PROTOCOL

Densitometric analysis of protein gels

Band quantitation using the chemiSOLO: Image acquisition and verification of calibration

chemiSOLO

Chemiluminescence | Visible Imaging | Densitometry

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I. Materials and reagents

- Gels stained with colorimetric total protein stain (e.g., Silver Stain, Coomassie Blue)
- chemiSOLO instrument
- chemiSOLO Densitometry Package
- AzureSpot Pro Analysis Software for band quantitation

II. Objective

This note describes a standard procedure for easy and reliable densitometric quantification of proteins separated on 1D and 2D protein gels using the chemiSOLO.

This note includes instructions to:

- verify the calibration (linearity of detection) of the chemiSOLO unit using the 21-Step Calibration Target included with the chemiSOLO Densitometry Package.
- measure optical density (OD) for quantification and identification of proteins separated by gel electrophoresis and stained with colorimetric dyes.

III. Introduction

The identification and quantification of target proteins in complex mixtures is broadly performed through the analysis of images of gels stained with reagents for total protein detection. Among the most-used colorimetric reagents are several formulations for silver staining and many commercial preparations of Coomassie blue dyes.

Different silver stain formulations stain protein spots and bands in a variety of colors including black, blue, brown, yellow, or red, depending on their charge and experimental conditions, which is useful for differentiating overlapping spots in 2D gels.

Some of the advantages of densitometry conducted on the chemiSOLO are:

- High-resolution imaging for small spot identification
- Direct proportionality of the signal over a range of 2.1 OD
- Even transillumination across the imaging area (15 x 10cm)
- Color and grayscale imaging for both qualitative and quantitative analysis
- Verification of the unit calibration

The chemiSOLO offers high resolution imaging to allow for the identification of small bands or spots in 1D or 2D gels. The powerful optics capture signal with direct proportionality using 16-bit data collection to ensure the linearity, sensitivity, and overall accuracy of quantitative analysis.

With an imaging field of view of 15 x 10cm, the chemiSOLO can accommodate most typical gel sizes. The LEDs imbedded in the inner lid provide even transillumination across the imaging stage to ensure consistent sensitivity across the entire field of view.

White light is emitted from the LEDs in the inner lid of the chemiSOLO and passes through the sample to the detector. The absorbance of light by the colorimetric stain in the sample is directly proportional to the concentration of stain bound to the target. Therefore, by detecting light absorbed by the sample, the concentration of stain and the concentration of the target can be detected and quantified.

IV. chemiSOLO verification procedure

Accurate quantification relies on the instrument's OD calibration. The following procedure describes how to verify the chemiSOLO's densitometric performance using the 21-Step Tablet included in the chemiSOLO Densitometry Package. The following instructions explain how to acquire an image of the 21-Step Tablet using the chemiSOLO capture software and analyze it using AzureSpot Pro.

1. 21-Step Tablet Image Acquisition	1. Turn on the chemiSOLO and connect a device to the unit, either through direct ethernet connection or via the unit's specific Wi-Fi. Open the chemiSOLO software by navigating to either its ethernet IP: 192.168.40.1 or its Wi-Fi IP: 192.168.12.1 on any web browser. Alternatively, if connecting by Wi-Fi, access the software by scanning the QR code on the inside of the lid.
	2. Place the 21-Step Tablet centered and face-down on the clean glass surface of the chemiSOLO.
	3. Close the chemiSOLO lid completely and activate grayscale imaging in the Visible Imaging section of the Home Screen. Select START to collect an image of the Tablet.
	4. When the image is visible in the Gallery, select the 'Save to Device' button and save the image as a 16-bit tiff directly to the connected device.
	5. Remove the 21-Step Tablet from the sample stage and clean the surface with a soft, lint-free tissue. Distilled water or ethanol can also be used to clean the glass if necessary.
2. 21-Step Tablet Data Analysis using AzureSpot Pro	1. Launch the AzureSpot Pro Analysis Software. Select the 1D module.
	2. Open the grayscale image of the 21-Step Tablet by selecting Open under the "Image" tab. Adjust the contrast to visualize the differences of signal intensities of the steps. Do not invert the signal.
	3. Create an individual lane to represent the background signal. a. Go to the "Lanes" tab in AzureSpot Pro. b. Under "New Box," select a box with one lane. c. On the image, place the box in the background signal surrounding the 21-Step Tablet. • **Note:** *The box can be any size because the following process will adjust for area.*

