

Saturation Transfer Difference (STD) NMR

Setup for STD experiments

1. Acquire a ^1H spectrum of your ligand.
2. Make a solution of ligand and binding partner at EL (excess concentration of ligand) between 50 to 100. Use appropriate solvent, e.g., binding buffer, containing 10% deuterated solvent. Incubate if necessary. For proteins, working concentrations are generally $\sim 50\mu\text{M}$.
3. Acquire a ^1H spectrum of the mixture to determine the **O1** of the solvent. Shim for solvent suppression (convcomp if on cryoprobe, ordmax=6, tune all).

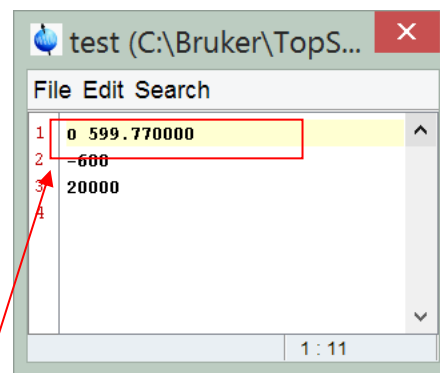
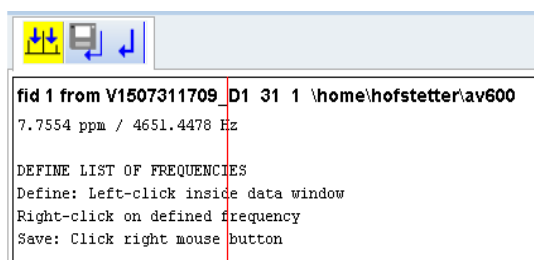
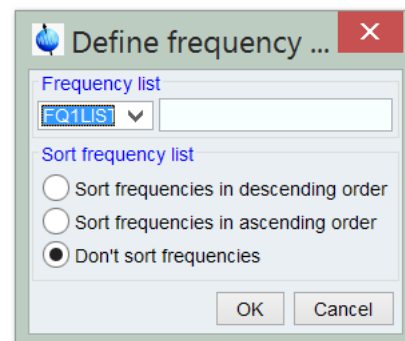
Note that for buffer and/or salt containing samples 90° pulses are usually very different from the ones stored in our instruments. Calibrate the pulse using “pulsecal”.

4. Read in the parameter set **H1_std-diff.UW** from /home/topspin.3.2/uwchem/par. Set **O1** to value of solvent. Set corrected pulses using “getprosol 1H ‘p1 [us]’ ‘pL1 [db]’.
5. In **ased** set the following other parameters:
 - **SPW9** – selective saturation pulse on F2. Sets the power for the saturation pulse **p42**. The default value is 0W|1000dB (meaning no power). It should be set between $1 \times 10^{-4}\text{W}$ [40dB] and $1 \times 10^{-6}\text{W}$ [60dB]. For larger proteins, set lower ($1 \times 10^{-6}\text{W}$ [60dB]).
 - **D29** – spin lock time for protein background suppression: 10 to 50 ms. Set to longer times for larger proteins.
 - **D20** – saturation time. Usually between 0.25s and 5s.
 - **D1** – relaxation delay, should be $1-5 \times T_1$, but make sure it is $\geq D20$. If $\text{DELTA1} = d1 - d31$ (saturation time as executed) < 0 , the experiment will not run, negative DELTAs are not allowed.
 - **NS** – should be multiple of 8
 - **DS** – should be 8 or 16

• **L4** – loop counter; enables interleaved acquisition: if **ns**=8 and **l4**=40, then 8 scans are acquired both on- and off-resonance before the next 8 scans are acquired, etc. until all 320 scans of each are acquired. When **ns**=320 and **l4**=0, then 320 scans are acquired on-resonance, then 320 scans off-resonance. Interleave may be important for longer experiments if the protein is not very stable.

6. Set on- and off-resonance frequencies to generate the frequency list. Two ways to do this are:

A. Type **.freqlist** (or use the user-defined icon) into the command line. Choose FQ1list and name your list. Click “ok” to save. In the new window define an on-resonance frequency (for proteins choose the very upfield methyl-groups at -3 to 0 ppm). Define an off-resonance frequency



far away from protein or ligand frequencies (± 30 to 40 ppm). Save parameters. In ased go to “lists”. Under FQLIST set newly created list as FQ2LIST, then edit. Remove the 1st line so only the entered frequencies are left in the file.

