NH DyeAGNOSTICS

Product Guide

Smart Protein Layers SPL Kit Blue

Product no. PR916, PR925

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Rev. 03/2018 (1)

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1 Products and content

	SPL Kit Blue 20W	SPL Kit Blue 40W
Product no.	PR916	PR925
Smart Label Blue Reagent A*	1x 200 Rcts	2x 200 Rcts
Smart Label Blue Reagent B*	1x 200 Rcts	2x 200 Rcts
SPL Buffer	1x	2x
SPL Smartalyzer basic red size S (SMA; 25 kDa)	1x	2x
SPL Smartalyzer basic red size L (SMA; 80 kDa)	1x	2x
SPL Calibrator A (CAL A)**	1x	2x
SPL Calibrator B***	3x	6x

* sufficient for 20 bzw. 2x20 Minigel Western Blots or 200 Smart Label reactions of up to 100 µg protein each
** for experiment-to-experiment comparisons
*** antibody specific SPL Calibrator B (CAL B) for experiment-to-experiment comparisons of antibody signals (mouse, rabbit and goat; for 20 blots each)

2 Storage and stability

Store all kit components at -20°C to -80°C
Store the Smart Label Working Solution at -20°C to -80°C

Best before: see kit packaging
The Smart Label working solution is stable for up to 6 month.

3 Safety instructions

The Smart Label reagent B contains dimethylformamide (DMF, $HCON(CH_3)_2$, CAS No: 68-12-2) and is harmful by inhalation, ingestion or skin contact.

4 Additional materials required:

- DTT (per kit: ca. 500 µl of a 60 mM DTT solution)
- gel electrophoresis and blotting device
- Low-fluorescent Blotting Membrane (Prod. no. PR811, PR812)
- primary and secondary antibody, blocking ad washing solutions for immuno detection
- detection of sec. antibody: e.g. conjugated to red fluorescence dye
- Imaging system for detection of blue and red fluorescence

5 Introduction

Smart Protein Layers (SPL) is a technology for stain-free, quantitative and standardized analysis of protein gels and Western blots. For the first time, target protein and total protein expression can be detected at the same time. In addition, SPL allows to precisely compare data derived from different experiments.

SPL offers:

- Stain-free, highly sensitive and standardized protein visualization on gels and blots
- Protein content equalizer (load by volume, inaccurate protein determination, loss of sample)
- Monitoring of every step of the Western blot analysis
- Accurate quantification of target protein expression
- Precise comparison of different experiments

SPL is based on three components:

The fluorescent Smart Label visualizes total protein in the gel and on the blot with high sensitivity (detection limit less than 1 ng, dynamic range 10^4 - 10^5) within seconds. Smart Labels are chemically bound to the protein prior protein separation (3 min hands-on).

The bi-fluorescent Smartalyzer (SMA) is a multi-functional standard added to every sample prior separation. For precise normalization, standardization and quantification of total protein. SMA is available in size S (12.5 kDa) and size L (80 kDa).

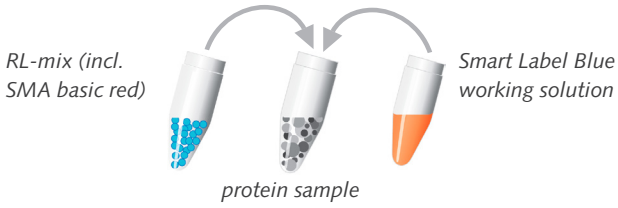
The bi-fluorescent Calibrator (CAL) is the standard for comparison between different gels or blots. CAL works also as bi-fluorescent molecular weight marker (80 kDa; 25 kDa; 12.5 kDa).

