Dot-it-Spot-it Total Protein Assay

INSTRUCTIONS
Issued March 2024

Dot·it·
Spot·it

Dot-it-Spot-it Total Protein Kit.
Store reagents refrigerated.
Shipping at ambient temperature.

TABLE OF CONTENTS

INTRODUCTION

- Dot-it-Spot-it measures the total protein concentration.
- One µL of sample mixture (application mixture) is applied on the Detection Sheet, which has positions for 20 samples.
- Sample proteins are binding to the Detection Sheet during a short incubation time.
- A black Detection Solution is flowed over the sheet and binds to the proteins, followed by a washing step.
- Finally the sheet is dried and mounted on a template, and the blackness intensity is quantified by the use of an image scanner.

REQUIRED ITEMS

Included in the Dot-it-Spot-it kit Store the reagents refrigerated. * The vial with Dilution Buffer needs to be warmed before use to dissolve the precipitates.	• Detection Sheet • Detection Solution • Washing Solution • Dilution Buffer* • Mounting Template
Equipment included in the Start-up package	• Light source • Dispensing Template with clips • Reservoirs with lids • Tape roller
Other equipment	• Pipettes • Hair dryer - or similar • Scissor
Detection equipment	• Image Scanner (Epson Perfection V600 Photo - or similar)
Software	• ImageJ is recommended
Files to download: dot-it-spot-it.com/method	• Template for Scanner (xlsx) • Detection recommendations….

Start-up package:

1. Reservoirs with lids
2. Dispensing Template with clips

WARRANTY

The information presented here is accurate to the best of our knowledge. It is the responsibility of the user to verify the suitability of the supplied materials and procedures for a particular purpose. In this respect further processing made by the user may affect the results, in which event Maplestone AB disclaims all warranties expressed, implied or statutory, including the implied warranty of merchantability and fitness for use. Maplestone AB and its authorized distributors, in such event, shall not be liable for damages indirect or consequential. The product is intended for research only.

TECHNICAL ASSISTANCE

If you experience any problems or difficulties with the products, please contact us.

Web: dot-it-spot-it.com
Email: info@dot-it-spot-it.com

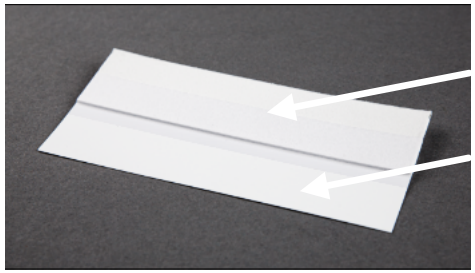
MANUFACTURE

Maplestone AB, Högåsvägen 205, SE-74141 Knivsta, Sweden

PROCEDURE

Precautions Only handle the Detection Sheet by the mounted thicker pad. Do not touch the thin nitrocellulose sheet. It will directly capture proteins from your hands, traces on your gloves and any settled dust.

Even tiny drops of saliva can be seen on the sheet after a good laugh - it really is a protein sensitive technique! See Note 1.



Detection Sheet

Thick pad: You can touch and write on it.

Thin sheet: A film of nitrocellulose on a polyester backing. Be careful, do not touch – it adsorbs proteins.

1. Preparations

Calibration standard
Bovine serum albumin (BSA) is often used as standard. Other proteins, or protein mixtures, with similar properties as the sample proteins might be preferred, see Note 2. It is important that the standard protein is in the same buffer as the samples. Prepare a dilution curve of the standard to each daily run.

Sample buffers
Dot-it-Spot-it assay can be used for a range of buffers containing both reducing substances and detergents. However, some additives in the sample buffer may restrict the percentage of sample you can use in the application mixture that you dispense to the Detection Sheet. Please refer to note 3 for a compatibility table featuring commonly used complex buffers and single buffer additives. In note 3, we refer to the compatibility for our Nano and Micro method.

Method	% sample	Application mixture	standard µg BSA/mL
Nano:	50	1 part of sample + 1 part DB	0.2-20
Micro:	5	1 part of sample + 50 part DB	2-200

Additionally, visit dot-it-spot-it.com/Results, Specificity and Compatibility, for updated information regarding sample buffer compatibility.

