Statistical Analysis – How to assess significance!

When doing differential HDX-MS analyses (e.g. of a protein-ligand interaction), it is important to assess the significance of each analyzed site. The individual measurements e.g. in the absence and presence of a ligand should be carried out at least in triplicates, to allow thorough statistical analysis.

In general, two different approaches can be followed:

1) Calculate significance of every single measurement, e.g. for each peptide at each time point.
2) Calculate a common cutoff value for all significant differences.

Overview

1. *Single measurement comparison*
    - *Significance in GraphPad*
    - *Significance in Excel (spreadsheet "Significance in Excel")*
    - *Significance in Excel (spreadsheet "Deuterium Uptake Curves V9_t-test_F-test")*
    - *Explanation of the Student's T-Test in the spread sheet "Deuterium Uptake Curves V9_t-test_Ftest"*
2. *Defining statistical significance using a common cutoff value for all significant differences*

1. Single measurement comparison

To do this statistical analysis, you need triplicate data for all the states and time points, you want to compare.

The absolute deuterium uptake of each proteolytic peptide in both states should be compared using an unpaired, two-tailed t-test (according to Schriemer et al. *Chem Biol* **2010**, 17, pp. 725-734). This can be done either in Excel (using the Data Analysis Add-in), or in GraphPad.

Significance in GraphPad (GraphPad document "Significance BL151")

