GLYCOTwinning
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Summer School
25th—28th September '23
Scientific Hands–on course
in protein/glycan interactions:
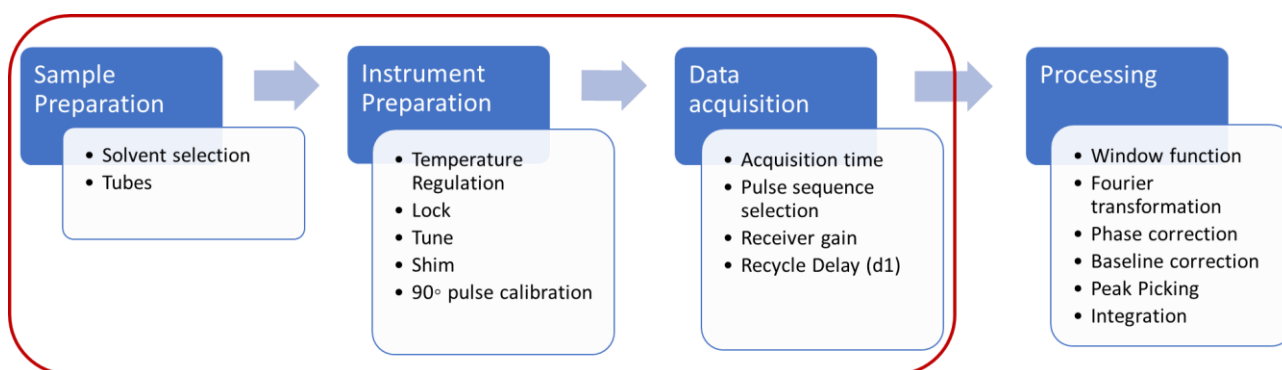a structural perspective.

NMR HANDS-ON PROTOCOLS – ACQUISITION

Workflow NMR experiments

Sample Preparation

Solvent selection

Historically, for liquid state NMR the sample is dissolved in deuterated solvents, in which ^1H atoms are replaced with ^2H atoms. The main reasons include deuterium lock, shimming, and providing an "invisible" solvent signal that will avoid a solvent-dominated spectrum.

The choice of solvent depends on the solubility and/stability of your sample, the location of the solvent chemical shift (is it near a region where my sample has resonances) and the structural information you want to obtain.

D_2O or H_2O/D_2O, that is the question: The choice of using D_2O or mixture of H_2O/D_2O (usually 90/10% (v/v)) depends on if you want to observe labile/exchangeable protons or not, which is particularly important for proteins if you want to observe NH of the peptide backbone. In this case, you should use H_2O/D_2O. For structural elucidation of carbohydrates, the choice usually is D_2O, since the anomeric protons are present near the solvent chemical shift.

NMR tubes

The choice of the proper NMR tubes depends on the amount of sample available, the field of the spectrometer and the type of experiments you want to run. For cryoprobes and/or high–field magnets do not use "disposal" and "economy" tubes. Cheap low-quality tubes not only will give worse data, due to the presence of paramagnetic metal impurities, but also due to their poor mechanical tolerance can cause disastrous damage to the probe. Use clean, unscratched NMR tubes and clean caps.

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Table 1: Types of NMR tubes and recommendations.
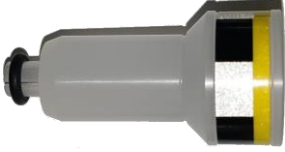
Tubes	Diameter	Sample volume	Comments
High-resolution	5 mm	500 µL	Most common
High-resolution	3 mm	200 µL	—Low sample amount — Diluted samples
Shigemi	5mm	250-300 µL	Precious samples (e.g. biological samples)

Dissolve your sample in an appropriate deuterated NMR solvent. Make sure there is no undissolved substance. If there is, you will need to either centrifuge the tube or filter your sample.

Insert sample into the Spinner

The sample is inserted into the probe in a piece of Kel-F, POM or ceramic designed to hold the NMR tube. This is called a spinner. The choice of the material depends on the working temperature.

Table 2: Types of NMR spinners.

Material	Appearance	Characteristics
POM		Blue color Temperature: 0 to +80°C
Kel F		white color Temperature: up to +120°C
Ceramic		white color Temperature: entire temperature range

Remember to wipe your NMR tube before inserting your sample in the spinner.