6 General protocol of the SPL analysis

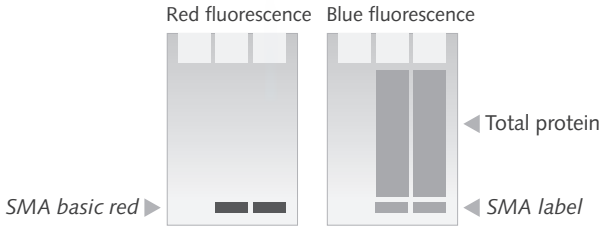
1. Smart labeling and sample preparation for gel electrophoresis
    a. Preparation of the Smart Label working solution (SLW)
    b. Preparation of the SPL reaction & loading mix (RL-mix)
    c. Labeling of protein samples
    d. optional: usage and preparation of the SPL Calibrator
2. Gel electrophoresis and detection of fluorescence
3. Western blot analysis and detection of fluorescence
4. Processing and evaluation of the acquired data

Overview Workflow SPL-Analysis

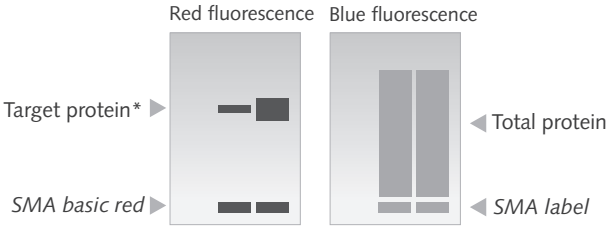
1 Add *RL-mix (incl. SMA)* & *Smart Label* to your protein.



2 Run SDS-PAGE. Detect red and blue fluorescence.



3 Perform Western and detect fluorescence of target (red) and normalized total protein (blue).



**E.g. using red-fluorescent 2nd antibody*

4 Data processing and evaluation.

- Detection and determination of band and lane raw volumes
- Normalization of gel load (based on *SMA basic*)
- Normalization of *Smart Label* (based on *SMA label*)
- Normalization of target protein signal (based on total protein)
- Experiment-to-experiment normalization (based on *CAL*)

7 Detailed SPL protocol

Please read the complete manual and ensure that all required reagents and materials are ready for use before starting work.

7.1 Sample preparation and Smart labeling of protein samples

7.1.1 Preparation of the Smart Label working solution

- Allow vials containing Smart Label reagent A and Smart Label reagent B to warm up to ambient temperature. Smart Label reagent B contains molecular sieves to avoid water pollution.
- Spin down briefly.
- To solubilize Smart Label add 15 µl of Smart Label reagent B to Smart Label reagent A, mix and spin down briefly.
- Transfer all liquid from Smart Label reagent A to Smart Label reagent B, mix and spin down briefly.

    Note: Step 4 ensures optimum stability of Smart Label working solution for up to 6 months.

Now Smart Label reagent B contains ready-to-use Smart Label working solution. Store unused Smart Label working solution for up to 6 months at -20°C to -80°C.

7.1.2 Preparation of the SPL reaction & loading mix (RL-mix)

For preparation of the RL-mix, mix

6 µl of SPL buffer,
2 µl of 60 mM DTT (newly prepared) and
2 µl of SMA basic size S or L

and spin down briefly. The RL-mix is now ready-to-use.

Note: Preparation of the RL-mix as a master mix is recommended. n protein samples = n+1 x mastermix

Note: SPL buffer contains SDS, which may precipitate during freezing. Allow SPL Buffer to warm to ambient temperature and mix until SDS is redissolved completely. Precipitation does not influence SPL buffer quality.

7.1.3 Labeling of protein samples

- Transfer up to 10 µl of protein sample (containing up to 100 µg of protein) in a fresh microcentrifuge tube.

  Note: If your sample contains more than 10 µl or 100 µg of protein scale up the labeling assay.

- Add 10 µl of RL-mix (see step 1b), mix and spin down briefly.

- Add 1 µl of Smart Label working solution (reagent B; see step 7.1.1), mix and spin down briefly.

- Perform denaturation and labeling of proteins by incubation for 5 min at 95°C. The protein samples are now ready for gel electrophoresis.

  Note for compatibility of protein samples:
  Smart labeling is compatible with all commonly used buffer systems. Please avoid amines or ammonium salts with higher concentrations than 400 mM.