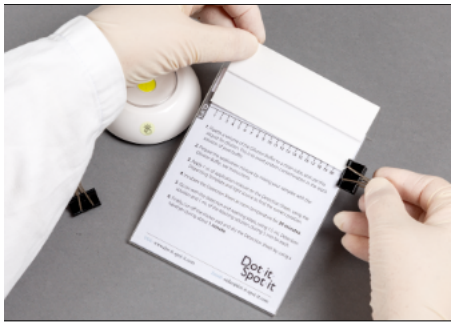
Application mixture
Take out the Dilution Buffer from the refrigerator and warm the vial until the precipitates are dissolved. Prepare the application mixture by mixing the sample/standard with the included Dilution Buffer (DB). Only 1 µL of application mixture is used for each dot replicate. The kit volume of Dilution Buffer is 15 mL, which is sufficient for using 50 µL per sample for dilution.

For a sample buffer that is fully compatible, such as TBS, you will achieve a more sensitive assay by increasing the amount of sample used in the application buffer. See the table below for details.

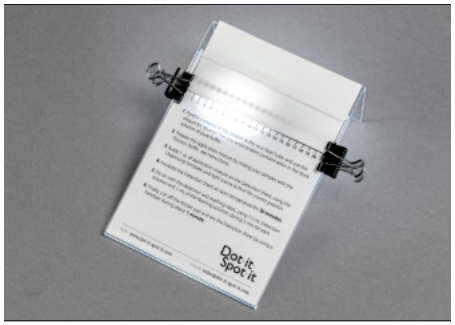
Sample in application mix	recommended low BSA standard µg/mL
50%	0,2
25%	0,4
10%	1,0
7%	1,4
5%	2,0
4%	2,5
2%	5
1%	10

2. Dispensing using a Template

1) Position the Detection Sheet on the Dispensing Template and use the clips to fasten it.



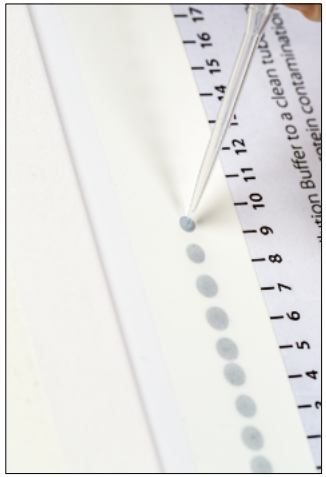
2) Place the light source under the Template and you will see the 20 circles through the thin nitrocellulose sheet.



3. Dispense and incubate

Dispense 1 µL of your application mixture on the nitrocellulose sheet in the designed position shown by the template.

Incubate the Detection Sheet at room temperature for 30 min.
The required incubation time may vary due to the temperature, humidity and air circulation in the laboratory.

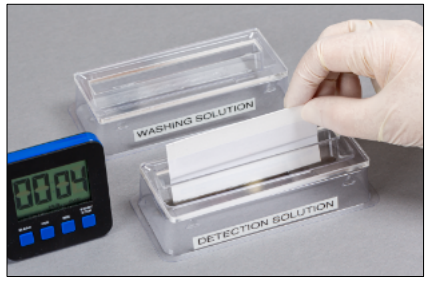


4. Detect and wash

This step requires the Detection and Washing Solutions. Invert the bottles before use. Fill up the two reservoirs, one with 1.5 mL of Detection Solution and the other with 1 mL of Washing Solution.

Take the Detection Sheet and press the tape and the thick pad together to make sure that liquid will be transported between nitrocellulose and the thick pad.

Place the nitrocellulose end of the Detection Sheet in the reservoir with the Detection Solution.



After 5 min, take up the Detection Sheet. Wipe off the surplus liquid at the end of the Detection Sheet and move the Detection Sheet to the Washing Solution reservoir.

After 5 min, take up the Detection Sheet. Wipe off the surplus liquid at the end of the Detection Sheet.

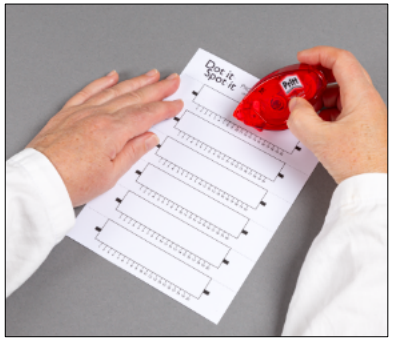
The reservoirs are washed with water and dried before next use.

