Application note

NH DyeAGNOSTICS

Stain-free and standardized analysis of protein 1D gels including absolute quantification using Smart Protein Layers technology

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Smart Protein Layers (SPL) is a standard-based technology for stain-free, quantitative and standardized analysis of proteins separated by 1D gel electrophoresis. SPL is based on rapid fluorescent labeling of protein samples using Smart Labels and a bi-fluorescent standard, the Smartalyzer (SMA).

The relation between fluorescent labeled protein sample and the SMA allows highly sensitive, stain-free protein visualization as well as for precise protein normalization and quantification.

A second standard named Calibrator (CAL) can be applied to the gels. CAL allows for the comparison of data derived from different gels and is also a bi-fluorescent protein molecular weight marker.

Introduction

Quantitative and reliable protein 1D gel analysis demands a standardized and reproducible system. Proteins of samples separated by SDS- or IEF-PAGE are usually visualized by using protein stains. Yet, protein stains are limited in their capabilities: Due to the low detection sensitivity of Coomassie high amounts of sample are required. Both Coomassie and Silver stains provide a linear range of signals of two orders of magnitude only {1}. Fluorescent stains provide much better sensitivity and linear range, however, all stains suffer from limitations in terms of reproducibility and require additional time and labor for staining and destaining.

Gels containing a tri-halogen compound, which binds to tryptophan containing proteins upon UV-irradiation, are clearly limited in terms of sensitivity of protein visualization {2}. Fluorescent pre-labeling of proteins allows for highly sensitive protein visualization of less than 1 ng of protein over a linear range of up to 5 orders of magnitude. Due to the pre-labeling of the samples no further staining and destaining is required for protein visualization.
However, protein labeling is a chemical process which can be affected by certain compounds of the protein extract or the buffers. Therefore, this approach requires the monitoring of labeling results and if necessary the normalization of the total protein.

The SPL technology offers the solution for this challenge. It combines a sample protein pre-labeling step for fast and easy protein visualization with a bi-fluorescent standard named Smartalyzer (SMA) which is added to every protein sample before sample labeling to monitor labeling reaction and gel load. *SMA* is available in size S (12.5 kDa) and in size L (80 kDa) as shown in Figure 1. Using an adequate fluorescence imaging device, protein can be visualized with high sensitivity (less than 1 ng) and a broad range of linearity (10^4 - 10^5).

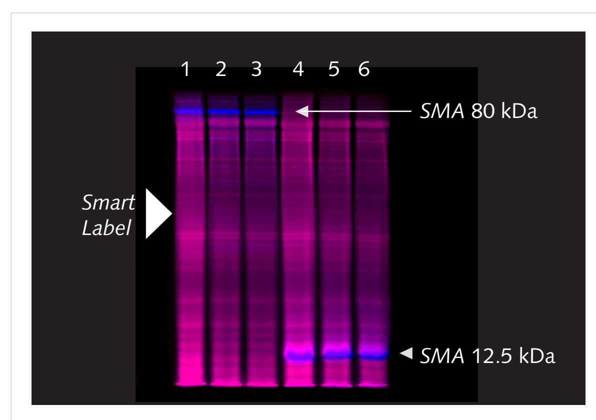


Fig. 1. Overlay of red and blue fluorescence images of a 1D gel containing six lanes of *E. coli* protein pre-labeled with Smart Red (20 µg of protein each). Lanes 1-3 show the red and blue bi-fluorescent SMA in size L (80 kDa), lanes 4-6 show the red and blue bi-fluorescent SMA in size S (12.5 kDa).

SPL buffer containing DTT and blue fluorescent SMA is added to a protein sample. Then, reactive red fluorescent Smart Label is added to the sample resulting in red fluorescent labeled protein sample and red/blue fluorescent SMA (Figures 2A, 2B). After electropho-retic separation sample protein and SMA is rapidly visualized using a fluorescence imager (Figure 2C). The red fluorescence visualizes the label of sample protein and SMA. The blue fluorescence visualizes the SMA by its basic fluorescence properties (blue).

