Test for Nippon Health Innovations Europe
IE NEO – Western Blot Application Protocol

Introduction

Western blotting is a simple and powerful approach to investigate the presence and relative abundance of proteins, as well as the presence of post translational modifications (PTMs). This method, first described by Towbin *et al.*[1] and Burnette[2], relies on the specific interactions of antibodies with target antigens in the sample mixture[3]. In our experiments, we look at one of the many PTMs that have been identified, i.e., the acetylation of α-tubulin[4]. As our compounds are designed to inhibit one of the proteins that deacetylates α-tubulin, we expect to see an increase in the acetylation status. However, we find that the difference between the various band intensities is indiscernible, and quantification is a necessity. Fortunately, Nippon's IE NEO enhancing agent promises to address this problem, in addition to some other problems like long incubation times and durability. During this experiment, we will test the IE NEO using our experiment set up, mostly looking at a potential increase in resolution while taking note of ease of use and durability.

Materials & methods

HeLa cells were cultured in DMEM (Gibco, 41966-029) supplemented with 15% FBS (Gibco, A3160801), and 1% Pen/Strep (Gibco, 15140-122) at 37°C, in a humidified atmosphere of 5% CO2.

HeLa cells ($5x10^5$ cells/2mL/well) were treated with compounds (Table 1) at a concentration of 10µM for 6h[5]. Hereafter, the cells were placed on ice before adding 1x sample buffer (300µL) and detaching with a cell scraper. The cell lysates were transferred to a tube and stored at -20° for later use. The protein lysates were boiled for 10min at 95°C before analysing by SDS-PAGE (±20min at 60V followed by ±1.5h at 120V) and Western blotting (70min at 100V). Probing was carried out using two different methods. Antibodies and dilutions used can be found in table 2.

Table 1 List of conditions used in this experiment. This table contains an overview of the compounds/conditions that the cells were exposed to. For simplification purposes, every condition has gotten a code that will be used in further mentioning. CC = control compound, TC = test compound.

Compound/condition	Code	Stock concentration
Negative control	NC	-
DMSO 0.1%	DMSO	100%
Positive control compound	CC1	50mM
Negative control compound	CC2	50mM
Negative control compound	CC3	50mM
Active inhibitor 1	TC1	50mM
Active inhibitor 2	TC2	50mM
Active inhibitor 3	TC3	50mM

1. **Traditional method:**
   The blot membrane was blocked (1h, RT) with 5% BSA in TBS with 0.1% Tween (TBS-T), followed by overnight incubation (4°C) with the primary antibody in blocking buffer. Hereafter, the membrane was washed (3x 10min.) with TBS-T followed by incubation with the secondary antibody in blocking buffer (1h, RT). Finally, the membrane was washed (3x 10min.) with TBS-T.

2. **IE NEO method:**
   The blot membrane was immersed with TBS-T for 4min, followed by incubation with both primary and secondary antibody in 3mL IE NEO for 1.5h. Hereafter, the membrane was washed (3x 10min.) with TBS-T.

Table 2 List of antibodies used in this experiment. This table contains an overview of all the antibodies used during this experiment, including the manufacturer and product number, and dilutions used for the two methods.

Origin	Antigen	Manufacturer Product#	Dilution traditional	Dilution IE NEO
Mouse monoclonal	α-tubulin	Sigma #T8203	1:1000	1:2000
Mouse monoclonal	Acetyl-α-tubulin	Sigma #T6793	1:1000	1:2000
Mouse monoclonal	GAPDH	ThermoFisher # 39-8600	1:2000	1:4000
Goat polyclonal	Mouse (Alexa Fluor® 488)	Invitrogen A11001	1:5000	1:10000

Following the final wash step, the western blots were developed using the Amersham Typhoon, the figures were processed by adjusting minimal pixel values using Adobe Photoshop and Illustrator. Band intensity was determined using the ImageJ (v1.53c) Gel Analysis method[6]. Figures were made using Adobe Illustrator and Microsoft Excel.