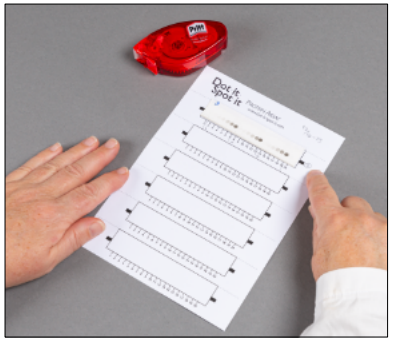
5. Dry and mount

Cut off the thicker pad using a scissor. Place the Detection Sheet on the backside of the Detection Template.

Dry the nitrocellulose sheet using a hair dryer (or compressed air), to avoid migration of the colour in the dot.

Use the tape roller to mount the nitrocellulose sheet on the Mounting Template.





Make sure the nitrocellulose sheet is completely dry before scanning.

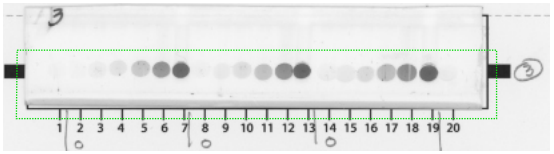
After scanning and calculating, dispose the Detection Sheet (thin nitrocellulose film on a polyester backing) with dots of protein in accordance with your biohazard waste disposal protocol.

6. Scanning

Make sure that both scanner area and nitrocellulose sheet are free of dust.

Position the Mounting Template with the nitrocellulose sheet against the glass surface. We have found that a position in the middle of the A4 short side, with the top of the Template against the short side, showed the lowest imprecision.

See Note 3 ,showing the required settings for Epson software



Scan the indicated area of the nitrocellulose sheet and save the tiff-file for further processing.

Follow Note 4 for quantifying the signal intensity of each dot using ImageJ and Excel.

7. Concentration estimation

Calculate the concentration for unknown samples by comparing their signal intensity values with the values for the calibration standard.
If plotting results by hand, a point-to-point fit is preferable to a linear fit.
Any software capable of four parameter logistic curve-fit, like the Rodbard function or similar, will work well for Dot-it-Spot-it.

A convenient curve-fitting can be obtained by using ImageJ – curve fitting, together with Excel, see Note 5.

NOTES

Note 1: Before starting

The Dot-it-Spot-it Total Protein Assay is an ultra sensitive test, using very low amounts of protein. When you are working with 0.1 nanogram protein you need to be aware of some pitfalls.

Make sure that you have clean tubes, clean pipette tips, clean gloves! Plastic consumables, like tubes and tips, may be manufactured using slip agents and plasticisers that can leach into the sample. We are using Forensic DNA Grade Eppendorf tubes, and Eppendorf epT.I.P.S, with good results. The tubes and tips are handled with clean gloves, we order tips for 1 µL already dispensed in trays. The immersion depth recommendation for tips are 1 mm for 1µL volume..

Do you generate additional proteins? If you place a finger on the nitrocellulose part of the Detection Sheet you may finally see the fingerprint. Saliva contains about 1 mg protein per mL, so be careful if talking or laughing. If you work with protein stock solutions of 1 mg/mL or higher, remember that the concentration is several thousand times higher than the concentration you measure, and your gloves might be contaminated. We work wearing a laboratory coat, disposable gloves, mob cap for covering the hair, and a medical mask.

Avoid protein adsorption and protease activity. For protein concentrations below 0.1 mg/mL there will be a substantial loss of protein to tube walls. Detergents, like Tween 20, are commonly added to reduce loss of protein. Presence of proteolytic enzymes can in some seconds degrade the proteins. Protease inhibitors, like EDTA, might be necessary to add.

Note 2:

Calibration curve

The need for a calibration curve (standard). Calibrate with a protein or protein mixture that corresponds to your samples.

The concentration of pure proteins can be checked by their absorbance at 280 nm, using 1 cm pathway in the photometer.

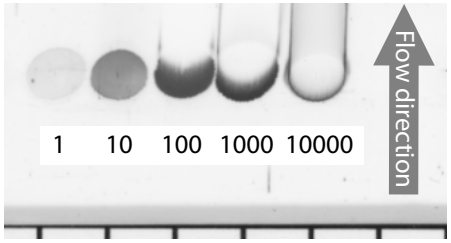
1 mg/mL BSA = 0.67 AU
1 mg/mL HSA= 0.53 AU
1 mg/mL IgG = 1.37 AU

Dilute your calibration protein in the same buffer as you have your samples in.