1. Copy your data into GraphPad

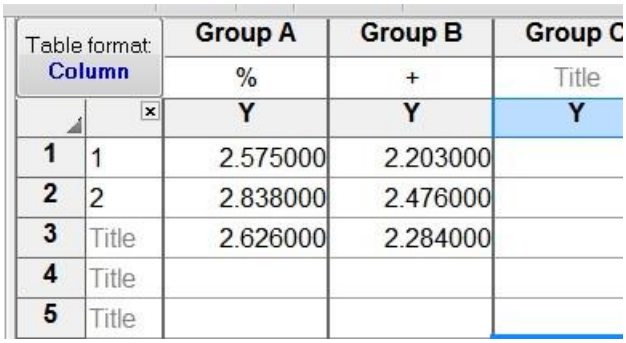
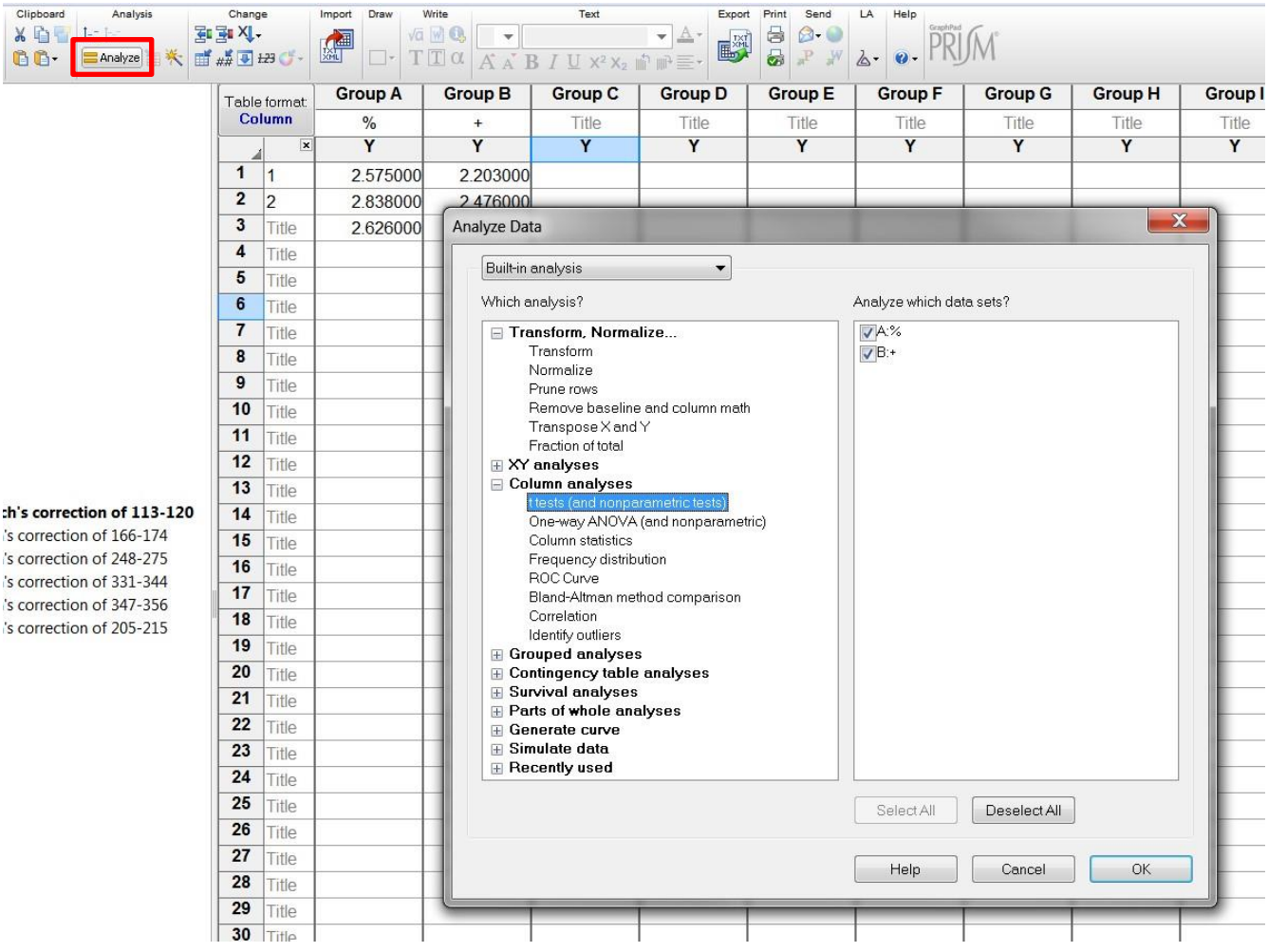
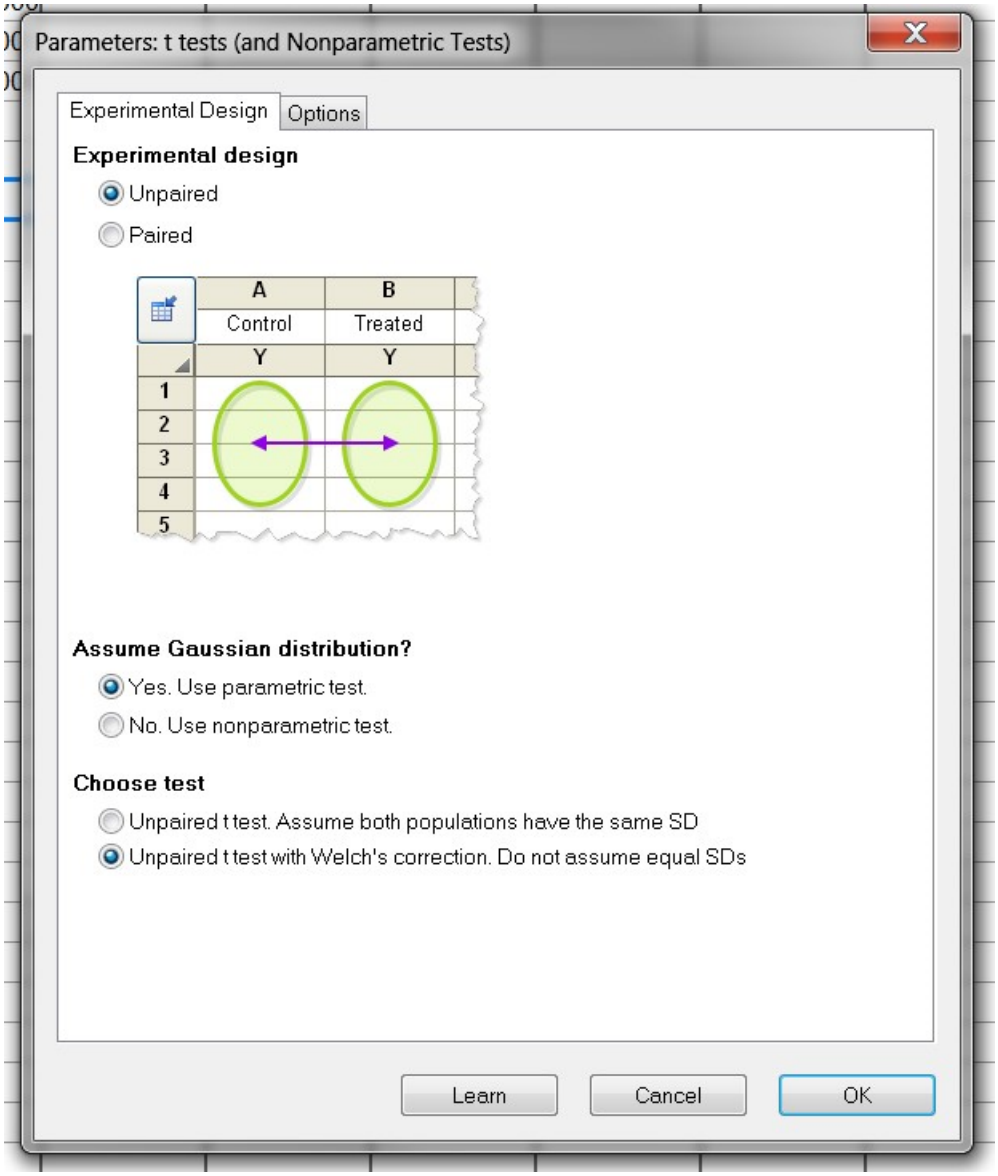