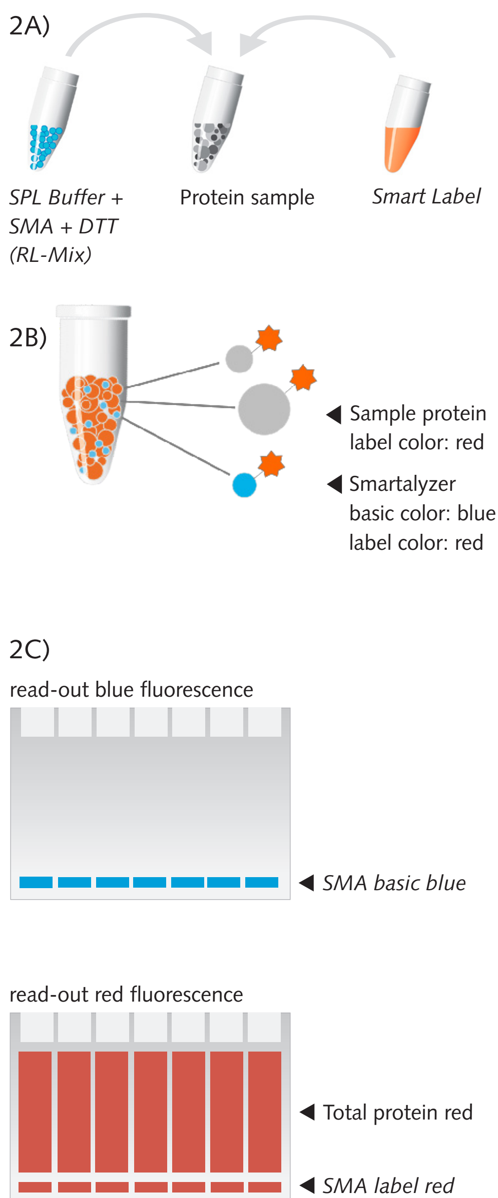


Fig. 2A. Procedure of Smart Labeling of a protein sample using e.g. Smart Label Red and Smartalyzer (SMA) basic blue. The label and the Smartalyzer are added to the protein sample prior gel electro-phoresis. **Fig. 2B.** The protein sample contains red labeled sample protein and red labeled Smartalyzer. The Smartalyzer is now bi-fluorescent (basic color blue, label color red). **Fig 2C**. Read-out of the gel by red and blue fluorescence imaging.

The combination of pre-labeled sample protein and the added standard SMA monitors protein content, sample loading and labeling reaction. Thereby, the SPL technology allows for precise normalization of sample protein.

Additionally the SPL technology enables the comparison of data derived from different gels or experiments. The SPL technology provides a gel-to-gel reference, called Calibrator (CAL), which also serves as a fluorescent protein molecular weight marker (80 - 25 -12.5 kDa; Figure 3).

In this paper we demonstrate that the SPL technology allows for absolute protein quantification even if varying amounts of protein have been applied to the gel - either by variations in the protein content or by loading different amounts of sample.

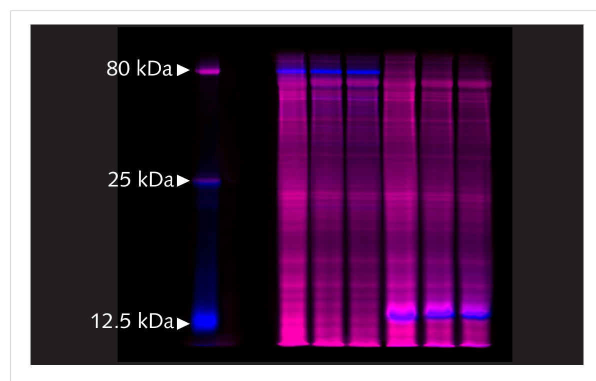


Fig. 3. Overlay of red and blue fluorescence images of a 1D gel with six lanes of *E. coli* protein pre-labeled with Smart Red. The lane far left show the red and blue bi-fluorescent Calibrator (CAL) with its three molecular weight bands of 80, 25 and 12.5 kDa, respectively.