  Note for choosing appropriate size of SMA basic:
  SMA is available in size S (12.5 kDa) and size L (80 kDa). For gels containing <15% AA/BisAA use SMA basic size L and for gels containing >15% AA/BisAA use SMA basic size S. Nevertheless, the size of SMA basic should differ from size of target protein as well as highly abundant proteins within your protein sample.

7.1.4 Usage and preparation of the SPL Calibrator

See appendix A.

7.2 Gel electrophoresis and fluorescence detection

7.2.1 Gel electrophoresis

Apply the appropriate amount of protein sample to your gel. Apply 12 µl of CAL (prepared according to appendix A). Perform electrophoresis as usual.

Note: When using SMA size S (= 12.5 kDa) make sure that electrophoresis is finished before the SMA size S bands migrated out of the gel.

Note: Apply the same amount of CAL to every gel.

7.2.2 Fluorescence Imaging

Imaging of fluorescence signals should be performed directly after electrophoresis without additional fixing or staining steps.

Detect fluorescence signal of total protein and SMA basic:

SMA basic: red
Total protein (incl. SMA label): blue

Optimal detection parameters (exposure time of camera or PMT of the photomultiplier): most intense signal of your samples (total protein or SMA basic or SMA label) should be marginally below saturation. Do not use CAL for optimization.

Excitation and emission parameters:

	max. excitation [nm]	max. emission [nm]
blue	496	520
red	650	665

Note: Indicate acquired imaging files according to appendix B.1. (processing and evaluation of acquired data) to enable fast and easy software processing of your data.

7.3 Western blot analysis / Target detection

7.3.1 Protein transfer

Perform transfer of protein as usual and check optionally for transfer efficiency by detection of fluorescence signal of total protein and SMA basic (see 2b). Use a low fluorescent blotting membrane (Nitrocellulose (PR811) or PVDF (PR818)).

7.3.2 Detection of target protein

Perform Western blotting including the detection of your antibody (AB) as usual. Detect fluorescence signals of total protein and target protein signal.

Note: For target detection of Smart Blue labeled protein we recommend red fluorescent 2nd AB.

Optimal detection parameters (exposure time of camera or PMT of the photomultiplier): The most intense signal of your samples (total protein or target protein, respectively) should be marginally below saturation. Do not use CAL for optimization.

Note: For the fast and easy software processing of your data we re-commend to name the acquired image files according to appendix B.1. (processing and evaluation of acquired data).

Note: If the detection of SMA basic and target is in the same fluorescence channel (e.g. SMA basic ired and fluorescence coupled antibody ired) a strong SMA basic signal can disturb the target detection. We recommend to excise the SMA basic bands before target detection. SMA *basic is not needed for calculation after blotting.*

7.4 Processing and evaluation of acquired data

See appendix B.

Appendix A

Usage and preparation of CAL

Use the Calibrator CAL A for experiment-to-experiment comparisons of fluorescence signals. Use Calibrator CAL B for comparisons of antibody (AB) signals derived from different experiments.

CAL A emits a red and blue fluorescence and is applied to every gel in equal amounts. CAL A comprises 3 polypeptides (12.5 kDa, 25 kDa and 80 kDa) in different amounts (to match various imaging settings e.g. exposure time). It can be used for experiment-to-experiment comparisons as well as a molecular weight marker.

The non-fluorescent CAL B is a 50 kDa polypeptide, specifically designed for secondary AB recognition. Add CAL B to every gel for subsequent experiment-to-experiment comparisons of AB signals.

Note: Depending on the sensitivity of your secondary AB, a second polypeptide at 20 kDa may be detected. However, this does not interfere with the calibration results.

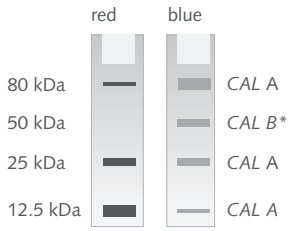
Choose the appropriate CAL B for your secondary AB, which depends on the host organism of your primary AB (mouse, rabbit, goat):

e.g. anti-rabbit IgG secondary AB: use CAL B rabbit

The two Calibrators CAL A and CAL B can be combined within one lane.