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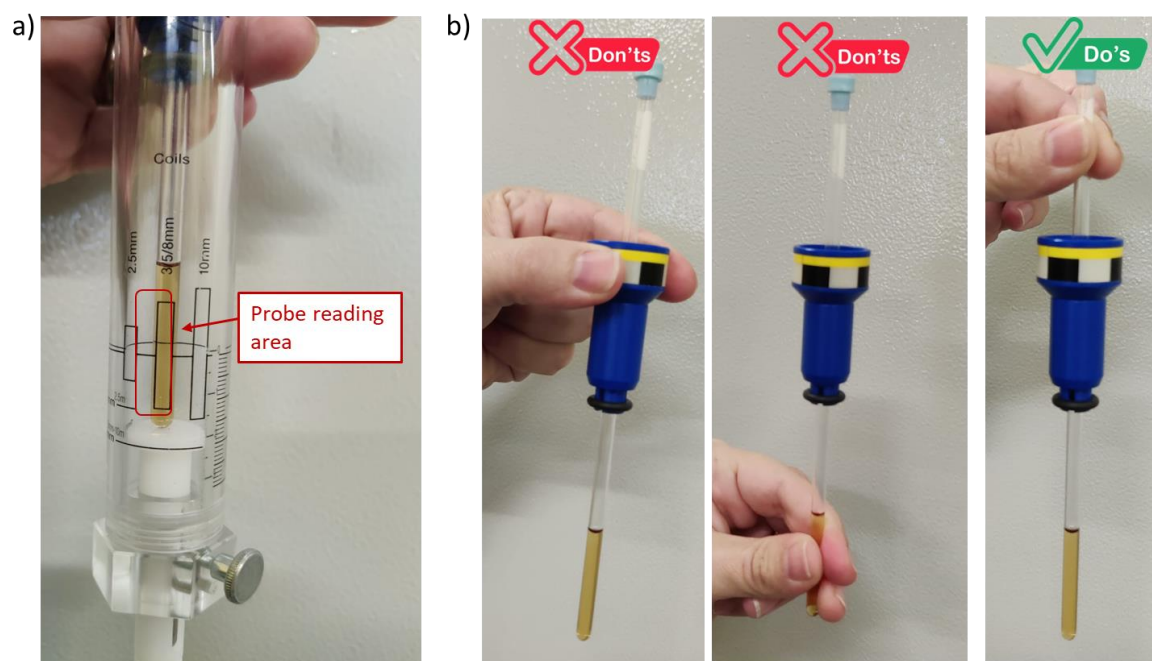in protein/glycan interactions:
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Figure 1: a) Correct depth adjusting of an NMR tube using the depth gauge; b) Correct way to grab an NMR tube.

Slide your sample tube gently into the spinner. The depth of the sample is crucial to acquire good data. To ensure that the sample volume is centered about the line, and not just pushed down using the depth gauge. It is important to expel air bubbles from the sample.