Table format: Column	Group A	Group B	Group C
	%	+	Title
	Y	Y	Y
1	2.575000	2.203000	
2	2.838000	2.476000	
3	2.626000	2.284000	
4			
5			

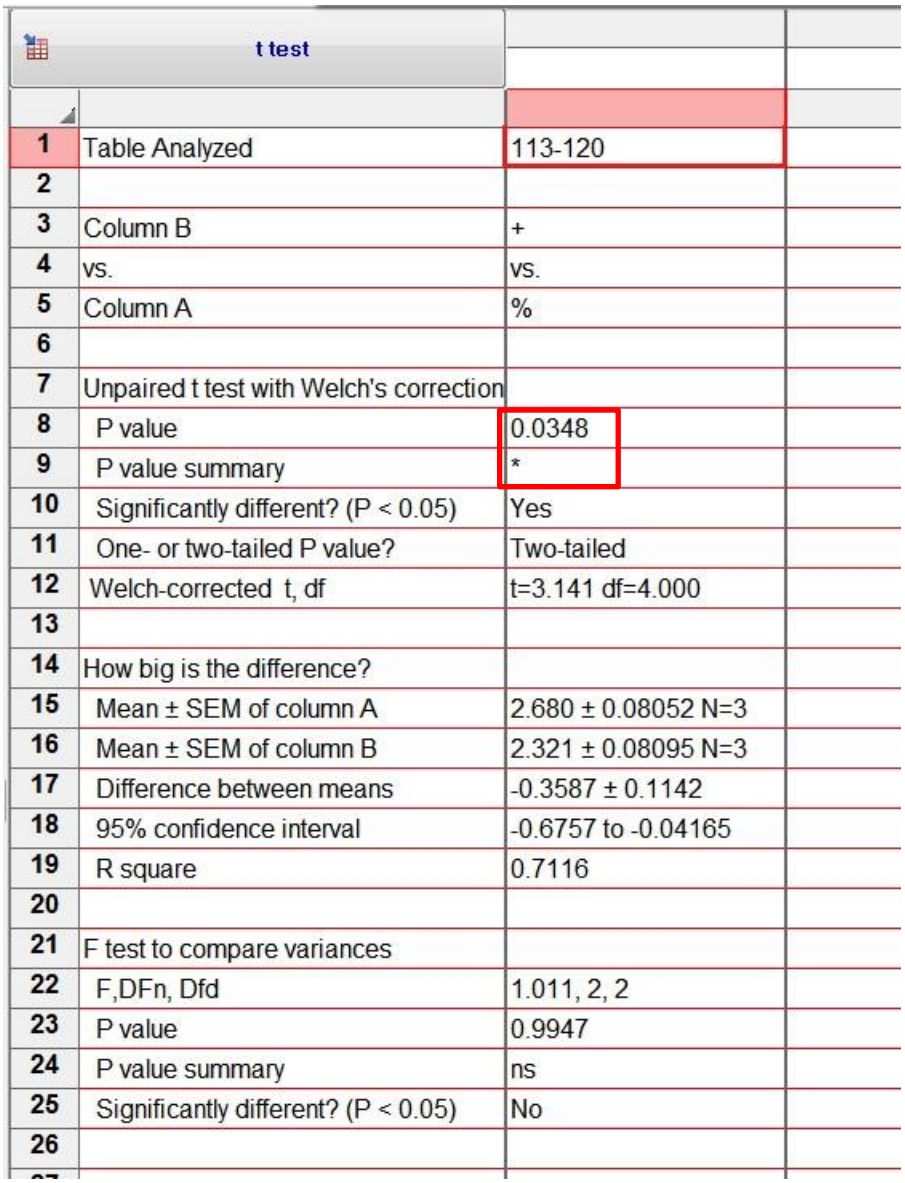
2. Click "Analyze" and choose "Column Analyses" > "t-tests"



3. Choose "unpaired, parametric t-test with Welch´s correction"



4. Check your p-value!



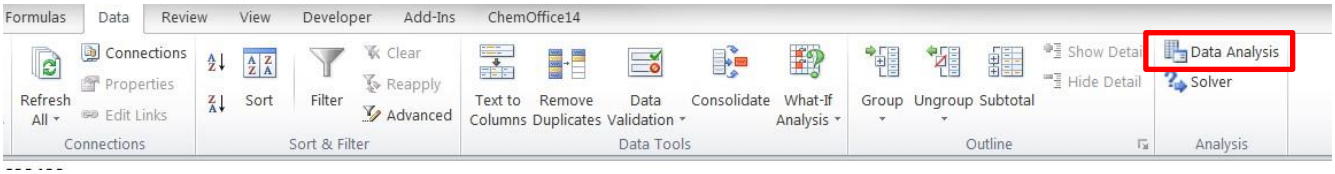
t test		
1	Table Analyzed	113-120
2		
3	Column B	+
4	vs.	vs.
5	Column A	%
6		
7	Unpaired t test with Welch's correction	
8	P value	0.0348
9	P value summary	*
10	Significantly different? (P < 0.05)	Yes
11	One- or two-tailed P value?	Two-tailed
12	Welch-corrected t, df	t=3.141 df=4.000
13		
14	How big is the difference?	
15	Mean ± SEM of column A	2.680 ± 0.08052 N=3
16	Mean ± SEM of column B	2.321 ± 0.08095 N=3
17	Difference between means	-0.3587 ± 0.1142
18	95% confidence interval	-0.6757 to -0.04165
19	R square	0.7116
20		
21	F test to compare variances	
22	F,DFn, Dfd	1.011, 2, 2
23	P value	0.9947
24	P value summary	ns
25	Significantly different? (P < 0.05)	No
26		

Significance in Excel (spreadsheet "Significance in Excel") 1.