Note: A charge depending normalization factor is available for the usage of different charges of CAL.

Calibrator A and B:



* detection of CAL B using red-fluorescent sec. antibody

Preparation of CAL

Preparation of CAL as a master mix is recommended.
n x gels = n x master mix.

- Transfer 8 µl CAL A per gel into a fresh microcentrifugation tube.
- Add 2 µl of CAL B per gel and mix.
- Add 2 µl of 60 mM DTT (newly prepared), mix and centrifuge briefly.
- Perform denaturation of proteins by heating for 4 min at 95°C). CAL A+B are now ready for gel electrophoresis.
- Apply 12 µl of the prepared CAL to each gel.

Note: We recommend to pretest the compatibility of used amount of CAL with detection of your target protein experimentally:

Signal intensity of CAL B >> target protein = use less CAL B per gel (or dilute)

Signal intensity of CAL B << target protein = use more CAL B per gel

Note: For the use of various CAL B on one blot, apply 1 µl of each CAL B directly onto the blotting membrane subsequent to protein transfer. Do not apply CAL B onto your protein lanes. Perform Western blot analysis as usual.

Appendix B

Processing and evaluation of acquired data using LabImage 1D SPL

Appendix B provides an overview of data processing using SPL-LabImage. However, all data can be processed and evaluated by using other 1D- and Western blot software. Find a detailed description of LabImage 1D SPL software usage and of all normalization steps in the LabImage 1D SPL product guide and product guide Data Processing and Evaluation at: www.dyeagnostics.com/site/en/products/spl/

B.1 Nomenclature of the accquired images

The SPL analysis requires 4 fluorescence images. These are:

- Total protein (incl. SMA label) after gel electrophoresis (GTO = gel total protein)
- SMA basic after gel electrophoresis (GLO = gel loading control)
- Total protein after Western blot analysis (BTO = blot total protein)
- Target protein after Western blot analysis (BTA = blot target protein)

Important!
Indicate acquired image files as GTO, GLO, BTO and BTA, respectively and add a tag for the experiment (e.g. date). Separate abbreviation, experiment tag and other information by underscores:

e.g.: GTO_Experiment1_April15_2014.tif

This nomenclature allows for an automated normalization of target protein signal using LabImage 1D SPL software.

B.2 Determination of lane and band volumes

Detect lanes and bands using LabImage 1D SPL.
Following data are required for normalization of target protein signal:

- Band volume of SMA basic (determined using GLO)
- Band volume of SMA label (determined using GTO)
- Lane volume (excl. band volume of SMA label; determined using BTO)
- Band volume of target protein (determined using BTA)
- For experiment-to-experiment comparisons (optional): band volume of CAL (determined for each experiment)

B.3 Normalization of target protein signal

When the images (called projects in the SPL software) have been named according to the proposed nomenclature (see appendix A, step 1), the LabImage 1D SPL software will perform all calculations automatically. Proceed as follows:
a) open all projects, that have to be analyzed. Ensure that all projects are named according to B.1 and all lanes and bands are detected according to B.2.
b) open the SPL project comparator. Normalization factor (SPL factor) and normalized lane and band volumes (SPL value) are calculated automatically.

B.3.1 Normalization of gel load (based on SMA basic):

Since SMA basic is added in equal amounts to each protein sample, loss of protein during sample preparation or gel load result in differences in band volumes of SMA basic (in GLO) between different lanes. The normalization factor for gel load (NF load) is determined using GLO and is applied to GTO, BTO and BTA. Since usually a marker is applied to the gel in the first lane, the SMA basic in the second lane in GLO is set as reference* (default setting).

B.3.2 Normalization of Smart Labeling (based on SMA label)

Since SMA basic is added in the same amount to each protein sample and is labeled with Smart Label to the same extend than the protein sample, unequal labeling results in differences in band volumes of SMA label (in GTO) in different lanes. The normalization factor for labeling efficiency (NF label) is determined using GTO and is applied to BTO. SMA label in the second lane far left in GTO is set as reference* (default setting).