4. Then, create a lane for each step of the 21-Step Tablet.
    a. Still in the "Lanes" tab in AzureSpot Pro, select a box with 21 lanes under "New Box."
    b. On the image, align the 21-lane box with the 21 lanes on the step tablet.
        - The 21-lane box can be adjusted to fully encompass the lanes using the "Edit Box" tab.
    c. Select 80% under "Def. Lane Width."

5. In the BG (Background) tab, go to the measurements table in the lower left.
    a. Select the table's displayed measurements to include "Name," "Width," "Length," and "Volume."
    b. Then, copy the measurements table using the "Copy" command ( ).

6. Open the provided "chemiSOLO Densitometry Calculator" Excel sheet and follow the instructions both in the sheet or as specified below:
    a. **Note:** *It is recommended to first make a copy of the chemiSOLO Densitometry Calculator Excel file to allow for repeated use.*
    b. Switch to the Excel tab labeled "21-Step Tablet Confirmation."
    c. Paste the previously copied table after selecting the cell labeled "Name."

7. This will fill out the graph on the right automatically, which will display the linearity of the detected 21-Step Tablet. If the R^2 value is above 0.98, the calibration of the chemiSOLO unit is verified.

For more information about the theoretical basis of densitometry and chemiSOLO verification, please see section VII.

V. Sample analysis procedure

The following procedure describes how to use the chemiSOLO capture software to acquire images of 1D or 2D gels for densitometric analysis.

Sample images may be analyzed using one of the following:

- AzureSpot Pro Analysis Software: 1D gel analysis
- SameSpots 2D Analysis Software: 2D single stain and DIGE analysis
- SpotMap Analysis Software: Host Cell Protein (HCP) Coverage

Analysis of the image using AzureSpot Pro Analysis Software for 1D gel analysis is described below. For 2D gel analysis, follow the instructions in the SameSpots or SpotMap software manuals.

1. Sample Image Acquisition	1. Turn on the chemiSOLO and connect a device to the unit, either through direct ethernet connection or via the unit's specific Wi-Fi. Open the chemiSOLO software by navigating to either its ethernet IP: 192.168.40.1 or its Wi-Fi IP: 192.168.12.1 on any web browser. Alternatively, if connecting by Wi-Fi, access the software by scanning the QR code on the inside of the lid.
	2. Place the sample (e.g., silver-stained protein gel, Coomassie-stained protein gel) centered face-down on the clean glass surface of the chemiSOLO. A broad variety of colorimetric stains and samples can be analyzed.
	3. Close the chemiSOLO lid completely and activate grayscale imaging in the Visible Imaging section of the Home Screen. Select START to collect an image of the sample.
	4. When the image is visible in the Gallery, select the 'Save to Device' button and save the image as a 16-bit tiff directly to the connected device.
	5. Remove the gel from the sample stage and clean the surface with a soft, lint-free tissue to prevent water marks or residue. Distilled water or ethanol can also be used to clean the glass if necessary. • DO NOT leave a gel sitting on the glass sample stage for an extended period of time as it may cause permanent staining of the glass. Additionally, do not use solvents to clean the glass surface as doing so can damage the water-tight seal between the plastic surfaces and the glass.
2. Sample Data Analysis using AzureSpot Pro	1. Launch the AzureSpot Pro Analysis Software. Select the 1D module.
	2. Open the grayscale image of the visible-stained sample by selecting Open under the "Image" tab. Adjust the contrast to visualize the differences of signal intensities. Do not invert the signal.
	3. First, create an individual lane to represent the background signal. a. Go to the "Lanes" tab in AzureSpot Pro. b. Under "New Box," select a box with one lane. c. On the image, place the box in the background signal surrounding the 21-Step Tablet. • **Note:***The box can be any size because the following process will adjust for area.*

4. Then, create a lane for each lane of the standard curve.
    a. Go to the “Lanes” tab in AzureSpot Pro.
    b. Under “New Box,” select a box with the correct number of lanes on the sample.
    c. On the image, align the box with the lanes of the sample.
        - The box can be adjusted to fully encompass the lanes using the “Edit Box” tab.