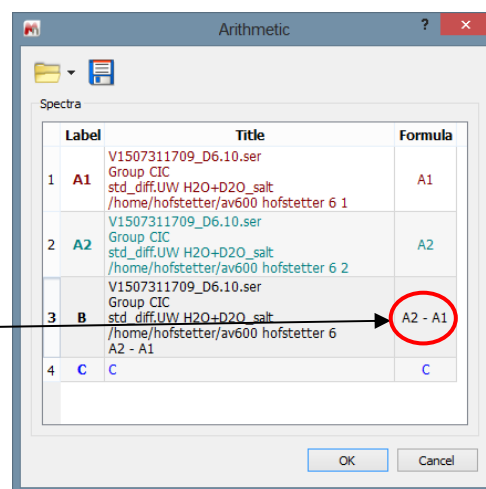
B. Go to lists \rightarrow FQLIST and choose the list displayed in FQ2LIST (in ased). Enter your known values manually to the list and save under a different name. Make sure list is chosen as irradiation frequency list.

Note: The on-resonance frequency needs to be optimized for each protein. This can be done varying the on-resonance in separate STD experiments or in an automated fashion using the above frequency list. Enter all on-resonance frequencies to be acquired first and then the off-resonance frequency. In the pseudo-2D setup change TD F1 to number of frequencies. Follow the instructions below and “stdsplit” will give you an STD spectrum for each on-resonance frequency.

7. Run **rga** and **zg** to acquire the spectrum. It will run as a pseudo-2D experiment. Use lb=1 for processing.

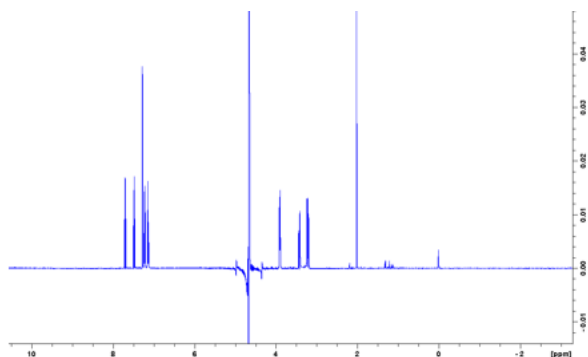
8A. Workup in MNova: The reference spectrum (off-resonance) and saturated spectrum (on-resonance) are created as a stack upon pulling the dataset into MNova.

After normal workup, subtract spectra from each other using Analysis \rightarrow Arithmetic..., and define the subtraction in the 3rd column.

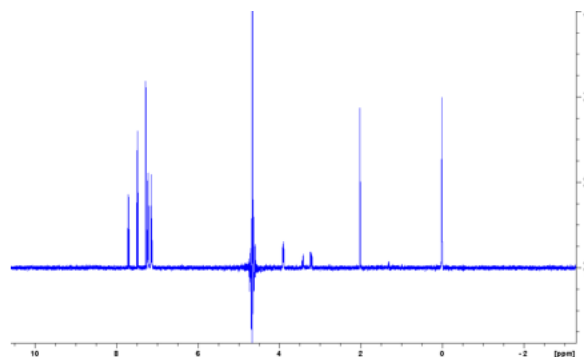


8B. Workup in Topspin: Run the au routine **stdsplit**. The routine will ask for expno to place the data: if you respond, e.g., with 1000, the off-resonance reference spectrum will be placed there, and the STD (difference) spectrum will go into 1001.

reference spectrum (off-resonance)



STD spectrum (difference)

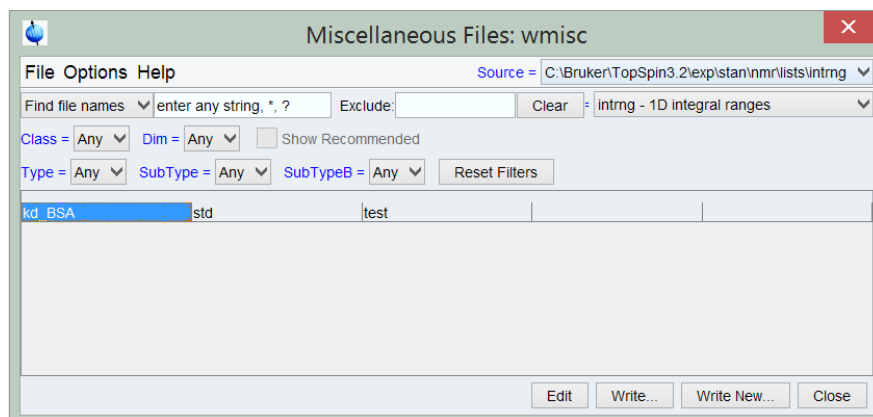


9. Optimize the STD experiment for values for on-resonance in **FQ2LIST**, **SPW9**, **D20** and **D29**.

Ligand mapping (epitope mapping) [analysis in TopSpin]

