[Letter to the Editor]

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Sequential use of milk and bovine serum albumin for streptavidin-probed western blot

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The extraordinarily strong interaction between streptavidin (SA) and biotin (the dissociation constant $K_d \sim 10^{-14}$ M) has been exploited extensively in biotechnologies [1]. One such application is using horseradish peroxidase (HRP)-conjugated SA to detect biotinylated proteins in western blot (WB). For routine WB using an antibody to detect the target protein, 5% (w/v) low-fat dry milk dissolved in tris-buffered saline/Tween 20 (TBS/T) solution is the most common blocking and antibody incubation buffer. However, milk contains biotin, which will competitively inhibit the interaction between SA and biotinylated proteins [2]. For WB using SA-HRP, common practice is to use either bovine serum albumin (BSA) to substitute milk, or reduce milk to 1%; less commonly, a specialized commercial blocking agent absent of biotin is used. However, each of these methods has a drawback: replacing milk with BSA not only increases the cost but also results in heavy background, while using 1% milk results in insufficient blocking and loss of specific signals (see Figure 1); the loss of signals could be overcome by increasing the concentration of SA-HRP, but this will increase the cost and potentially further increase the background; a specialized blocking reagent is generally not readily available for many laboratories and will increase the cost markedly compared with milk.

It appears that BSA is not as efficient as milk for blocking. If we use milk only for blocking, and sequentially use BSA only for SA-HRP incubation (after a short wash to get rid of the biotin from milk), we might solve the problem. We therefore compared four combinations of blocking and SA-HRP incubation conditions for WB with lysates from 293 and 293T cells

(Figure 1). Samples 1–3 were from cells overexpressing biotinylation-irrelevant proteins, while sample 4 was from 293 cells overexpressing a promiscuous biotin ligase BioID2 [3]. BioID2-expressing cells have been supplemented with 0.1 μ M biotin to promote promiscuous biotinylation. Indeed, sequential use of milk and BSA outperformed all the other tested conditions, giving relatively low background and specific signals (Figure 1). Three bands were detected from the BioID2-negative samples, while additional bands were detected from the BioID2-positive sample, with BioID2 itself being the predominant band (corroborated by an epitope-tag antibody, not shown). There are five known naturally biotinylated proteins in mammals: acetyl-CoA carboxylase 1 and 2 (ACC1/2, ~265/280 kDa), pyruvate carboxylase (PC, ~130 kDa), propionyl-CoA carboxylase subunit alpha (PCCA, ~80 kDa), and 3-methylcrotonyl-CoA carboxylase subunit alpha (MCCA, ~80 kDa) [4,5]. According to the size, ACC1 or 2 was not detectable from 293 or 293T cells, whereas the identity of the ~60 kDa band is unclear, as the size does not match any known biotinylated protein. Although using 5% BSA for both blocking and SA-HRP incubation resulted in a stronger signal, the high background is unacceptable. In addition, it gave multiple nonspecific bands; surprisingly, the 130-kDa band was not intensified, but rather masked for an unknown reason. By contrast, SA-HRP incubation in 1% milk resulted in dramatic loss of the signal. Thus, only sequential use of milk and BSA gave a satisfying result.

It is worth noting that conditions could be optimized by extending the blocking time (e.g., to 2 h) and varying the concentration of SA-HRP to further reduce the

background and to contrast specific signals. As the blocking effect is largely exerted by the milk, the BSA in the incubation buffer functions rather as a carrier protein than a blocker. Since BSA is relatively expensive, the cost could be reduced by decreasing the concentration of BSA to 1% or even to 0.1% (corresponding to 1 μ g/ μ l, a commonly used concentration for BSA as a carrier), if sufficient prior blocking has been done. We do not recommend omitting BSA completely; however, we have not tested the conditions extensively.

It is said that some preparations of BSA may contain biotin from serum. However, it appears that most biotin, if not all, is lost during commercial BSA preparation; thus commercial BSA is generally compatible with SA-probed WB, even if not stated as being 'biotin-free', like the BSA we are using (#A4503; Sigma-Aldrich, MO, USA). We compared the information for two BSA products, #8076 and #0163 (biotin-free) from another vendor Carl Roth (Karlsruhe, Germany); the only difference we could identify is that an additional test for the absence of biotin has been conducted for #0163. Thus, presence of biotin in BSA is not usually a concern. However, if loss of signals happens, another BSA product or biotin-free blocking agent should be used for the SA-HRP incubation step.

In summary, we present a simple solution to the common SA-HRP WB problem, which can lead to a satisfying result. Also, the protocol could serve as a starting point for further optimization.

Author Contributions

Y Cui conceived and designed the study; L Ma and Y Cui performed the experiments; Y Cui and L Ma wrote the manuscript.

Figure 1. Comparing blocking and SA-HRP incubation conditions for western blot. Lysates from 293 (RRID: CVCL_0045) and 293T (RRID: CVCL_0063) cells overexpressing biotinylation-irrelevant proteins, and from 293 cells overexpressing BiolD2 were loaded for four replicates for western blot (WB). The proteins are annotated according to size.

Sample 1–4: 293 transfected with NF2 isoform 2 (S518A; unpublished data and refer to [6]); 293T transfected with the same NF2 construct; 293T transfected with EGFP; 293 transfected with BiolD2. The BiolD2 plasmid was transiently transfected into 293 cells; ~24 h later, 0.1 μ M biotin was added into the culture medium to promote promiscuous biotinylation; ~48 h after transfection, the cells were lysed with PBS/0.6% CHAPS supplied with a protease inhibitor cocktail; ~0.5% of the lysate from confluent cells in a six-well plate (one well) was loaded per lane. For the other samples, the cells were lysed with 2 $\times$ SDS sample buffer ~24 h after transfection; for each sample, 5% of the total lysate from confluent cells in a 12-well plate (one well per sample) was loaded per lane. Nitrocellulose membranes and TBS/0.1% Tween 20 were used for WB. All the WB steps were performed at room temperature. The membranes were blocked for 1 h, washed shortly three times, and incubated with SA-HRP (Thermo Scientific Pierce #21130, 1:25,000) for 1 h; the membranes were washed 4 $\times$ 5 min before adding ECL substrate (Thermo Scientific Pierce #32106). See also Supplementary Figure 1.

PC: Pyruvate carboxylase; PCCA: Propionyl-CoA carboxylase subunit alpha; MCCA: 3-methylcrotonyl-CoA carboxylase subunit alpha.

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: <https://www.future-science.com/doi/suppl/10.2144/btn-2018-0006>

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