B.3.3 Normalization of target protein (based on normalized total protein)

To compensate differences in protein content of the respective protein samples (resulting from unequal loading or insufficient protein concentration determination) target protein signal is normalized against total protein. SPL factor is determined using BTO and is applied to BTA. The second lane far left is set as reference* (default setting).

** Note: To use other bands or lanes as reference for determination of all normalization factors choose "settings" of SPL factor or SPL value and select appropriate band or lane.*

B.3.4 Output and export of data

LabImage 1D SPL is able to export all calculated values as csv file and all images as jpg or tiff file.

B.4 Experiment-to-experiment comparisons

In order to compare fluorescence and/ or target protein signals derived from different gels or blots a point of origin is required. This is given by the Calibrator (CAL). Choose one gel or one blot as your reference. Use always the same out of the three possible fluorescent CAL A bands or CAL B band for comparison of different experiments. Choose such a CAL A band which can be visualized/ detected in all of your experiments and which is below signal saturation.
For each fluorescence channel the factor of normalization of CAL (NF CAL) can be determined by:

$$\text{NF CAL experiment X} = \frac{\text{band volume CAL experiment X}}{\text{band volume CAL reference experiment}}$$

The generated NF CAL now serves for the normalization of signals derived from different experiments:

$$\text{Normalized band volume target protein of experiment X (lanes Y to Z)} = \frac{\text{band volume target protein exp. X (lane Y to Z)}}{\text{NF CAL target protein experiment X}}$$

Note: When using different lots of CAL the lot-dependent CAL factor must be taken into account. CAL-normalized band volumes of the target proteins must be divided additionally by this factor.

Appendix C
Applications post electrophoresis

Fixation and storage of gels
Acquire the fluorescent image of Smart labeled protein gels after finishing the SDS-PAGE. Gels stored within low fluorescent glass cassettes can be scanned up to 6 h after finishing the SDS-PAGE. Otherwise, fix the gel for 30 min in 40% (v/v) ethanol / 10% (v/v) acetic acid and store in 25% (v/v) ethanol / 3% (v/v) glycerol in the dark. For pre-cast gels, please see manufacturers' recommendations.

Stains
Smart Labels are compatible to all conventional gel staining methods like silver or Coomassie brilliant blue. However, visible stains like Coomassie may mask fluorescent signals.

Mass spectrometry
Smart Labels do not interfere with subsequent protein digestion neither protein identification by mass spec.

Antibody recognition
Smart Labels do not interfere with the protein recognition by antibodies.

Appendix D
Example of the SPL Western blot analysis

Experiment

Protein was extracted from E. coli expressing recombinant HSP90. 3 aliquots of 20 µg of protein each were transferred to a fresh vial. To each of the three vials equal amounts of the standard SMA basic and the Smart Label working solution were added and labeling was performed according to the SPL Red or SPL Blue manual[1]. For sample 2 (figure 1 +2: lane 2 of all 4 images) and sample 3 (figure 1+2: lane 3 of all 4 images) a labeling inhibitor was added in order to artificially decrease labeling efficiency.

Abbreviations of the fluorescent images

GTO = Gel Total Protein + *SMA label*
GLO = Gel Load Protein (= *SMA basic*)
BTO = Blot Total Protein
BTA = Blot Target Protein

Gel electrophoresis, Western blot and image acquisition

After gel electrophoresis fluorescent images (red and blue detection) were captured and named GTO_exp1.tif and GLO_exp1.tif (figure 1: images GTO and GLO).
The protein was transferred using a Beo Dry Blotter onto a low fluorescence blotting membrane. The membrane was blocked using commercial milk powder. As an first antibody anti-HSP90 was used. As a secondary antibody a HRP-conjugated anti-goat in combination with Immuno Blue Fluorescent Substrate was used in order to detect the target protein. All washing and antibody incubation steps were performed by an usual WB procedure.
After the Western blot, both total protein (named BTO_exp1.tif) and target protein (named BTA_exp1.tif) were detected by fluorescence imaging (figure 2: images BTO and BTA).