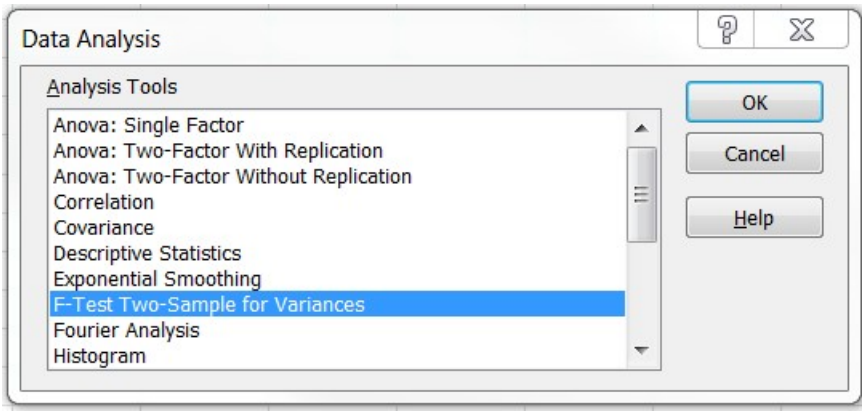
Copy your data into Excel.

	A	B	C
1	without ligand	with ligand	
2	2.575	2.203	
3	2.838	2.476	
4	2.626	2.284	
5			
6			

2. Open the *"Data Analysis"* Add-in, in the **Data** tab in Excel.



3. Choose "F-test Two-sample for Variances"



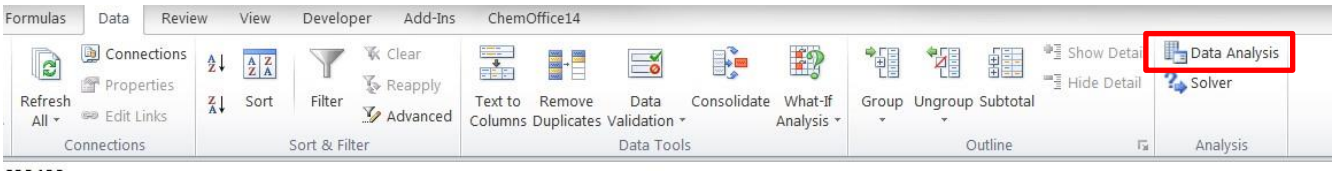
4. Check the p-value!

F-Test Two-Sample for Variances

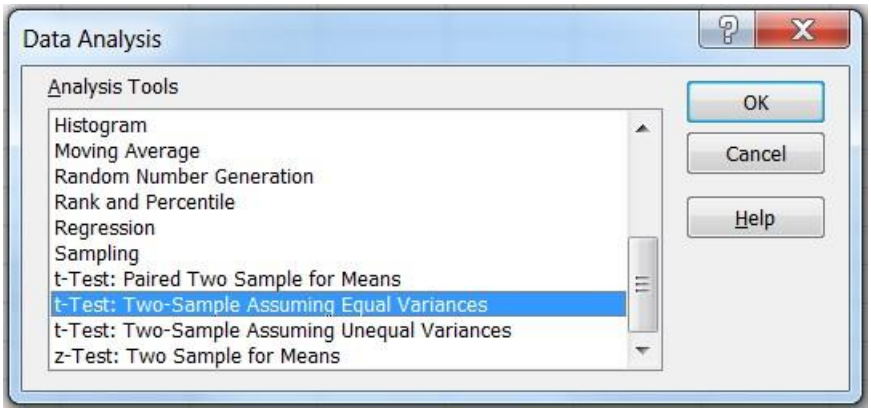
	Variable 1	*Variable 2*
Mean	2.321	2.679667
Variance	0.019659	0.019452
Observatic	3	3
df	2	2
F	1.010624	
P(F<=f) on	0.497358	
F Critical o	19	

If the P-value is below 0.05 perform a *"t-test: Two Sample Assuming Equal Variances"* if the P-value is above 0.05 perform a *"t-test: Two Sample Assuming Unequal Variances"*.

