Using Dolphin-1D microtiter assay to establish protein concentration with the Lowry protein assay

MATERIAL

- BSA (Sigma-Aldrich, St Louis, MO, U.S.A.) 1 mg/ml diluted in 0.01 M PBS pH 7.4.
- Lowry reagents: 0.1 M NaOH, 2 % Na_2CO_3 in 0.1 M NaOH (reagent A), 1 % Na-tartrate in ddH_20 (reagent B), 0.5 % CuSO4 in ddH_20 (reagent C), 1:1 Folin-Ciocalteu's phenol reagent in ddH_20 (Protein and chemicals all from Sigma-Aldrich).
- Standard curve with concentrations 1 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml. Diluted in 0.1 M NaOH.
- Mouse J774 cells (macrophage cell line). 10 µl added→ D.F. 500.
- 96-well plate.
- Dolphin-Doc Pro Image system with UV-transilluminator (Wealtec Bioscience, Taipei, Taiwan)).
- UV/White light converter plate (Wealtec).
- Dolphin-1Dsoftware (Wealtec).
- Vortexer (Pantec Instruments, Taipei, Taiwan)

PROCEDURE

- **Preparation of assay:** Samples were diluted in 0.1 M NaOH up to 600 µl. 4 ml of a mixture of solutions (96% reagent A, 2% reagent B and 2% reagent C) was added to each protein sample. Samples were left for incubation in RT for at least 10 minutes. Then 400 µl of a 1:1 dilution of Folin-Ciocalteu's phenol reagent in ddH_20 was added followed by immediate vortexing. The samples were left at RT for 1 hour before measurements.
- 300 µl of both standard curve samples and protein samples were pipetted in quadruples into a 96-well plate.
- The 96-well plate was placed on top of a white plate covering the UV-transilluminator in the image system.

- **Image capture:** Dolphin Doc Pro was turned on (visible light) and the software was opened.
- The image was focused and zoomed in Live-mode, until the image covered most of the image window. For this particular image, the following settings were used; iris 2, zoom 12 and focus 1.
- The door was closed and the illumination was turned to UV.
- The image was captured and saved and the UV-illumination was turned off.
- **Analysis:** "Microtiter Plate Image" was chosen under "Edit", and the box for white background was ticked (*fig 2*). To open the microtiter assay tool, the monkey-wrench symbol (in the row of buttons above the image) was pressed. This tool can also be opened by choosing "Microtiter Plate tool" under "analysis" or pressing "Microtiter plate tools" in the quick guide window to the right of the captured image.
- The region of interest (ROI) was chosen by pressing the blue square in the tool-box and then marking the interesting rows and columns (*fig 3*). Grid format was chosen (8x12 96-well), and the number of wells (rows and columns) used was keyed in (*fig 4*). The radius size was adjusted until most of each well was caught in a circle. For fine-tuning the position of individual circles, the arrow-tool was used. By pressing a circle and dragging the pointer outwards, the radiuses of all circles become enlarged simultaneously. By clicking on the edge of the blue square and dragging the pointer to either direction, the whole square can be moved. When every well was covered by a circle of suitable size, the "OK"-button was pressed.
- *To use the mass-standard function in assessing the concentration of an unknown sample using Dolphin 1D, press the "Std"-box in the row above the captured image. Place the pointer and click on the well containing the smallest concentration. The well becomes marked by a red arrow. Update the concentration of that particular well by keying in the concentration in the empty field and then pressing "update". Continue to the next sample in your standard (fig 5). When all the standards are updated, press "OK" to enter the report window, where the concentration in each well has been updated according to the keyed in standard values. The report data can be exported to Microsoft Excel for further analysis by pressing the "Excel"-symbol*
- To compare O.D values of every well in the marked area, the report-button was pressed after the region of interest was chosen and all wells were covered by red

circles. The O.D. values of all wells in the chosen area are listed in the order of appearance, even empty wells.

- By pressing the Excel-sign in the upper left corner, the data was exported to Microsoft Excel (*fig 6*). The concentrations of the unknown sample was calculated accordingly; 1. The mean O.D. values of every quadruple sample in the standard curve as well as the unknown sample were calculated. 2. The mean value represented by the 0-sample of the standard curve was subtracted from every other mean value. 3. The graph guide of excel was used to create a graph of the standard curve values. 4. A trend-line was inserted and the equation of that line was used to calculate the concentration of the unknown sample.