Materials and methods

Extraction of total protein

Total protein was extracted from *E. coli* (Sigma-Aldrich). Lysis buffer (30 mM Tris-HCl (pH 8.5), 7 M urea, 2 M thiourea and 4% (w/v) CHAPS) was added to lyophilized *E. coli* cells. Cells were lysed by repeated freeze-thaw-cycles and cell debris was removed by centrifugation. The total protein content of the supernatant was measured using the EZQ™ Protein Quantitation Kit (Thermo Fisher Scientific).

SPL labeling

First, the reaction and loading mix (RL-mix) was prepared by mixing 6 µl SPL buffer, 2 µl SMA basic blue size S respectively size L, and 2 µl 60 mM DTT (freshly prepared). In order to minimize the pipetting error the RL-mix was prepared as a master mix (n samples = n x RL-mix). 10 µl of the RL-mix was mixed with 10 µl sample containing 2-100 µg of protein. To start the labeling reaction 1 µl Smart Label working solution containing *Smart Label Red* reagent A and B was added, and the samples (each with a total volume of 21 µl) were heated for 5 min at 95 °C.

SDS-PAGE and fluorescence detection

The SPL labeled protein samples were loaded on a 1D gel containing 12.5% acrylamide/bis-acrylamide and than separated by gel electrophoresis. Total protein and SMA were detected by red and blue fluorescence detection using the Octoplus QPLEX Imager (NH DyeAGNOSTICS).

Coomassie staining

The gel was fixed and stained for 30 min with Coomassie blue and destained overnight. The image of the stained gel was captured by the Octoplus QPLEX Imager using the white light transmission module.

Data processing and evaluation

Data processing and analysis was performed using LabImage-SPL 1D and Western blot software (NH DyeAGNOSTICS). Lane and bands were detected, background was removed and the raw volumes of lanes (representing total protein) and individual bands (SMA basic and SMA label) were calculated and SPL-normalized by SPL-LabImage software.

Results and discussion

To evaluate the performance of the SPL technology for quantitative protein analysis in 1D gels four different experiments were performed. The SPL technology was evaluated in terms of a) sensitivity; b) reproducibility; c) linearity and d) load control and absolute protein quantification.

Highly sensitive Smart label protein visualization

The comparison of protein visualized by Coomassie staining and Smart labeling is shown in Figure 4. Each lane contained 20 µg of *E. coli* protein which is visualized in a much higher sensitivity by Smart labeling then by Coomassie. Detection limits of Coomassie stains are in the range of 10 - 100 ng the linear range is 1-2 orders of magnitude {1}. The detection of the Smart Labels can be as low as in the pictogram range (depending on the imaging device). The Octoplus QPLEX Imager is capable to detect 0.1 ng of Smart labeled protein {3} over a linear range of 5 orders of magnitude (Figure 5).

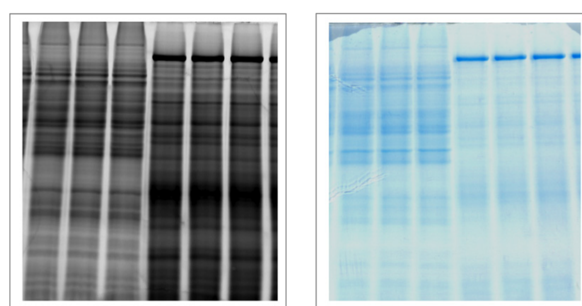


Fig. 4. 1D gel showing 6 lanes with 20 µg of *E. coli* protein per lane (2 different strains), labeled with Smart label red (left) or stained with Coomassie (right).