5. Open the *"Data Analysis"* Add-in, in the **Data** tab in Excel.



6. Choose *"t-test: Two Sample Assuming Equal Variances"*.



7. Check the p-value!

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	2.679667	2.321
Variance	0.019452	0.019659
Observations	3	3
Pooled Variance	0.019556	
Hypothesized Mean Difference	0	
df	4	
t Stat	3.141234	
P(T<=t) one-tail	0.017403	
t Critical one-tail	2.131847	
P(T<=t) two-tail	0.034806	
t Critical two-tail	2.776445	

An example showing a complete significance analysis of a full protein is shown in the Excel spreadsheet "Summary Triplicate & Duplicate Analysis".

Significance in Excel (spreadsheet "Deuterium Uptake Curves V9_t-test_F-test")

If you do statistical analysis using t-test in the spreadsheet *"Deuterium Uptake Curves V9_t-test_Ftest"* follow the steps below

1) Click on the **Statistics** chart. The background of the statistical analysis is described in the protocol named **Statistical Analysis of Differential HDX Data**. OBS!! The spreadsheet is only able to compare State 1 and State 2. If you want to compare more than two states a one-way ANOVA analysis is the correct statistical analysis to perform.
2) Define the sample size of state 1 and state 2 in G2 and G3. (Figure 5)
3) Define the number of tails of the perform student's T-test. Should for all general purposes be 2 as a perturbation of conformation can results both in a decrease or an increase in deuterium uptake.
4) Set the α-levels of T-test (G5) and the F-test (G7). The default and recommended levels are 0.01 and 0.05, respectively.
5) You are now ready to assess the statistical results. All time points showing a significance difference in deuterium uptake have a p-value below 0.01 and the **Significance** cells turns green for the time points.
6) A peptide showing a significant difference in deuterium incorporation at two consecutive time points are considered to have underwent a significant conformational perturbation.

	A	B	G	H	M	N	S	T	Y	Z	AE	AF
1												
2			3	Sample size of state 1 (n1)								
3			3	Sample size of state 2 (n2)								
4			2	tails								
5			0.05	T-test Alpha								
6												
7			0.05	F-test Alpha								
8												
9												
10	Peptide		0.25		1.00		10.00		60.00		240.00	
11	Start	End	Significance	P-value	Significance	P-value	Significance	P-value	Significance	P-value	Significance	P-value
12	2	10	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
13	9	23	No	0.103258349	Yes	0.00958	Yes	2.69621E-05	Yes	0.000544434	Yes	0.00068766
14	10	23	No	0.055592094	No	0.42498	Yes	2.03561E-05	Yes	9.95947E-05	Yes	0.000651365
15	24	33	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
16	0	34	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
17	35	60	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
18	38	60	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
19	0	61	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
20	42	60	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
21	61	82	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
22	0	84	Yes	0.001442135	Yes	0.00015	Yes	8.29905E-07	Yes	3.2743E-05	Yes	9.68218E-08
23	62	84	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
24	85	99	Yes	6.08607E-48	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Yes	1.16411E-47	#DIV/0!	#DIV/0!
25	0	103	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
26	104	110	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
27	111	123	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Yes	1.04771E-48	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
28	124	131	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
29	128	139	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
30	132	139	No	0.159195108	No	0.85004	Yes	0.003306069	Yes	0.001390858	No	0.223313299
31	140	156	Yes	1.37939E-50	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	Yes	1.51744E-50
32	0	157	Yes	0.002970981	Yes	0.00023	Yes	0.000441827	Yes	2.53481E-06	No	0.06237096
33	0	159	Yes	1.1544E-05	Yes	0.00472	Yes	1.09547E-05	Yes	7.25727E-06	Yes	0.000557509
34	169	180	Yes	0.00680199	Yes	0.00246	Yes	0.000415277	Yes	0.00083963	Yes	0.001086843

Figure 4. Example of the **statistics** sheet. The red box marks the changeable parameters for the statistical analysis.

Final preparation of supporting figure.

7) Open the saved pdf-file of the powerpoint slides and add a star above every significant time point at each peptide. Export the file as a PNG-file.

Advantages, Disadvantages and Alternative Method

Advantages
-relatively fast
-little copy and pasting of data
-generates to Powerpoint so it can be used for publication

Disadvantages
-error bars disjoined from the original data set
-works with Excel and Powerpoint but Macro might have some problems with different versions
-the graphs used for publication may need to be redone in illustrator as Excel may not give high resolution images

Alternative Method

-One simple method is to utilize OriginLab or GraphPad Prism.

-After making the Pivot Table, copy and paste data into a worksheet

-Graph different deuterated sequences and overlay stacks.

-Make an array to make multiple graphs

Explanation of the Student's T-Test in the spread sheet "Deuterium Uptake Curves V9_t-test_F-test"

By performing three technical replicates of a measurement we can apply statistical analysis to determine a significant difference instead of using gut-feelings, (e.g. >0.5 da) or pseudo statistical analysis (>2 x S_D). Furthermore, the sensitivity of the HDX method increases markedly as SD<0.1 Deuterium is reachable by manual sample-handling.