RESULTS

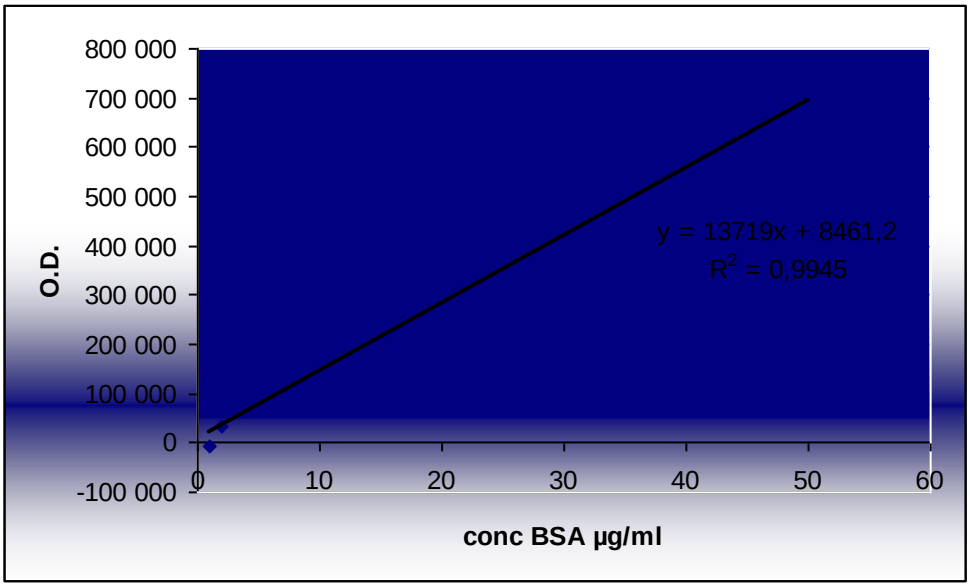

Figure 1. *Linearity of Lowry/BSA standard curve measured in O.D. using Dolphin Doc Pro and Dolphin 1D-software.*

The concentration of the unknown sample was calculated accordingly;
Y = 13719x + 8461.2 →
421781 = 13719X + 8461.2
X = ((4217181 – 8461.2)/13719) x 500 (D.F.)
X = 15 µg/µl

REMARKS

- Since the concentrations in the 100 µg/ml wells generate O.D. values that lie outside the linearity of the Lowry-method, these values have been excluded from the standard curve calculations.
- The microtiter plate assay is useful for assessing concentrations and comparing O.D. values generated by colorimetric samples, however all colorimetric assays, e.g. Bradford, are not suited for this application due to the complexity of the colours generated when staining proteins with this dye. CCD-camera only distinguishes between O.D. values.
- For colorimetric microtiter plate analyses, an image generated by UV is to prefer, since epi-illumination generates light-impressions in the images of the solutions in the wells.
- If the microtiter plate used is translucent, a white light converter plate should be placed on top of the UV-translilluminator.

APPENDIX

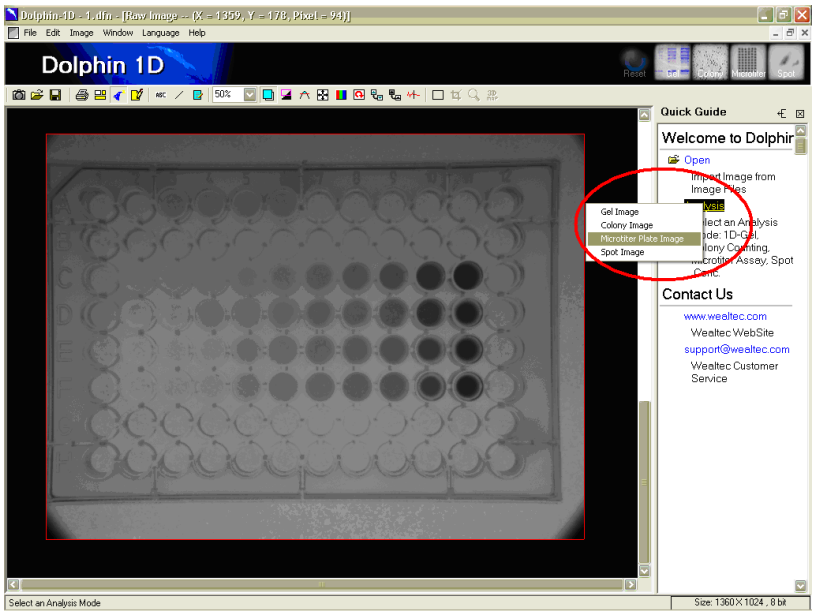

Figure 2. *After capturing the image, choose "Microtiterplate Image" to enter the microtiter plate analysis mode*