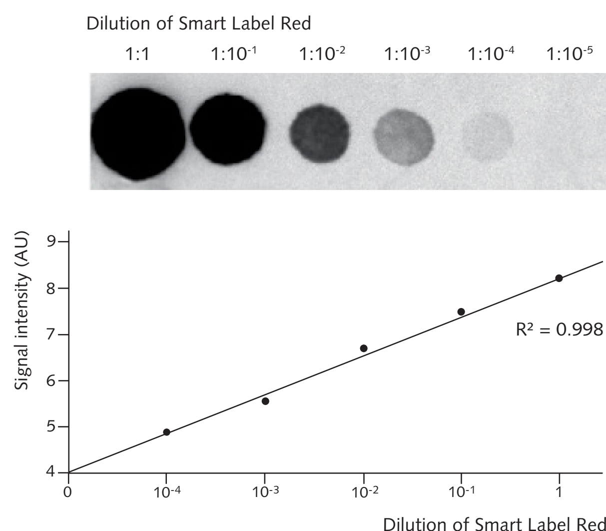
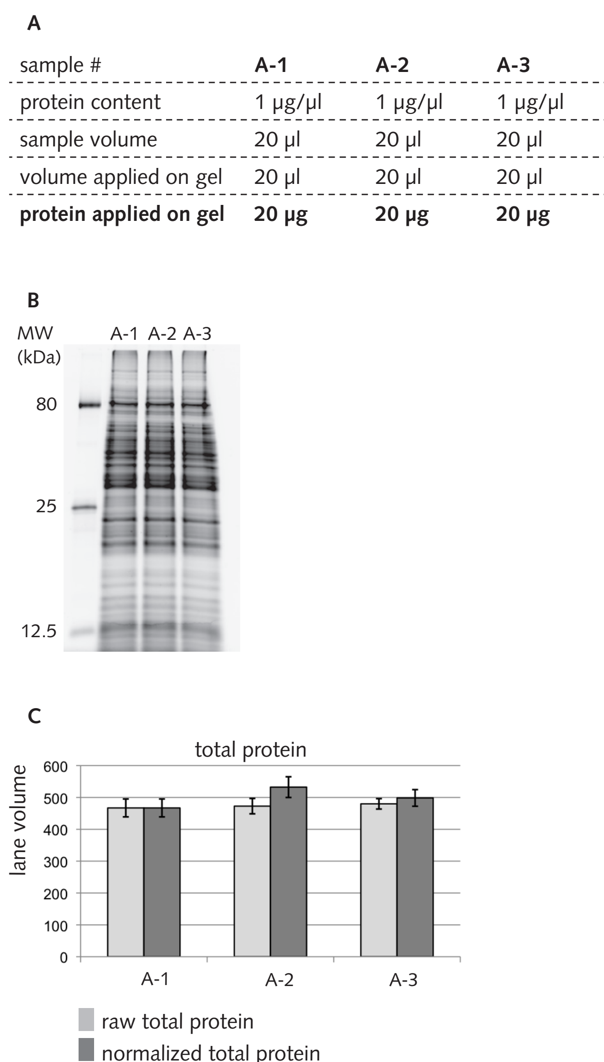


Fig. 5. 10fold serial dilution of Smart Label Red. The dye was directly spotted onto a low-fluorescent blotting paper and imaged by Octoplus QPLEX Imager. Signal intensities were analyzed by SPL-LabImage analysis software.

Reproducibility of the Smart label reaction

To evaluate the reproducibility of the Smart Labeling reaction, 20 µg of *E. coli* protein was labeled using one reaction of Smart Label Red and SMA basic blue size S (12.5 kDa). Nine independent labeling reactions were separated within three 1D gels. Figure 6 shows the labeling set-up for one of the three replicate gels, the fluorescent image of one gel replicate and the determined average lane volumes including standard deviations of all three gel replicates (raw and normalized, respectively) representing the total protein amount. The comparison of the nine Smart labeling reactions show a high degree of reproducibility as the standard error is below 5%.