5. Select the specific bands from the standard curve.
    a. Select the “Bands” tab and manually select the bands from the Lane boxes. For the background, select the entire box as one band.
    b. Select the table’s displayed measurements to include “Volume” and “Band Area (px)” only.
    c. Then, copy the measurements table using the “Copy” command ( ).

6. Open the provided “chemiSOLO Densitometry Calculator” Excel sheet and follow the instructions both in the sheet or as specified below:
    a. **Note:** *It is recommended to first make a copy of the chemiSOLO Densitometry Calculator Excel file to allow for repeated use.*
    b. Switch to the Excel tab labeled “Sample Analysis.”
    c. Paste the previously copied table after selecting the cell labeled “Lane Number.”
    d. Manually input the corresponding loaded standard values in the “Loaded Standard” column. Since the first row refers to the Background, make sure to fill the value of cell E9 as “0.”

7. This will fill out the graph on the right automatically, which will display the linearity of the detected standard curve.
    - Using the line of best fit, input the slope and intercept into the labeled cells under the graph.

8. Last, specify the lanes and bands for the unknown sample lanes in the same way as described in Steps 3 and 4.
    - After copying the measurement table with the “Copy” command ( ), paste the table after selecting the cell labeled “Sample Number.”

9. The final loading values for the unknown sample, calculated through the standard line of best fit, will be displayed to the right, highlighted in green.

VI. Conclusions

The chemiSOLO was designed for sensitive densitometric quantification of colorimetric gels. With the ability to linearly detect a broad dynamic range of 0 to 2.1 OD, densitometric quantification of visible-stained gels is possible on the chemiSOLO.

VII. Theoretical basis

Verification of the scanner's OD calibration (analytical instrument qualification, AIQ)

For Operational Qualification (OQ), the verification of the scanner linearity for densitometry measurements is performed using a neutral density filter array (21-Step Tablet, delta OD 0.10 per step). The user should decide the frequency of this procedure. Some laboratories perform the verifications every 6 months, while others repeat it every time a densitometry experiment is performed.

a. Light Transmittance (T):

$$T_i = \frac{I_i}{I_0} \qquad \textit{[Equation 1]}$$

$$T_i\% = T_i * 100 \qquad \textit{[Equation 2]}$$

Where:

T_i : Transmittance of the sample for an individual pixel

I_i : Light intensity of the transmitted beam (after passing through the sample)

I_0 : Light intensity of the incident beam

b. Optical Density (OD):

$$OD_i = -\log(T_i) \qquad \textit{[Equation 3]}$$

Where:

OD_i : Optical density of the sample for an individual pixel

Thus, relating *[Equations 1–3]* shows the relations between T%, T, and OD as:

T% = 100%	→ T = 1	→ OD = 0
T% = 10%	→ T = 0.1	→ OD = 1
T% = 1%	→ T = 0.01	→ OD = 2
T% = 0.1%	→ T = 0.001	→ OD = 3

c. By substituting *[Equation 1]* into *[Equation 3]*,

$$OD_i = -\log\left(\frac{I_i}{I_0}\right) = \log(I_0) - \log(I_i) \qquad \textit{[Equation 4]}$$

d. The OD values of the calibrated neutral density filter array (21-Step Tablet) supplied in the chemiSOLO Densitometry Package correlate with experimental optical density over a range of 0.10 – 2.10 OD. The linearity is verified in a scatter plot of the experimental ODs over the theoretical ODs with an R^2 value > 0.97.