High samples

If you see very dark half-filled dots on the nitrocellulose sheet after testing, you might have too high protein concentration in your sample.

Below you can see the results for 1 and 10 µg/mL BSA in the application mixture, which are OK to measure. Samples with 100-10.000 µg/mL needs to be diluted.



Note 3: Sample Buffer Compatibility Tables

In addition to proteins, sample buffers contain various essential additives for extracting, dissolving, and storing proteins from biological samples. These additives may affect the results of total protein assays.

The tables below indicate the recommended maximum percentage of sample in the application buffer when using a particular sample buffer. They also specify the recommended BSA concentration of the lowest standard point.

Table 1 displays the results for several complex buffers.

Table 2 presents some individual additives tested in TBS buffer with 1 mM EDTA. Additionally, except for the Detergent group, 0.03% Tween 20 was included. Both zero and BSA samples were analysed. The additive was considered satisfactory if the results with and without the additive were similar.

Please use the information in the tables as a reference only. It is the user's responsibility to confirm the compatibility of their specific sample buffer.

Table 1

	Application mixture	Recommended low standard µg BSA/mL
Complex buffers that can be directly applied *Method Nano-special*		
Laemmli-B buffe mixed with PBS 33 mM TRIS, 5 mM phosph., 75mM NaCl 13.2% glycerol, 1.1% SDS, 0.005% BFB	100% sample	0,15
Laemmli-S buffer mixed with PBS 63 mM TRIS, 5 mM phosph, 75 mM NaCl 10% glycerol, 2% SDS, 0.004% BFB	100% sample	0,15
OGP buffer mixed with PBS 10 mM TRIS-buffer, 16 mM phosphate 1% OGP, 1 mM EDTA, 150 mM NaCl	100% sample	0,15
Complex buffers with limits *Method Micro with different percentage of sample in the application mixture*		
RIPA buffer Pierce 25 mM TRIS-buffer, 150 mM NaCl 1% NP-40, 1% DOC, 0.1% SDS	4% sample	2,5
Cell Lysis buffer 25 mM TRIS-buffer, 150 mM NaCl 1% Triton X-100, 5% glycerol, 1 mM EDTA	7% sample	1,4
Buffer systems *Method Nano, Method Micro*		
TBS: 20 mM TRIS buffer pH 7.5 0.15 M NaCl, 0.02% NaN3	50% sample	0,2
PBS: 20 mM phosphate buffer pH 7.5 0.15 M NaCl, 0.02% NaN3	50% sample	0,2

Table 2

recommended low BSA standard	Method Nano 0.2 µg/mL	Method Micro 2 µg/mL	unit
Detergents			
Tween® 20	0,1	1,0	%
Triton™ X-100	0,1	1,0	%
SDS	4	20	%
Reducing agents			
TCEP	40	400	mM
DTT (fresh)	20	200	mM
BME	5	50	%
Sugars			
Lactose	40	400	mM
GlcNAc	20	200	mM
Protease inhibitors			
cOmplete™, EDTA free, Roche	0.5 x	1 x	w.s.
w.s.= working solution			
EDTA	1	1	mM
PMSF	1	1	mM
Buffer additives			
Glycerol	5	50	%
EDTA	20	200	mM
urea	6	10	M
thiourea	2	2	M

Abbreviations

BFB:	Bromophenol blue
BSA:	Bovine serum albumin
BME:	2-Mercaptoethanol, beta-Mercaptoethanol
DOC:	Deoxycholic acid
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
GlcNAc:	N-Acetylglucosamine
PMSF:	Phenylmethylsulfonyl fluoride
SDS:	Sodium dodecyl sulphate
TCEP:	Tris(2-carboxyethyl)phosphine

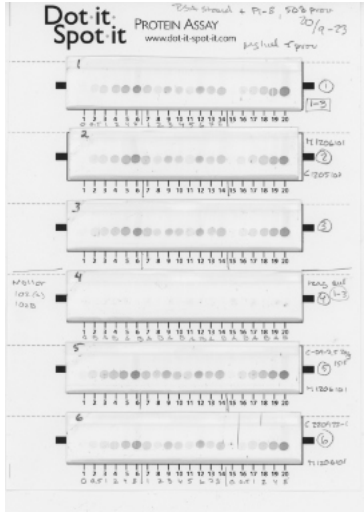
Note 4: **Scanning**

Instructions for using the software Epson Scan or SilverFast.