A

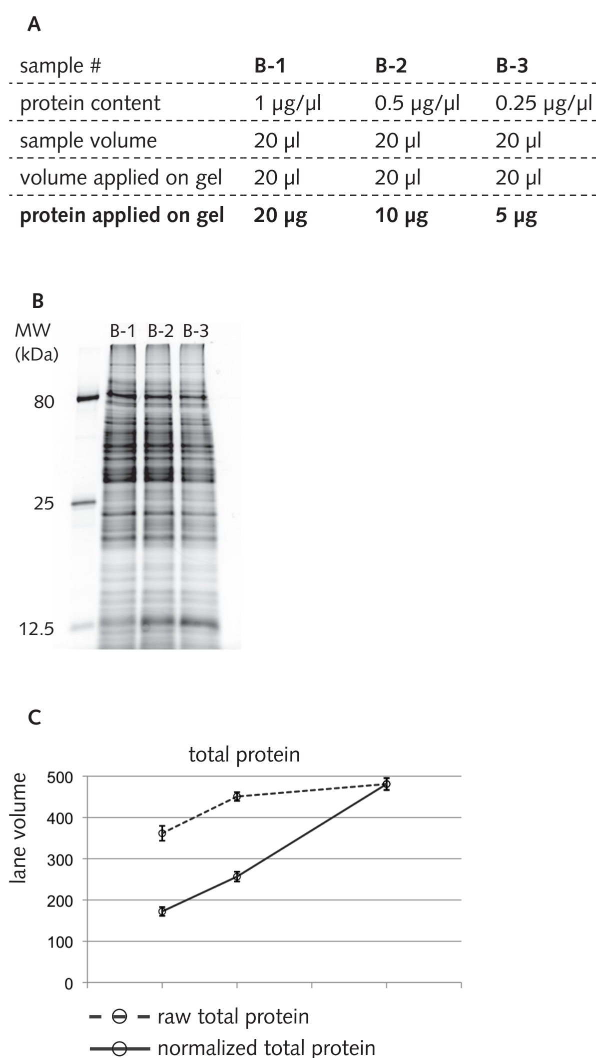
sample #	A-1	A-2	A-3
protein content	1 µg/µl	1 µg/µl	1 µg/µl
sample volume	20 µl	20 µl	20 µl
volume applied on gel	20 µl	20 µl	20 µl
protein applied on gel	**20 µg**	**20 µg**	**20 µg**

Figure 6. Labeling set-up of the samples and applied protein to determine the reproducibility of Smart Labeling **(A)**, fluorescent image of gel **(B)** and the average raw and normalized lane volumes of the total protein amount **(C)**.

Linearity of Smart Label and protein amount

For quantitative analysis of 1D gels the linearity between fluorescence label (lane volume) and the used protein amount is essential. To test for linearity 20 µg, 10 µg and 5 µg of *E. coli* protein was labeled using Smart Label Red and SMA basic blue in a total velum of 20 µl. The three labeling reactions were separated within one 1D gel, which was performed in three replicates.

Figure 7 shows the labeling set-up for this experiment, the acquired gel image of one gel replicate and the determined average lane volumes including standard deviations of all three gel replicates (raw and normalized, respectively) representing total protein amount.



A

sample #	B-1	B-2	B-3
protein content	1 µg/µl	0.5 µg/µl	0.25 µg/µl
sample volume	20 µl	20 µl	20 µl
volume applied on gel	20 µl	20 µl	20 µl
protein applied on gel	**20 µg**	**10 µg**	**5 µg**

Figure 7. Labeling set-up of the samples and applied protein to determine the linearity of Smart Labeling **(A)**, fluorescent image of one gel **(B)** and the average raw and normalized lane volumes of the total protein amount **(C)**.

The non-normalized lane volumes show poor linearity between fluorescence signal/lane volume and the amount of protein used for the labeling reaction. Labeling efficiency depends for example on the protein concentration (ratio of protein/ fluorophore). However, after SPL normalization based on the Smartalyzer (labeled under the same conditions as the sample protein) the normalized fluorescence signal/lane volume shows excellent and reproducible linearity to the used amount of protein as the coefficient of determination (R^2) is 0.996. Therefore the SPL technology provides a tool for the analysis of samples without determination of protein concentration before electrophoresis.