1. Run STD experiments on one sample (usually EL 50-100).
2. Choose a range of different saturation times **D20** between 0.25s and 5s. Make sure **D1** is either 0.5s longer than **D20** (this will speed up your overall experiment time – data will be ok) or set **D1** to $3 \times T_1$ (this will lead to longer overall experiment time, but provide more consistent data).
3. Work up spectra, carefully phase, correct the baseline and calibrate (if needed) all spectra.

4. Integrate one spectrum. In the TopSpin command line type **wmisc**. Select WRITE NEW and name this set of integration values.

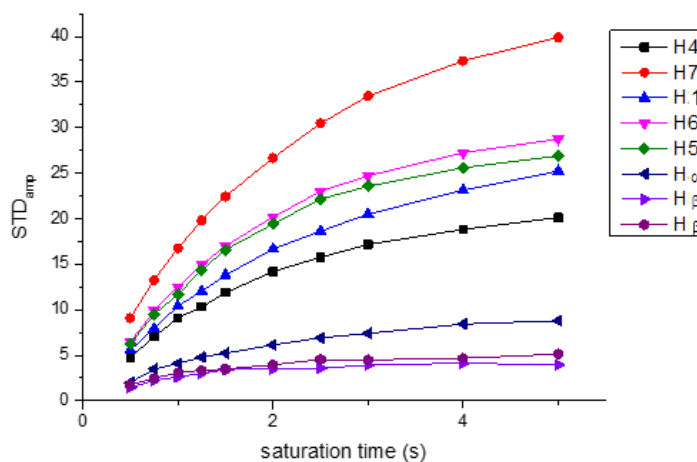


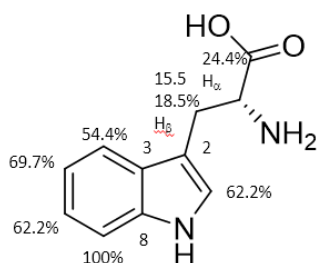
5. Work up all other spectra using the same integration file. Type **rmisc** into the command line to read in your integration file.
6. Make sure to go into the integration menu, right click on an integral and select “use lastscale for calibration” and save integrals.
7. Integrals can be found in processed data in the file “integrals”.
8. Determine the STD amplification factor (STD_{amp}) for the integrated protons using the following equation:

$$STD_{amp} \text{ of resonance} = \frac{STD_{diff} \text{ intensity of resonance } x}{STD_{off} \text{ intensity of resonance } x} \times \text{ligand excess}$$

9. Plot STD_{amp} versus saturation time for all protons.

10. At one saturation time, select STD_{amp} values for all protons. Set the highest STD_{amp} to 100% and calculate $STD_{\%}$ of maximum. This will allow you to easily compare the proximity of protons on your ligand to the binder.





L-Trp resonance	STD _{amp}	STD % of maximum
H4	9.11	54.37
H7	16.76	100
H1	10.42	62.17
H6	12.49	74.56
H5	11.68	69.70
H α	4.09	24.43
H β	2.60	15.49
H β	3.10	18.49

Determination of K_D

1. Run STD experiments on multiple samples with different EL. Prepare samples using the same stock solutions of ligand and protein. Keep the protein concentration constant but use different amounts of ligand.
2. Choose an optimized saturation time **D20** to run all the samples (see above for **D1** considerations).
3. Work up spectra, carefully phase, correct the baseline and calibrate all spectra.
4. Follow the above described work-up for Topspin, or use MNova for integration.
5. Determine STD_{amp} for the integrated protons as described above. Keep in mind that EL is now changing for each sample.
6. Plot STD_{amp} against the ligand concentration.
7. Determine K_D using a hyperbolic Michaelis-Menten type fit or linearize similar to Lineweaver-Burk plot.

$$K_D = \frac{\alpha_{STD}[L]}{STD_{amp} + [L]}$$