Ensure correct settings in the scan software:

- Document type: Photo,
- Output image: tiff-file.
- Bit depth: 16-bit gray scale.
- Pixel resolution: 600 dpi.
- Adjustments: It is important that the image is not adjusted by the software.
- Configuration/Colour: No colour correction.
- Gamma gradation: Epson scan 1.8, SilverFast 1.0.

If you like to have an overview image , save a pdf file of the complete Mounting Template..



Note 5: To get intensity values for each dot

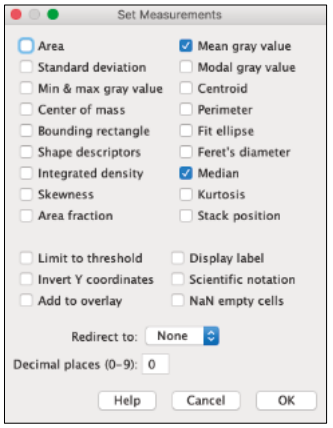
Download the free software **ImageJ**:
https://imagej.nih.gov/ij/download.html

Download the Excel file **Template for scanner**:
See dot-it-spot-it.com/method

Set the conditions for ImageJ measurement *(Set measurement... can be found under Analyze in the menu).*

Use measurement of Median and Mean gray value.

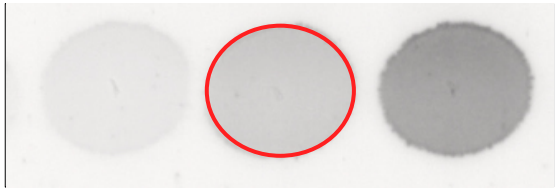
Set Decimal places (0–9) to 0.

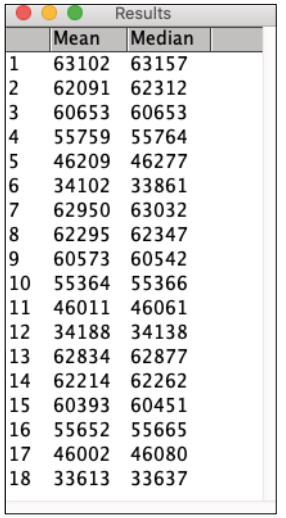


Open your tiff image of the Detection Sheet in Image J

Use the oval tool and measure the dot - use "Analyze/ Measure" or use the rapid command "M". An ImageJ results table will collect the values,..

We recommend to measure the whole dot area, but not outside this area, as shown in the image. However, if you see tiny black spots inside the dot you can limit the measurement area to avoid the contamination spots..





	Mean	Median
1	63102	63157
2	62091	62312
3	60653	60653
4	55759	55764
5	46209	46277
6	34102	33861
7	62950	63032
8	62295	62347
9	60573	60542
10	55364	55366
11	46011	46061
12	34188	34138
13	62834	62877
14	62214	62262
15	60393	60451
16	55652	55665
17	46002	46080
18	33613	33637

The values for the 20 dots are collected in an ImageJ results table.

The value represents the median reflected light per pixel. Reflected light: a dark dot gives low value, whilst a white dot gives a high value.

Copy the Image J results table into worksheet tab "1. Paste from ImageJ" in the Excel file **Template for scanner**. The median blackness per pixel (bpp) will be calculated as RESULTS. Blackness per pixel: a dark dot will have a higher bpp value than a light dot.

Dot-It-Spot-It Protein Assay — **Blackness per pixel (bpp), 16-bit** greyscale= 0, 1-65.535 levels

