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Optimized semi-quantitative blot analysis in infection assays using the Stain-Free technology

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ABSTRACT

Western blots are a commonly used method for protein detection and quantification in biological samples. Compensation of loading variations is achieved by housekeeping protein (HKP) normalization and/or total protein normalization (TPN). However, under infection conditions, HKP normalization, traditionally used in cell biology for quantification of western blots, can be problematic. Binding of microbes to target cells via specific receptors can induce signal transduction events resulting in drastic changes in the level of expression of HKPs. Additionally, samples collected after infection assays will include cellular and microbial proteins altering the analysis with TPN. Here we demonstrate under experimental infection conditions, how a reliable semi-quantitative analysis of proteins in western blots can be achieved using the Stain-Free technology.

1. Introduction

Semi-quantitative analysis of western blots is a common method to evaluate the quantity of proteins of interest in biological samples (Heidebrecht et al., 2009). In cell biology studies the reliability of the analysis relies on the uniformity of sample loading, which is attempted by measuring protein concentration in each lysate to diminish the variability between sample loads. However, for semi-quantitative analysis of western blotting it is necessary to apply normalization parameters to signals. Two methods commonly used in cell biology studies, dealing with quantification of proteins, include the housekeeping protein (HKP) normalization and the total protein normalization (TPN). Comparison of several studies using the HKP or the TPN method has shown that Stain-Free technology provides the most reliable, most robust and most sensitive results (Gurtler et al., 2013). Stain-Free staining of proteins relies on the UV light-induced crosslinking of tri-chlorethanol to the amino acid tryptophan present in proteins, resulting in increased fluorescence of tryptophan at wavelengths of around 300 nm (Ladner et al., 2004). With this method, TPN is able to address problems such as oversaturated protein band signals, or variations in the level of housekeeping proteins directly on the loaded SDS-acrylamide gel. However, in the field of infection biology, the presence of proteins from bacteria, viruses or parasites additional to the host cell proteins can strongly impede the mentioned normalization methods. Additionally, any interference with the host cellular protein expression caused by the pathogen, which can include changes in the housekeeping proteins, cannot be ruled out when using standard HKP normalization. Consequently, we evaluated the use of the Stain-Free technology as a standard for semi-quantitative analysis in infection biology and its possible applications.

To adapt and validate the known methods we performed infection assays using several eukaryotic cell lines in contact with the human pathogen *Helicobacter pylori* (*H. pylori*) and the signals for phosphorylation of the *H. pylori* toxin CagA. Upon contact of *H. pylori* with host cells, the bacterium adheres strongly and injects the CagA toxin, encoded by the cytotoxin associated gene A (CagA), into the host cell. Once inside, CagA is phosphorylated on its tyrosine residues (EPIYA motifs) by cellular kinases (Odenbreit et al., 2000, Poppe et al., 2007, Selbach et al., 2002). The efficiency of CagA translocation is usually determined by western blot analysis of cell lysates detecting the tyrosine-phosphorylated form of CagA. Our attempts to determine variations in the CagA translocation efficiency by *H. pylori* using western blots led to the analysis and validations of signals presented here.

2. Materials and methods

2.1. Infections

Infection assays using human gastric epithelial cells (AGS cells, ATCC CRL 1739a) and the *H. pylori* strain P12 and P217 were performed as specified before (Jimenez-Soto et al., 2013). Bacteria were grown on GC agar (Oxoid) plates as described before (Jimenez-Soto et al., 2012).

2.2. Preparation of cell- and bacterial lysates and western blots

For the generation of host cell lysates, approx. 1×10^6 cells were collected by centrifugation in a swing rotor centrifuge at $500 \times g$ for 10 min,

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at 4 °C. For bacterial lysates, approx. 6×10^7 bacteria were collected by centrifugation in a swing rotor at 1500 ×*g* for 20 min, at 4 °C. Cellular and bacterial pellets were resuspended in 20 μl PBS containing proteinase inhibitors (1 mM PMSF, 10 μM Leupeptin and 10 μM Pepstatin) as previously described (Jimenez-Soto et al., 2009). To the bacterial and cell suspension 25 μl of a 2× SDS sample buffer was added, and the mixture boiled at 95 °C for 10 min in non-reducing conditions.

In the case of infection assays, whole-cell lysates (1×10^6 cells) containing target cells and bound bacteria were collected, mixed and prepared as described above. For the separation of proteins we used a 6% single gel (Ahn et al., 2001) containing trichlorethanol 0.01% (v/v) and let it run at 90 V for 10 min and 140 V for 55 min. Total protein amounts were visualized by activation of the gel under UV light (302 nm) for 1 min. The software ImageLab™ of the ChemiDoc MP (Bio-Rad Laboratories, Inc., California, USA) imaged the bands automatically, using different options for intense or weak protein bands as parameters for exposure times. Proteins were then transferred to PVDF membranes and protein detections were performed (Jimenez-Soto et al., 2013). As primary antibodies we used mouse α-phosphotyrosine 4G10 (Upstate, Millipore, Schwalbach, Germany) for phosphorylated CagA; rabbit α-CagA AK299 (Rojas et al., 2015) for detection of CagA protein; and mouse anti-Tubulin (Upstate, Millipore, Schwalbach, Germany) for detection of Tubulin as an example of housekeeping protein (HKP). As secondary antibody we used horseradish peroxidase-conjugated α-mouse or α-rabbit (Sigma-Aldrich, St. Louis, MO, USA) in dilutions of 1:10,000. For Stain-Free activation, signal detection and chemiluminescent antibody detection we used the Millipore Immobilon Western (Millipore) and ChemiDoc MP imaging system (Bio-Rad Laboratories, Inc., California, USA).