In a standard HDX-MS experiment where you want to look at you system in **two** different states (e.g with and with out ligand, stressed and native protein, protein at native vs. formulation conditions, etc.), the correct statistical analysis to perform is a *Student's T-Test* either assuming equal variance or not. If you have to compare more than two states the proper statistical analysis to perform is a one-way ANOVA analysis. This can be performed in the GraphPad Prism software, but will not be described in this paragraph.

The first thing you have to determine is whether the variance of the measurements can be assumed to be equal. This can be performed by using a F-test:

$$F = \frac{{S_D}_1^2}{{S_D}_2^2}$$

The largest S_D always has to be the numerator. Compare the calculated F-value with the critical F-value, which can be found by using the F.INV.RT-function in excel. The degrees of freedom for the numerator and the denominator are n_1*-1* and n_2*-1*, respectively. Set the α-value to 0.05.

If the calculated F-value is below the critical F-value, the null-hypothesis (S_{D1}=S_{D2}) **cannot** be rejected, and a *Student's T-Test* assuming equal variances (Homoscedastic) should be performed. If the calculated F-value is above the critical F-value, the hypothesis **can** be rejected, and a *Student's T-Test* assuming non-equal variances (Heteroscedastic) should be performed.The biggest difference between the two t-tests is that you have more statistical power in a *Students T-Test*, where you can assume equal variances.

T-value for Student's T-test assuming equal variances:

$$T = \frac{Mean_1 - Mean_2}{\sqrt{\frac{(n_1 - 1){S_D}_1^2 + (n_2 - 1){S_D}_2^2}{n_1 + n_2 - 2}} * \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Compare the T-value with a critical T-value with n_1+n_2-2 degrees of freedom. This value can be calculated by excel with the function *T.INV.2T.* The common recommendation is to set the α-value to 0.01.

T-value for student's T-test assuming non-equal variances:

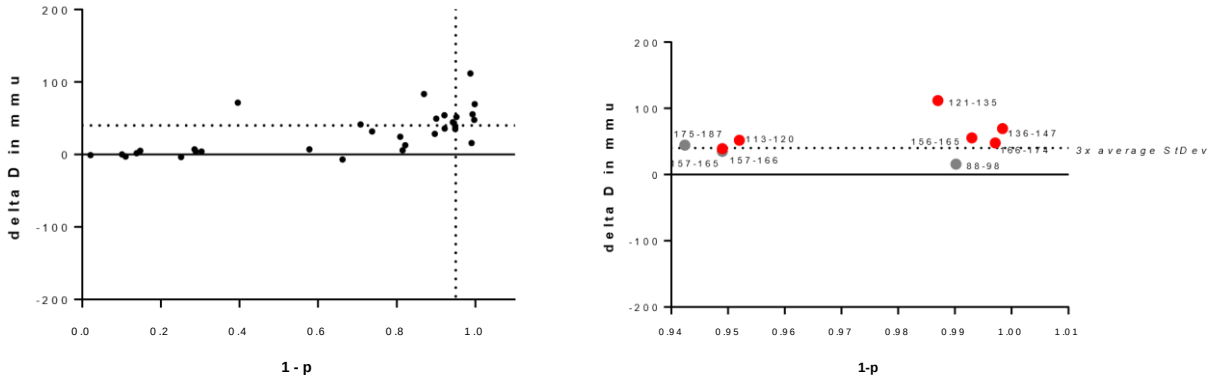
$$T = \frac{Mean_1 - Mean_2}{\sqrt{\frac{{S_D}_1^2}{n_1} + \frac{{S_D}_2^2}{n_2}}}$$

Compare the T-value with a critical T-value $\frac{\frac{1}{n_1}}{n_1 - 1} + \frac{\frac{2}{n_2}}{n_2 - 1}$ with $\frac{\left(\frac{{S_D}_1^2}{n_1} + \frac{{S_D}_2^2}{n_2}\right)^2}{\left({S_D}^2\right)^2 \quad \left({S_D}^2\right)^2}$ degrees of freedom. This value can be calculated

by excel with the function *T.INV.2T.* The common recommendation is to set the α-value to 0.01.

Lately a couple of papers have incorporated a second level of significant cut off by using an average SD as a second cut off (see MS studio) generating a plot similar to a volcano plot.

To present the data, a “1-p”-plot can be prepared, plotting the 1-p on the x-axis (for each peptide), and the difference in deuterium uptake in mmu (Da x 1000) on the y-axis. A horizontal line that indicates 3x the average standard deviation, and a vertical line indicating 1-p=0.95 can then illustrate which changes are significant / insignificant (Schriemer et al. *Chem Biol* **2010**, 17, pp. 725-734).



