 1 h to reduce the non-specific binding.

1. Bass, J.J., et al., *An overview of technical considerations for Western blotting applications to physiological research.* Scand J Med Sci Sports, 2017. **27**(1): p. 4-25.
2. Hirashima, K., et al., *Aberrant activation of the mTOR pathway and anti-tumour effect of everolimus on oesophageal squamous cell carcinoma.* Br J Cancer, 2012. **106**(5): p. 876-82.
3. Yaba, A. and N. Demir, *The mechanism of mTOR (mammalian target of rapamycin) in a mouse model of polycystic ovary syndrome (PCOS).* J Ovarian Res, 2012. **5**(1): p. 38.