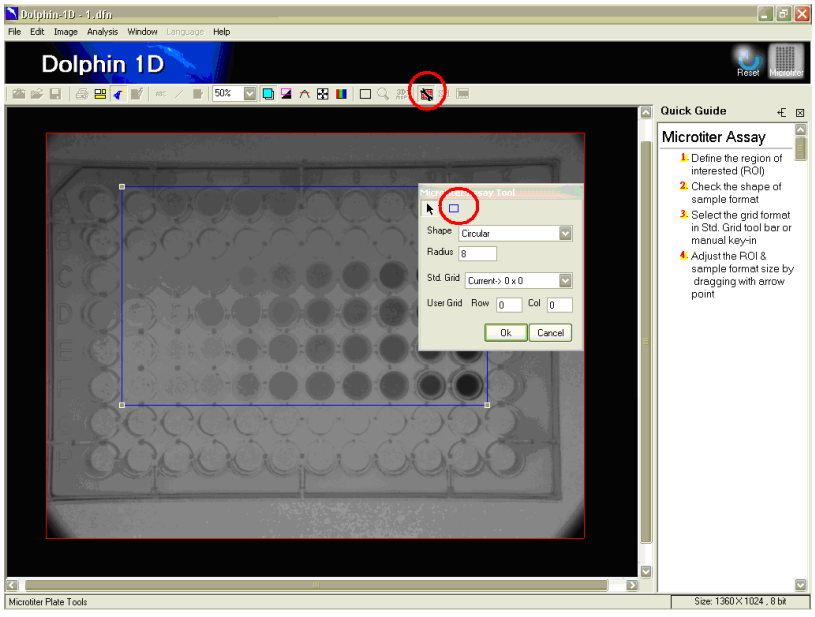

Figure 3. *Open the tool-box, either under "analysis" in the main menu, by pressing the monkey-wrench key in the row of buttons above in the captured image, or by clicking the designated area in the Quick Guide. Choose ROI by using the blue square in the tool-box and then marking the wells of interest. The position of the blue square can be adjusted by clicking either side of the square and then dragging the pointer in either direction.*

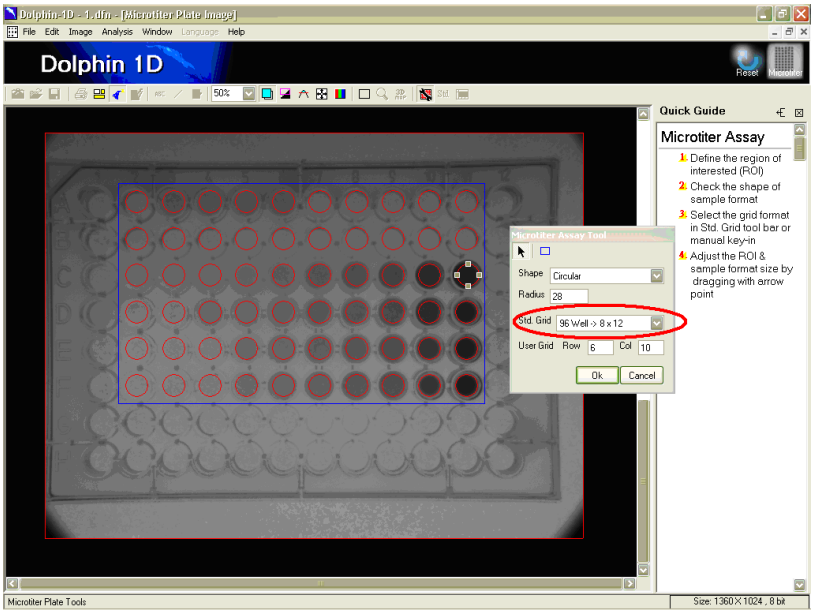

Figure 4. *Choose grid format (circular or square as well as layout of microtiter plate. Key in the number of used rows and columns. Adjust the radius of the circles covering the wells. The position of a single circle can be fine-tuned by placing the pointer on a circle and then dragging it in either direction. By pressing a circle and the dragging inwards or outwards, all circles change size simultaneously.*