v. 210202

Blackness per pixel = bpp

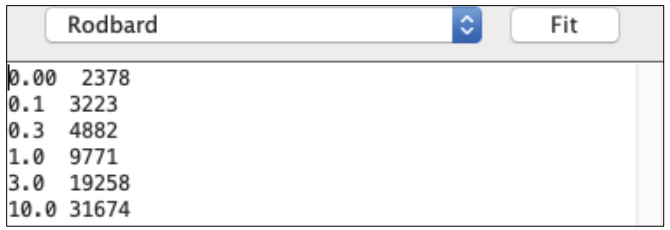
		RESULTS		bpp= 65.535-Reflected light			Reflected light PASTE FROM IMAGEJ	
Detection	Sample	Median					Mean	Median
SHEET	Position	bpp		Median bpp	Mean bpp	Mean/Median	Mean	Median
1	1	2 378,0	1	2 378,0	2 433,0	1,02	63102	63157
1	2	3 223,0	2	3 223,0	3 444,0	1,07	62091	62312
1	3	4 882,0	3	4 882,0	4 882,0	1,00	60653	60653
1	4	9 771,0	4	9 771,0	9 776,0	1,00	55759	55764
1	5	19 258,0	5	19 258,0	19 326,0	1,00	46209	46277
1	6	31 674,0	6	31 674,0	31 433,0	0,99	34102	33861
1	7	2 503,0	7	2 503,0	2 585,0	1,03	62950	63032
1	8	3 188,0	8	3 188,0	3 240,0	1,02	62295	62347
1	9	4 993,0	9	4 993,0	4 962,0	0,99	60573	60542
1	10	10 169,0	10	10 169,0	10 171,0	1,00	55364	55366
1	11	19 474,0	11	19 474,0	19 524,0	1,00	46011	46061
1	12	31 397,0	12	31 397,0	31 347,0	1,00	34188	34138
1	13	2 658,0	13	2 658,0	2 701,0	1,02	62834	62877
1	14	3 273,0	14	3 273,0	3 321,0	1,01	62214	62262
1	15	5 084,0	15	5 084,0	5 142,0	1,01	60393	60451
1	16	9 870,0	16	9 870,0	9 883,0	1,00	55652	55665
1	17	19 455,0	17	19 455,0	19 533,0	1,00	46002	46080
1	18	31 898,0	18	31 898,0	31 922,0	1,00	33613	33637
						1,01		

Note 6: Concentration estimation for unknown samples

A convenient curve-fitting and concentration estimation can be obtained by combining ImageJ – curve fitting, and the Excel file **Template for scanner**, worksheet tab "3. Concentration".

Calculate the mean bpp value for your standard replicates. Worksheet tab "2 Standard" might be useful, remember that ImageJ use dots and not comma".

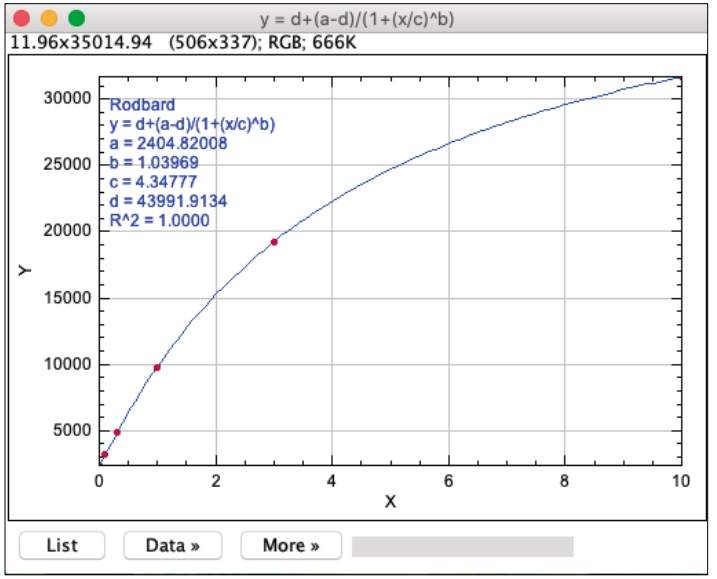
Curve fitting ImageJ: see Analyze – Tools – Curve Fitting, and choose Rodbard.

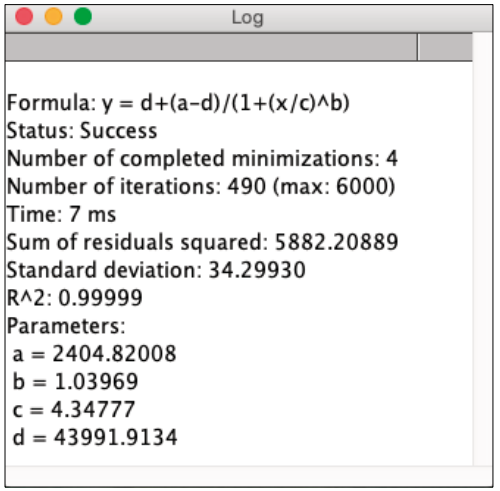


The first table row is for concentration values for your standard points, and the second for the obtained bpp values. Fill in your values and push Fit.