Parts of this approach are currently being implemented in the spread sheet “Deuterium Uptake Curves V9_ttest_F-test”

2. Defining statistical significance using a common cutoff value for all significant differences

As mentioned earlier, a critical step in differential HDX-MS data analysis, is the procedure to calculate a statistically-correct preset threshold to assign significance in D uptake, to use for subsequent coloring in Pymol. One way to do this, is to assign significance based on “confidence limit” (or confidence interval) as described in the literature. (Miller 2010, Harris 2010)

Confidence interval is defined as “a defined range, which we may reasonably assume that it includes the true value of a sample (assuming no systematic errors). Such a range is known as a confidence interval and the extreme values of the interval are called the confidence limits.” (Miller 2010, page 26) In the context of HDXMS data, 99% confidence limit =0.5 D implies that there is 99% probability that the true D uptake value is below 0.5 D confidence interval, thus any value above this limit indicates a meaningful change.

In order to calculate confidence interval, after exporting your data from DynamX to the template Excel macro using “DA_01_Generation of Uptake Plots in Excel-v 1.2.pdf” (available on G drive), choose the suitable approach to determine confidence interval based on your dataset. We classify possible dataset scenarios into 3 main cases, described below and summarized in Table 1:

Case 1. When you have **duplicate measurement** and no 100% control, then show your data based on the absolute difference in uptake: In this case you should (hopefully) have at least one single triplicate measurement of a "labeled sample" at a single timepoint and state. Calculate root-mean-square SD of that triplicate measurement and use this SD to calculate confidence interval.

A special case under Case 1: When you have triplicate measurement at a single time point of all states, and no 100% control, show your data based on the absolute difference in uptake. In this case, calculate root-mean-square SD in uptake in State A and B of the time point where you have triplicate measurement. Then the "SD of differences" is calculated as the pooled SD, $D = \sqrt{SD_A{}^2 + SD_B{}^2}$. If you present the data using the sum of deuterium uptake (i.e. uptake is independent of different measured time points), an accumulated SD is calculated,
$SD_{sum} = \sqrt{n * SD^2}$, here n is the number of all your time points. Use pooled SD (SD_{pool}) or accumulated SD (SD_{sum}) to calculate the confidence interval. See example on the G-drive.

Case 2. When you have **triplicate measurement and no 100% control**, again show your data based on the absolute difference in uptake. Here, calculate root-mean-square SD in uptake in State A and B across ALL timepoints. Then, the "SD of differences" is calculated as the pooled SD, $SD = \sqrt{SD_A{}^2 + SD_B{}^2}$.
Use this pooled SD to calculate the confidence interval.

Case 3. When you have **triplicate measurement with 100% control**, calculate the confidence interval based on "normalized difference". Note that the pooled SD used here, is "SD of subtraction of two normalized values" ($SD_{NormA-NormB}$).

Instead of taking the arithmetic mean of multiple SD from the same state, the SD of a state is found by use of root-mean-square (RMS):

$SD_{RMS} = \sqrt{\frac{\sum_N s_i^2}{N}}$, where s_i is all the SD's within the state and N is the total amount of peptides measured in replicates (e.g. if you have 67 peptides measured over 5 time points in triplicates, N=1005 (67*5*3) but if replicates are only for two time points, N = 402 (67*2*3)).

Table.1. Three approaches in determining confidence interval

Case	Replicates	Normalized deuterium content using fully labeled	Difference to show	SD for calculating confidence interval	SD to use
1	Duplicate	no	Absolute	Calculate root-mean-square SD from triplicates from a single time point	Root-mean-square SD $SD_{RMS} = \sqrt{\frac{\sum s_i^2}{N}}$
1a: A special case under case 1	Triplicate at a single time point of both state A and B	no	Absolute	Calculate root-mean-square SD from triplicate data of State A and B as in Case **1**, and use it to calculate a pooled SD Calculate accumulated SD across all time points using the pooled SD if the sum of deuterium uptake is used.	$SD_{pool} = \sqrt{s_A^2 + s_B^2}$ In cases where sum of deuterium uptake is used: $SD_{sum} = \sqrt{n \cdot SD_{pool}^2}$ See example on G drive
2	Triplicate for all time points	no	Absolute	Calculate root-mean-square SD from State A and B and use it to calculate a pooled SD Calculate accumulated SD across all time points using the pooled SD if the sum of deuterium uptake is used.	
3	Triplicate with 100% control	yes	Normalized	Calculate root-mean-square SD from State A, B and 100% control and use it to calculate a pooled SD	$SD_{Norm_A - Norm_B}$ (see below)

$$Norm_A = \frac{D_A}{D_{100\%}}, \quad Norm_B = \frac{D_B}{D_{100\%}}$$

$$SD_{Norm_A} = \sqrt{\left(\frac{SD_A}{D_A}\right)^2 + \left(\frac{SD_{100\%}}{D_{100\%}}\right)^2} \times Norm_A$$

$$SD_{Norm\,B} = \sqrt{\left(\frac{SD_B}{DB}\right)^2 + \left(\frac{SD_{100\%}}{D_{100\%}}\right)^2} \times Norm_B$$

Once you calculated the correct SD value, use it to calculate confidence limit from the equation for confidence interval determination for small sample sizes (n < 30):

$$Confidence\ limit = \frac{(t_{n\text{-}1}.SD)}{\sqrt{n}}$$ (Miller 2010)

where $t_{n\text{-}1}$ is the student t value for the sample size n and the desired confidence level for a two-tailed test. You can find t values for different confidence intervals here (Harris 2010).