Processing of the images

The 4 acquired fluorescent images (GTO_exp1.tif, GLO_exp1.tif, BTO_exp1.tif, BTA_exp1.tif) were processed using the 1D and Western Blot analysis software LabImage 1D SPL.
The region of interest (ROI), lanes and background were detected in GTO. These obtained data were than transferred to GLO. SMA basic was detected in GTO and this band than transferred back to GTO in order to detect the SMA label bands. The SPL normalization occurs automatically in LabImage 1D SPL as described in the manual SPL Data Processing and Evaluation[2].

Results

The effect of SPL normalization on the target protein signal is demonstrated in figure 3. Target protein signal is shown in relation to: (i) non-normalized total protein and (ii) SPL-normalized total protein. Non-normalization of decreased labeling efficiency of the 3 E. coli aliquots (caused by external addition of an labeling inhibitor) would lead to increasing target protein signal. SPL-normalization is able to compensate for unequal labeling. As a result, target protein signal do not differ as it is expected for the three aliquots originating from one E.coli sample.

[1,2] Manuals are available as PDF at the SPL product site in the section: Related Documents.

Link to the SPL product site:
http://www.dyeagnostics.com/site/en/products/spl/

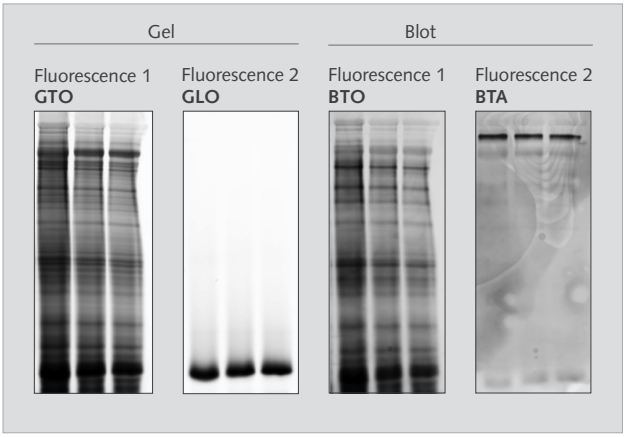


Figure 1. Acquisition of the fluorescent images

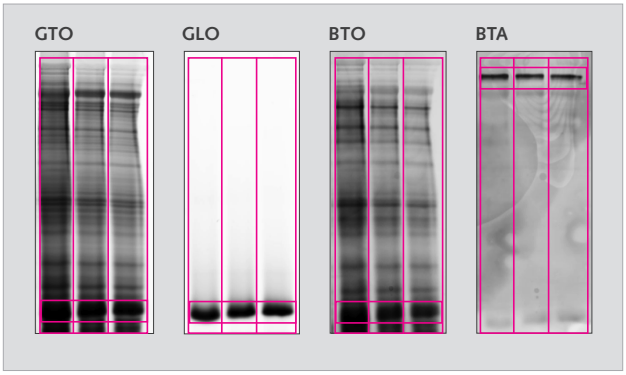


Figure 2. Processing of the fluorescent images

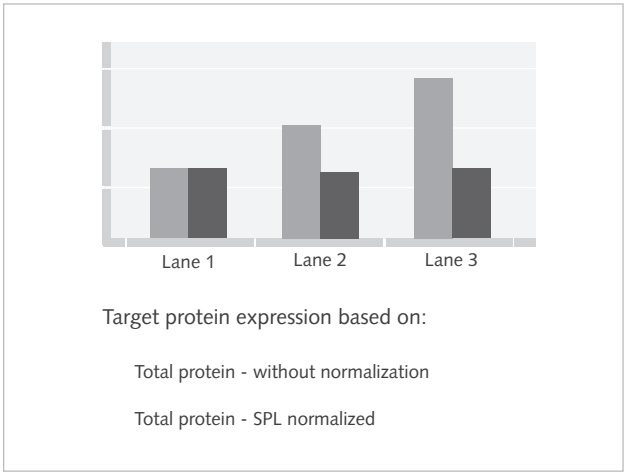


Figure 3. Evaluation of SPL-normalized target expression