Parameters from ImageJ

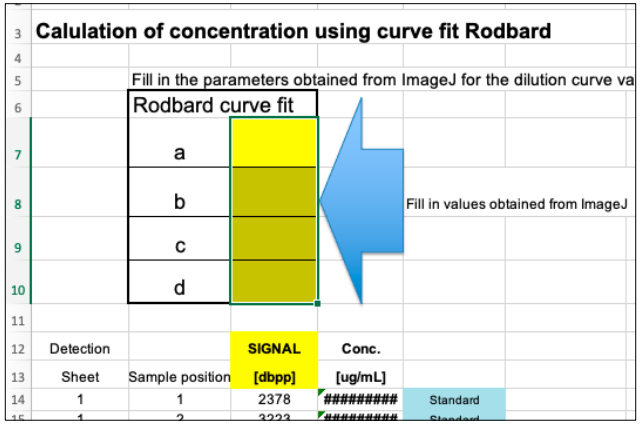
After fitting, you can see how the individual standard concentrations forms a standard curve. The parameters a-d will be used in Template of Calculation.



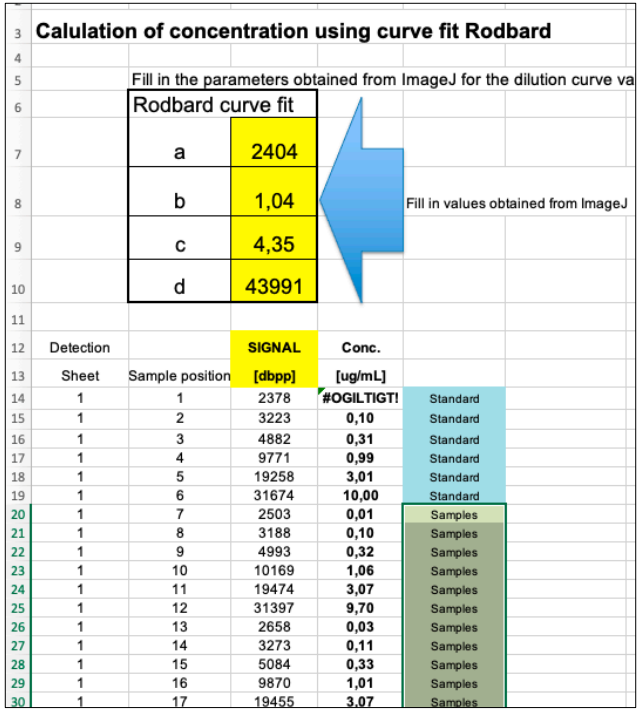


Paste parameters a-d from ImageJ into the Excel file

In **Template for scanner**, worksheet tab "3. Concentration", you can find the signal values from tab "1. Paste from ImageJ". Fill in the values for parameter a-d, and the concentration for the unknown samples will be calculated.



3	**Calulation of concentration using curve fit Rodbard**				
4					
5		Fill in the parameters obtained from ImageJ for the dilution curve va			
6		Rodbard curve fit			
7		a			
8		b		Fill in values obtained from ImageJ	
9		c			
10		d			
11					
12	Detection		SIGNAL	Conc.	
13	Sheet	Sample position	[dbpp]	[ug/mL]	
14	1	1	2378	#########	Standard



3	**Calulation of concentration using curve fit Rodbard**				
4					
5		Fill in the parameters obtained from ImageJ for the dilution curve va			
6		Rodbard curve fit			
7		a	2404		
8		b	1,04	Fill in values obtained from ImageJ	
9		c	4,35		
10		d	43991		
11					
12	Detection		SIGNAL	Conc.	
13	Sheet	Sample position	[dbpp]	[ug/mL]	
14	1	1	2378	#OGILTIGT!	Standard
15	1	2	3223	0,10	Standard
16	1	3	4882	0,31	Standard
17	1	4	9771	0,99	Standard
18	1	5	19258	3,01	Standard
19	1	6	31674	10,00	Standard
20	1	7	2503	0,01	Samples
21	1	8	3188	0,10	Samples
22	1	9	4993	0,32	Samples
23	1	10	10169	1,06	Samples
24	1	11	19474	3,07	Samples
25	1	12	31397	9,70	Samples
26	1	13	2658	0,03	Samples
27	1	14	3273	0,11	Samples
28	1	15	5084	0,33	Samples
29	1	16	9870	1,01	Samples
30	1	17	19455	3,07	Samples