The approach described above 1a is adopted from two papers from Houde et al and Arora et al.

Houde et al.

In the paper they conclude the following (Supporting figure 3):

1) There is no difference in SD for peptide length (max number of exchangeable BB amide hydrogens)
2) There is no difference in SD for different time points
3) There is no difference in SD for absolute uptake

They calculate the average mean SD from >1000 SD's

A confidence limit difference at a single time point with a 98% confidence interval is estimated by the following formula:

$$\pm\, t_{\propto/2} \times \frac{SD_{ave}}{\sqrt{n}}$$

Where the t-value used is for n-1 degree of freedom (n = 3).

Using the numbers from their experiments:

$$\pm 6.965 \times \frac{0.14Da}{\sqrt{3}} = \pm 0.56Da$$

$$SD_{Norm_A - Norm_B} = \sqrt{\left(\sqrt{\left(\frac{SD_A}{D_A}\right)^2 + \left(\frac{SD_{100\%}}{D_{100\%}}\right)^2} \times Norm_A\right)^2 + \left(\sqrt{\left(\frac{SD_B}{D_B}\right)^2 + \left(\frac{SD_{100\%}}{D_{100\%}}\right)^2} \times Norm_B\right)^2} =$$

$$\sqrt{\left[\left(\frac{SD_A}{D_A}\right)^2 + \left(\frac{SD_{100\%}}{D_{100\%}}\right)^2\right] \times {Norm_A}^2 + \left[\left(\frac{SD_B}{D_B}\right)^2 + \left(\frac{SD_{100\%}}{D_{100\%}}\right)^2\right] \times {Norm_B}^2}$$

They do round the number down to 0.5Da – why we do not know.

When they want to have a cut-off for the accumulated difference they use the standard propagation of error equation for a simple sum of variables for 5 time points:

$$SD_{sum} = \sqrt{{SD_{ave}}^2 + {SD_{ave}}^2 + {SD_{ave}}^2 + {SD_{ave}}^2 + {SD_{ave}}^2}$$

$$SD_{sum} = \sqrt{5 \times {SD_{ave}}^2}$$

Using the numbers from their experiments:

$$SD_{sum} = \sqrt{5 \times 0.14Da^2} = 0.31Da$$

They again calculate a confidence interval for the summed difference with equation above. Resulting in a summed cutoff to 1.1Da (I calculate 1.28, but again it is the rounding off).

With an average SD of 0.05Da, which is what we normally obtain, the cutoff is 0.2Da for a single time point and 0.45Da for the summed cutoff.

Arora et al.

In this paper, they follow the same methodology as Houde et al. to calculate a confidence interval for differences for a single time point, but they do not show the underlying equations.

The resulting limit they state for a 99% percentile of standard deviation is 0.28(same as the confidence interval described above). Calculating back this gives an average SD on 0.0489Da, which is similar to the average SD we normally obtain.

$$S_{99\%} = t_{99\%/2} \times \frac{SD_{ave}}{\sqrt{n}}$$

$$0.28Da = 9.925 \times \frac{SD_{ave}}{\sqrt{3}} \leftrightarrow SD_{ave} = \frac{0.28Da \times \sqrt{3}}{9.925} = 0.0489Da$$

They state that they are looking at differences and are then propagating the $S_{99\%}$ even further.

$$CI = \sqrt{{S_{99\%}}^2 + {S_{99\%}}^2} = \sqrt{0.28Da^2 + 0.28Da^2} = 0.396Da$$

With an average SD on 0.05Da, which is what I normally obtain, the cutoff is 0.405Da for a single time point.

NB.1. Note that if you have triplicate measurements (case 2, 3), you can also do the t-test. Use the "statistics" sheet in the template excel sheet for generation of deuterium uptake plots available on G drive (named as "Deuterium Uptake Curves V9_t-test_F-test"). Here, the significance threshold is defined based on the results of F-test and T-test, and you can use the green cells that indicate "Yes" (P-value <0.05) as significant changes.

NB.2. When you have more than 2 states, (fx. state A, B, and C) you can only use the t-test to compare state A with B and A with C. If you want to compare A with B, A with C and B with C, then you should use 1-way ANOVA.

References

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Protocol Log:

Date	Version	Comments/Changes	Responsible

XXXXXX	1.0	Original protocol	UL
2015-10-22	1.1	Protocol Log, Explanations of Statistical analysis in deuterium uptake curves V9	ET
2017-06-29	1.5	Defining statistical significance using confidence intervals	ZEN, ZW, ET
2017-07-10	1.6	Further integration of DA_01 and DA_10 into the current protocol	ZEN, ZW, ET
2019-09	1.7	Calculations updated to be based on RMSD and accumulated SD only calculated for sum of HDX	LT, KDR, TN, IRM