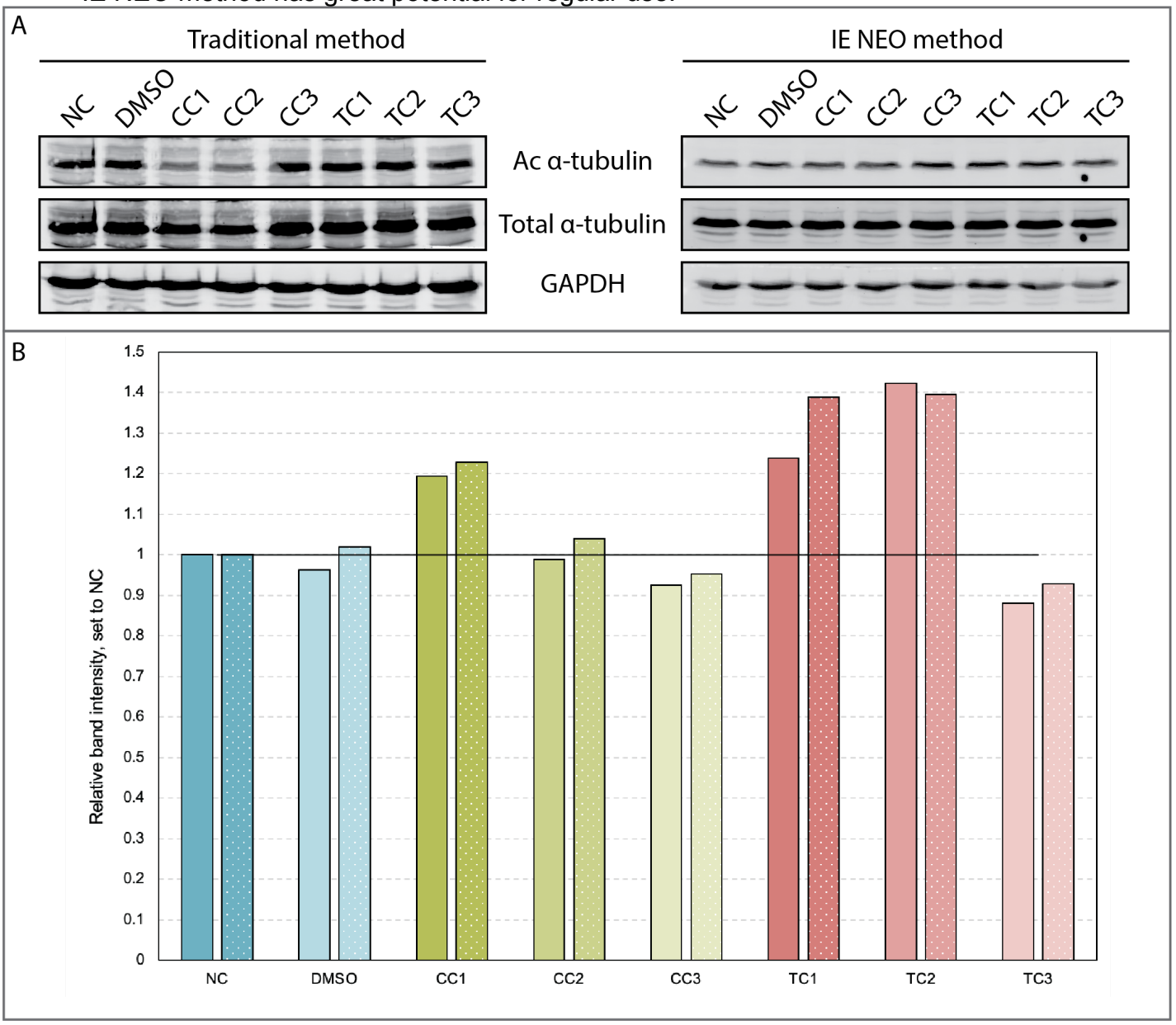
Results

To accurately assess the differences between the traditional Western blotting method and that using IE NEO, we performed a direct comparison. Here, we ran two SDS-PAGE gels with the same layout, of which some samples were expected to increase the acetylation status of α-tubulin (CC1, TC1 and TC2), while other were expected to have no effect (DMSO and CC2) or reduce the acetylation status (CC3 and TC3).

When comparing the actual western blot images (Figure 1A), it is quite clear that the traditional method creates a lot more background noise compared to the IE NEO method, however, it should be noted that longer exposure times were necessary for the IE NEO blot, as these bands were quite faint initially. That being said, there was no time for optimalisation of the IE NEO protocol and antibody dilutions, so optimalisation of the method before use would be strongly recommended.

In all our runs of this particular experiment we found that the differences in band intensity for the levels of acetylated α-tubulin are quite small and somewhat indiscernible. For this reason, we determined the intensity for each band using the ImageJ Gel Analyser tool. Using these numbers, the levels of total α-tubulin were normalised to the negative control, followed by normalisation of the levels of acetylated α-tubulin over total α-tubulin. Subsequently, the levels of acetylated α-tubulin were set to the corresponding negative control and plotted in a graph (Figure 1B). When comparing the results for the traditional method (complete fill) to that using IE NEO (pattern fill), we found that the overall trend between the two methods is the same and is as expected. Moreover, we do see a slightly more

discernible difference between the bands for the IE NEO method, indicating that the IE NEO method has great potential for regular use.



Conclusions

Overall, the IE NEO method was easy to use and relatively quick, especially given the shorter incubation times. Moreover, the results showcase the same general trend in comparison to the traditional method, with fewer background noise and higher antibody dilutions, resulting in cheaper antibody use overtime. All of this indicates a great potential for the use of IE NEO on a regular basis during Western blotting. However, it should be noted that optimalisation of the method prior to running actual experiments is recommended, and re-use of IE NEO-antibody mixtures has not yet been tested. To conclude, our results indicate a promising future for the use of IE NEO in regular Western blotting experiments.

References

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