

- 9) This example assumes that the first cell in your raw data table is at position B5, and the cell containing the AVERAGE positive growth calculation is in cell E14 - make sure to check that you insert the correct cell references for your own data as the locations may be different.
- 10) Note also that this formula uses the **absolute cell reference** for the positive control, so it is essential to place the dollar symbols before both letter and number of the positive control cell reference (but not the first cell reference).
- 11) Copy this formula and paste it into all 96 cells in a new table of 12 x 8 cells to yield the background corrected data.
- 12) This is done most easily by dragging the lower right green cross on the bottom right of the first cell down 8 rows, then letting go of the mouse button, then clicking the green cross again and dragging the cell 12 columns to the right to fill the table.
- 13) Your two blocks of data for the same 96-well plate should now look something like this, with the raw data in the upper table, and the background-corrected data in the lower table:

	A	B	C	D	E	F	G	H	I	J	K	L	M
1													
2	Phytotitre set 1: extracts 001 - 080												
3													
4		1	2	3	4	5	6	7	8	9	10	11	12
5	A	0.138	0.620	0.588	0.249	0.682	0.705	0.291	0.651	0.891	0.791	0.392	0.887
6	B	0.137	0.397	0.320	1.083	0.172	0.393	0.455	1.117	1.008	0.889	0.564	0.890
7	C	0.140	0.463	0.974	0.438	1.025	0.356	0.469	1.084	0.517	0.840	0.174	0.977
8	D	0.139	0.970	0.832	0.866	0.685	0.279	0.648	0.456	0.634	0.961	0.598	0.950
9	E	0.136	0.673	0.263	0.531	0.564	0.956	0.648	0.397	0.587	1.059	0.404	1.031
10	F	0.135	0.661	0.871	0.630	0.479	0.233	1.048	1.103	1.038	0.868	1.120	1.005
11	G	0.139	1.123	0.246	0.948	0.150	0.491	0.585	0.170	1.044	0.891	0.792	1.049
12	H	0.134	0.430	0.977	0.244	0.823	0.516	0.563	1.103	0.993	0.230	0.234	1.085
13													
14	Average of positive control values				0.137								
15													
16	Background corrected absorbance values												
17		1	2	3	4	5	6	7	8	9	10	11	12
18	A	=B5-\$E\$14	0.483	0.451	0.112	0.545	0.567	0.154	0.514	0.754	0.654	0.255	0.750
19	B	0.000	0.259	0.183	0.945	0.035	0.256	0.318	0.980	0.870	0.752	0.427	0.753
20	C	0.003	0.326	0.837	0.301	0.888	0.218	0.332	0.947	0.380	0.702	0.036	0.840
21	D	0.002	0.833	0.695	0.728	0.547	0.142	0.510	0.319	0.497	0.823	0.460	0.813
22	E	-0.001	0.535	0.126	0.394	0.426	0.818	0.510	0.259	0.450	0.922	0.267	0.894
23	F	-0.002	0.524	0.734	0.492	0.342	0.095	0.911	0.966	0.900	0.731	0.983	0.868
24	G	0.002	0.986	0.108	0.811	0.012	0.354	0.447	0.033	0.906	0.754	0.655	0.912
25	H	-0.003	0.292	0.840	0.107	0.685	0.379	0.425	0.965	0.856	0.093	0.097	0.948

- 14) Now repeat the same process for data from any other plates you have measured, ensuring that the absorbance values used for one background correction calculation are always from the same plate.
- 15) To help ensure you do not mix data from one plate with another plate during this process, you should insert a new worksheet in the same Excel file for each individual plate you have data for.
- 16) Double click the tab at the bottom of the page to change the name of the worksheet to indicate which plate each sheet refers to, e.g. "Plate-1 Exp 1", "Plate-5 Exp4" etc., as shown below:



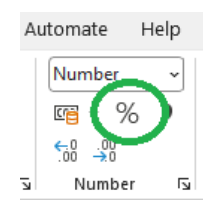
Normalisation of background-corrected data

- 1) Once you have background-corrected the raw absorbance values using the method above, you should progress to normalising the growth measurements to percentages of the control condition, which we define as the growth of cells in the absence of any TNF- α (i.e. the cells in column 12 of the plate).
- 2) We assume that cell growth in this condition is maximal, and we therefore assign it a value of 100% growth.
- 3) All other growth values are then calculated as a percentage of the maximum growth.
- 4) First we must calculate the mean (average) of the maximum growth values, which should be in wells A12 to H12 in the plate you set up.
- 5) Type "Max growth" in the first well of a row below the background corrected data table, and then in a separate cell to the right of it, insert the following equation:

=AVERAGE (M18 :M25)

- 6) This example assumes that the right-most column of values in your background corrected data table run from cell M18 to cell M25, but check your own data as the locations may be different.
- 7) Now, just below this, type a label for the normalised data table, for example: "Normalised cell growth data"
- 8) In the first cell of your new table, insert the following formula:

=B18/\$E\$27
- 9) This example assumes the first cell in your background corrected data table is at position B18, and the cell containing the AVERAGE max growth calculation is in cell E27 - make sure to check that you insert the correct cell references for your own data as the locations may be different.
- 10) Note also that this formula uses the **absolute cell reference** for the max growth value, so it is essential to place the dollar symbols before both letter and number of the max growth cell reference (but not the first cell reference).
- 11) Now click on the percentage icon in the Home ribbon near the top of the page to reformat the cell to show the new calculation as a percentage.
- 12) Now copy and paste, or drag and fill, this formula into every cell of a 12 x 8 table to give all of your normalised growth values.
- 13) Your max growth average calculation cell, and normalised data table should now look something like this:



	A	B	C	D	E	F	G	H	I	J	K	L	M
15													
16	Background corrected absorbance values												
17		1	2	3	4	5	6	7	8	9	10	11	12
18	A	0.001	0.483	0.451	0.112	0.545	0.567	0.154	0.514	0.754	0.654	0.255	0.750
19	B	0.000	0.259	0.183	0.945	0.035	0.256	0.318	0.980	0.870	0.752	0.427	0.753
20	C	0.003	0.326	0.837	0.301	0.888	0.218	0.332	0.947	0.380	0.702	0.036	0.840
21	D	0.002	0.833	0.695	0.728	0.547	0.142	0.510	0.319	0.497	0.823	0.460	0.813
22	E	-0.001	0.535	0.126	0.394	0.426	0.818	0.510	0.259	0.450	0.922	0.267	0.894
23	F	-0.002	0.524	0.734	0.492	0.342	0.095	0.911	0.966	0.900	0.731	0.983	0.868
24	G	0.002	0.986	0.108	0.811	0.012	0.354	0.447	0.033	0.906	0.754	0.655	0.912
25	H	-0.003	0.292	0.840	0.107	0.685	0.379	0.425	0.965	0.856	0.093	0.097	0.948
26													
27	Average of negative control values				0.847								
28													
29	Normalised cell growth data												
30		1	2	3	4	5	6	7	8	9	10	11	12
31	A	0%	57%	53%	13%	64%	67%	18%	61%	89%	77%	30%	89%
32	B	0%	31%	22%	112%	4%	30%	38%	116%	103%	89%	50%	89%
33	C	0%	38%	99%	35%	105%	26%	39%	112%	45%	83%	4%	99%
34	D	0%	98%	82%	86%	65%	17%	60%	38%	59%	97%	54%	96%
35	E	0%	63%	15%	46%	50%	97%	60%	31%	53%	109%	31%	106%
36	F	0%	62%	87%	58%	40%	11%	108%	114%	106%	86%	116%	102%
37	G	0%	116%	13%	96%	1%	42%	53%	4%	107%	89%	77%	108%
38	H	0%	35%	99%	13%	81%	45%	50%	114%	101%	11%	11%	112%
39													

- You may notice that some values show more than 100% growth - this is normal, as it reflects typical variation in the experimental methods and measurements.
- If your experiment and normalisation have gone well, you should see that the percentage growth values in column 1 of your table should all be close to 0%, the middle values in the table should be a mix ranging from 0% to 100%, and the values in column 12 should be around 100%.

Interpretation of the data

- Remember that high concentrations of TNF- α should kill the L929 cells in this assay.
- Macrophage cultures that produced high levels of TNF- α should therefore result in wells with LOW levels of blue colour, and percentage absorbance, in this assay.
- As we are seeking inhibitors of TNF- α production, those wells with HIGH levels of blue colour, and percentage absorbance, are the “hits” we want to find.

Modification of analysis protocol for the TNF- α quantification assay

- A more advanced version of the same L929 cell assay can be used to quantify the amount of TNF- α produced by macrophages, by including of a series of dilutions of recombinant TNF- α on the same plate receiving macrophage supernatants.
- In this version of the assay, the positive controls and negative controls will be in different places on the plate.
- Repeat the same analytical procedure as shown in the steps above, but modify the cell references to ensure the negative and positive controls point to the correct position on the modified plate layout for this version of the assay.

Notes

- If you are struggling to follow these instructions, you may find it helpful to first read the advice given in **Method sheet 101**, on Basic Data Handling techniques using Microsoft Excel.
- Remember to never modify the raw data files, but rather copy their values into a new, separate analysis file, which should be stored in a separate folder to further protect the integrity of the raw data.

- Don't worry if you see any values that are slightly below 0% growth, this is normal and reflects typical variation in the assay and measurements.
- If you do not see a clear difference between columns 1 and 12, the assay or calculation has failed, and you should repeat the Excel calculations first to check whether this was where the problem may have occurred.
- For further troubleshooting, if a blue colour is present in every well of the plate, including in column 1, even after careful washing, this suggests there was either no production of TNF- α by the macrophages, or the L929 cells were not properly sensitised to TNF- α by actinomycin-D treatment.
- If there is no blue colour remaining in any wells of the plate after crystal violet staining and washing, this suggests a very low number of L929 cells were plated initially (a cell counting or seeding error, easy to check by microscopy before adding macrophage supernatants).
- If there is very high variation between the different wells within column 1, or within column 12, this suggests that the washing technique was not sufficiently rigorous, when repeating the experiment be sure to wash the cell layer gently after staining and tap the plate gently on a pad of tissues to remove excess dye after each wash.

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