SPL Smartalyzer to control loading and for absolute quantification

The SMA in its basic fluorescence color (SMA basic) is added in equal amounts to every labeling reaction. Differences in band volume of SMA basic result from differences in volumes loaded on the gel. Therefore, SMA basic allows to control and normalize protein load. To demonstrate SMA´s ability to monitor gel load 20 µg of *E. coli* protein was labeled using one reaction Smart label red and SMA basic blue size S in a total volume of 20 µl and three replicates. 20 µl, 10 µl and 5 µl were loaded on the gel, respectively. SDS-PAGE was performed in three replicates.

Figure 8 shows the labeling set-up for this experiment, the acquired gel image of one gel replicate and the determined average lane volumes including standard deviations of all three gel replicates (raw and normalized, respectively) representing total protein amount.

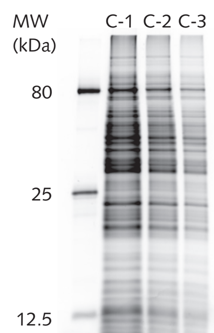
The non-normalized total protein is decreasing from lane C-1 to C-3 since decreasing sample volumes were applied. After SPL normalization the lane volume (representing total protein) is the same for the samples C-1, C-2 and C-3 demonstrating the ability of SMA to compensate differences in gel load.

Furthermore SMA allows for the absolute quantification of protein bands. The linearity of fluorescence signal and the corresponding labeled protein amount could be proven. A defined amount (µg) of SMA result in a defined amount of fluorescence signal/ band volume. Therefore the determined fluorescence signal/band volume of a sample protein can be used to calculate the amount of this sample protein.

A

sample #	**C-1**	**C-2**	**C-3**
protein content	1 µg/µl	1 µg/µl	1 µg/µl
sample volume	20 µl	20 µl	20 µl
volume applied on gel	20 µl	10 µl	5 µl
protein applied on gel	**20 µg**	**10 µg**	**5 µg**

B



C

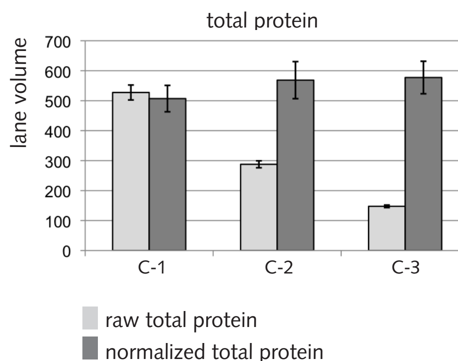


Figure 8. Labeling set-up of the samples and applied protein to monitor protein loading **(A)**, Fluorescent image of one gel **(B)** and the average raw and normalized lane volumes of the total protein amount **(C)**.

Conclusions

1. Smart Labeling is an ideal replacement for post-stains providing high sensitivity and broad linear range.

2. Smart Labeling allows to absolutely quantify protein samples even if they differ in protein content or in sample volume.

3. Smart Labeling offers the reproducibility and the comparison of results derived from different experiments.

References

{1} Miller I. et al. 2006. Protein stains for proteomic applications: Which, when, why? Proteomics 2006 6: 5385-5408

{2} Bio-Rad Laboraties Inc. 2010: Bulletin 5974

{3} NH DyeAGNOSTICS. 2012. Octoplus QPLEX eBrochure_170613.pdf. Page: 16

Ordering information

For detailed information, please visit https://www.dyeagnostics.com/site/products/spl-for-1d-gels/

Product overview

Product	Prod. No.
SPL Gel Kit Red - 400 rct.	PR902
SPL Gel Kit Blue - 400 rct.	PR905
SPL Gel Kit Red-IR - 400 rct.	PR907
VELUM Gold Precast Gels 25S	PR254
VELUM Gold Precast Gels 52S	PR255
VELUM IEF Precast Gels RTU 24S	PR232
LabImage-SPL 1D & WB Software	PR989
Octoplus QPLEX Imager	PR435

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SPL eBrochure

SPL Product Guide

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