To calculate the experimental OD values of the 21-Step Tablet, apply *[Equation 4]* as:

$$OD_i\ (Measured) = -\log\left(\frac{I_i}{I_0}\right) = \log(Signal_{(background\ (OD=0))}) - \log(Signal_{STEPi})$$

If the measurements of intensities are determined using differently-sized areas, normalize the signal by each box's area using:

$$OD_i\ (Measured) = \frac{Area_{STEPi}}{Area_{background}} * \log(Signal_{(background\ (OD=0))}) - \log(Signal_{STEPi})$$

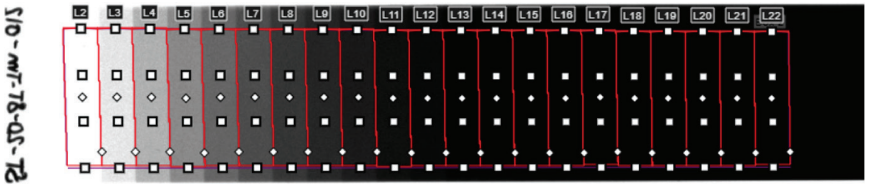

Figure 1. 21-Step Tablet Analyzed in AzureSpot Pro Analysis Software

Name	Width (px)	Length (px)	Volume
Lane 1	167	301	1738289763
Lane 2	93	373	896453441
Lane 3	93	372	729084470
Lane 4	93	373	583115580
Lane 5	93	372	469304158
Lane 6	93	372	372807163
Lane 7	93	372	303721248
Lane 8	93	371	237280723
Lane 9	93	370	194333914
Lane 10	93	368	158629638
Lane 11	93	369	127377355
Lane 12	93	368	101580691
Lane 13	93	368	78490091
Lane 14	93	367	66130761
Lane 15	93	367	54657748
Lane 16	93	367	45207059
Lane 17	93	364	38200274
Lane 18	93	364	32407513
Lane 19	93	360	26943384
Lane 20	93	360	22870467
Lane 21	93	361	20475671
Lane 22	93	361	18941511

Table 1. Raw Data from AzureSpot Pro

Lane number	OD of Steps Tablet	Measured Signal (area-corrected)	Ti (measured)	ODi (measured)
Lane 0	0	34581.13202	1	0
Lane 1	0.1	25842.58529	0.747303046	0.126503248
Lane 2	0.2	21074.24182	0.609414458	0.215087247
Lane 3	0.3	16809.81233	0.486097804	0.313276341
Lane 4	0.4	13565.27223	0.392273805	0.406410692
Lane 5	0.5	10776.01928	0.311615573	0.506380846
Lane 6	0.6	8779.085675	0.253869239	0.595389919
Lane 7	0.7	6877.104107	0.198868681	0.701433607
Lane 8	0.8	5647.599942	0.16331449	0.786975281
Lane 9	0.9	4635.040849	0.134033809	0.87278564
Lane 10	1	3711.78585	0.10733558	0.969256294
Lane 11	1.1	2968.112757	0.085830411	1.06635881
Lane 12	1.2	2293.422481	0.066320052	1.17835514
Lane 13	1.3	1937.557089	0.056029314	1.251584698
Lane 14	1.4	1601.410682	0.046308799	1.334336485
Lane 15	1.5	1324.5161	0.038301699	1.416781964
Lane 16	1.6	1128.449545	0.032631943	1.48635706
Lane 17	1.7	957.3293454	0.027683575	1.557777834
Lane 18	1.8	804.7605735	0.023271667	1.633172514
Lane 19	1.9	683.1083333	0.019753787	1.704349622
Lane 20	2	609.8850564	0.017636353	1.753591214
Lane 21	2.1	564.1888124	0.016314932	1.787414736

Table 2. Theoretical and Experimental OD Calculations

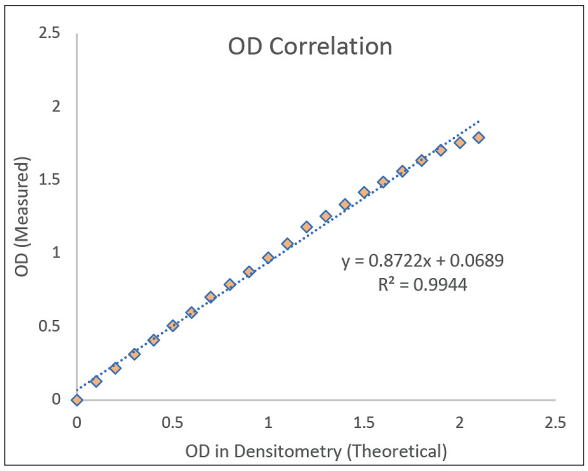

Figure 2. Scatter Plot for Validating chemiSOLO Calibration

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