2.3. Calculations used for semi-quantitative analysis of CagA phosphorylation signals

During infections, cells were co- or pre-infected as described before (Jimenez-Soto et al. 2013), which led to variations in CagA translocation efficiency. These results were used for the standardization of blots shown here. For each infection, a single bacterial strain was used as the standard for CagA translocation. The signal of phosphorylated CagA obtained in the control infection was defined as 100% of translocated CagA under normal conditions (100% Phosphorylated CagA). Using the ImageLab™ software the phosphorylated CagA (antibody 4G10) was imaged avoiding saturated signals. The procedure for quantification of phosphorylated CagA signals was followed according to manufacturer's instructions (see ImageLab™ manual). Normalized values, relative to control infection signals, were obtained by multiplication of the relative value and the normalization factor, given by the software, using each of the methods described in the Results section.

3. Results

3.1. Bacterial proteins interfere with the Stain-Free total protein normalization (TPN)

We repeatedly observed distinct protein patterns in gels during infection assays after staining with Stain-Free method and these depended on the treatment of the cells. Notably, samples from uninfected AGS cells (a human gastric adenocarcinoma cell line, epithelia-like) showed many differences to samples from infected cells in the band pattern between 25 kDa to 100 kDa (Fig. 1A, Supplement Figure A). After the harvest of infected cells, *H. pylori* remains tightly attached to them and results in the presence of bacterial proteins in the lysate. To determine how strong bacterial proteins can affect the cellular signals, we analyzed the Stain-Free signal pattern of a number of different bacterial lysates (Figure 1B). We observed that bacterial protein signals are mainly found in the lower half of a 6% gel (<100 kDa). This applied not only to different *H. pylori* strains (P217, P12 and 26695), but as well to *Campylobacter jejuni*. In contrast, *Streptococcus pneumoniae* shows almost no proteins containing tryptophan, with one strong protein around 85 kDa in size. Taking these results into account we anticipated that, for infections with *H. pylori*, only the area below 100 kDa would introduce variations that could interfere with the normalization by TPN. To validate this, we compared the Stain-Free signals of the different cell types used in our infection assays. The data show that cellular lysates of AGS cells, ST-3051 cells (both human gastric cancer epithelial cells), THP1 cells (human leukemia cell line) and primary human leukocytes show distinct patterns of tryptophan-rich proteins in the high molecular size region (over 100 kDa, upper region), possibly allowing the use of only these signals as an alternative to the whole lane Stain-Free analysis.

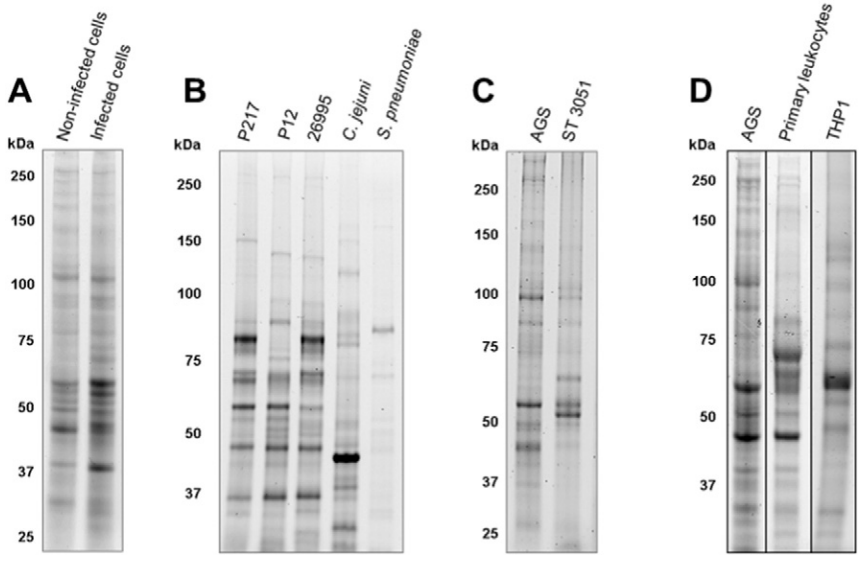

Fig. 1. Stain-Free signals from different eukaryotic and bacterial cell lysates. (A): Comparison of non-infected and with *H. pylori* infected AGS cells showing differences in the protein pattern in a 6% polyacrylamide single gel. (B) Comparison of different bacterial cell lysates. Strains P217, P12 and 26,695 are *H. pylori* strains. Non-*H. pylori* strains are *C. jejuni* and *S. pneumoniae* in a 6% polyacrylamide single gel. (C) Comparison of lysates from two eukaryotic epithelial cell lines, AGS and ST-3051, in a 6% polyacrylamide single gel. (D) Comparison of two immune cell lysates with standard AGS cell lysate in a 7% polyacrylamide single gel.