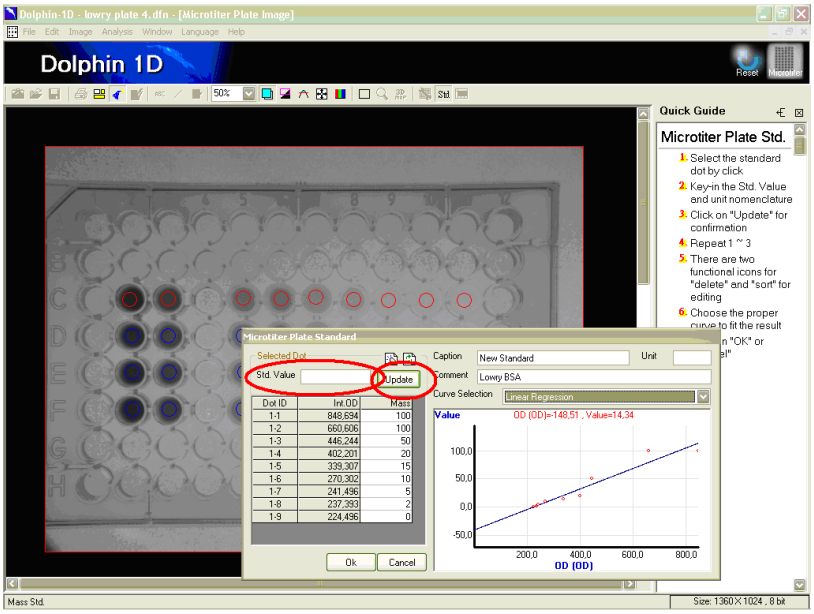

Figure 5. *In order to calculate protein concentration directly in Dolphin-1D, press the "Std"-box in the row above the captured image. Place the pointer and click on the well containing the smallest concentration. The well becomes marked by a red arrow. Update the concentration of that particular well by keying in the concentration in the empty field and then pressing "update". Continue to the next sample in your standard. When all the standards are updated, press "OK" to enter the report window, where the concentration in each well has been updated according to the keyed in standard values.*

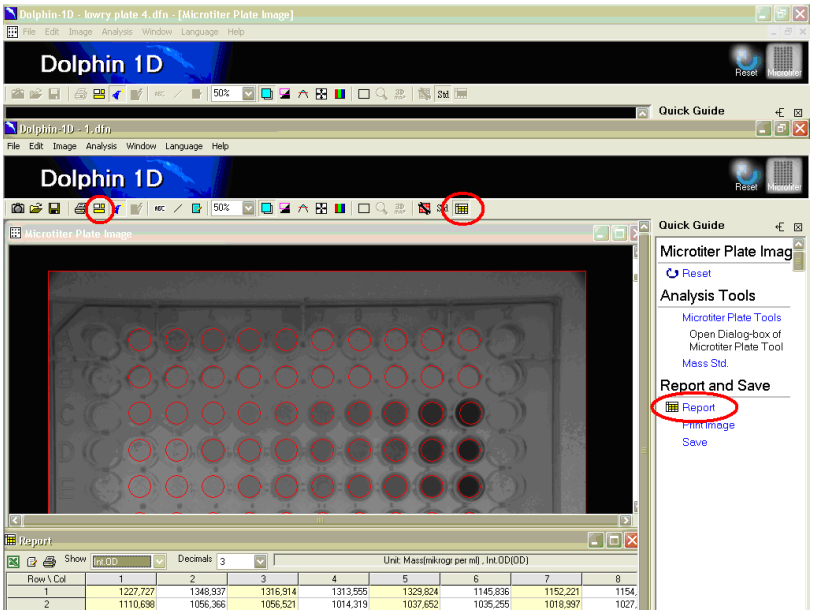

Figure 6. *When all wells are covered, the data can be viewed in the report window by pressing the "Report"-button. To export data to excel for further analysis, press the Microsoft Excel-sign in the upper left corner of the report window.*

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