General software considerations

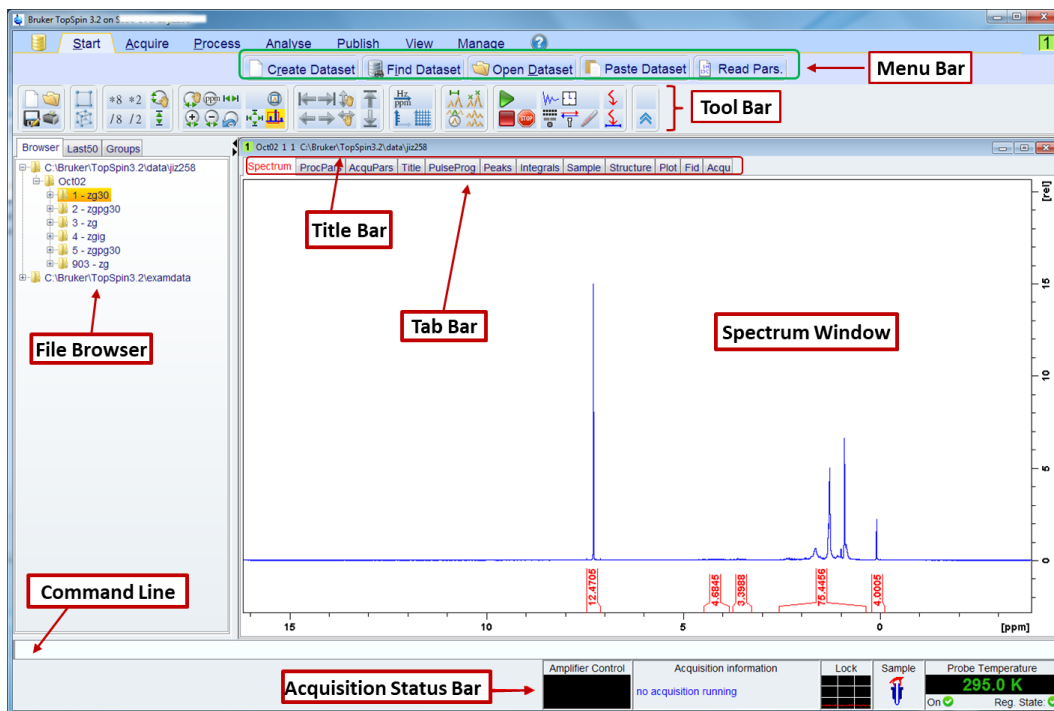## TOPSPIN Layout

Topspin 3.X interface

Figure 2: Overview of the Topspin 3.X interface

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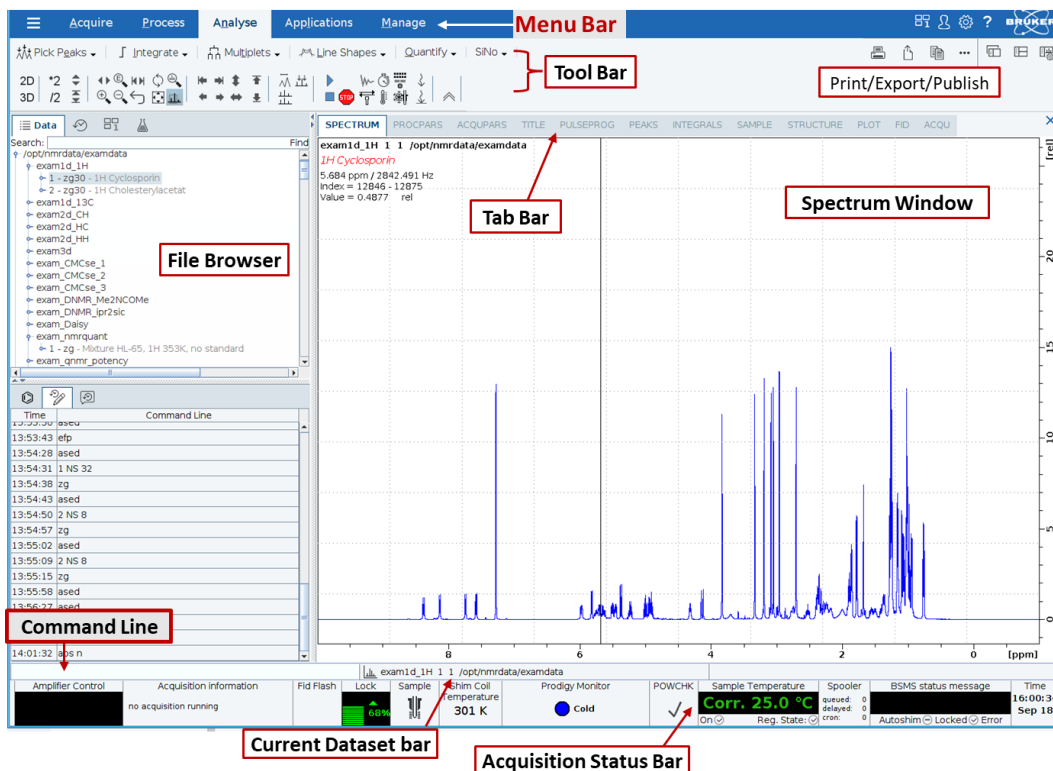*Topspin 4.X interface*

Figure 3: Overview of the Topspin 4.X interface

NOTES:

RED - commands written in the command line.

GREEN – icons to select in the software.

For NMR parameters (*e.g.* **o1, p1**, **sw**, **TD**, ...) see the parameters/commands document.

Instrument Preparation

Temperature regulation

Before you load a new sample into the magnet check the actual temperature of the probe. We can do this looking into the Status bar in the Topspin interface.
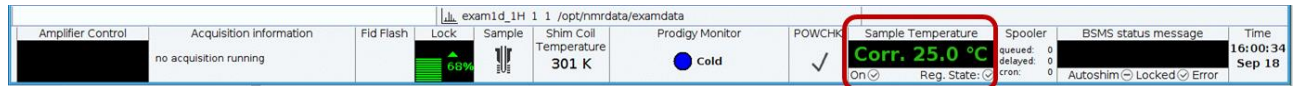

Figure 4: Status bar in Topspin.

If the temperature is not set to the temperature you want for your measurement, you need to open the Temperature Control window. This can be done by **i)** Double clicking the Sample Temperature window in the status panel; **ii)** in the command line:
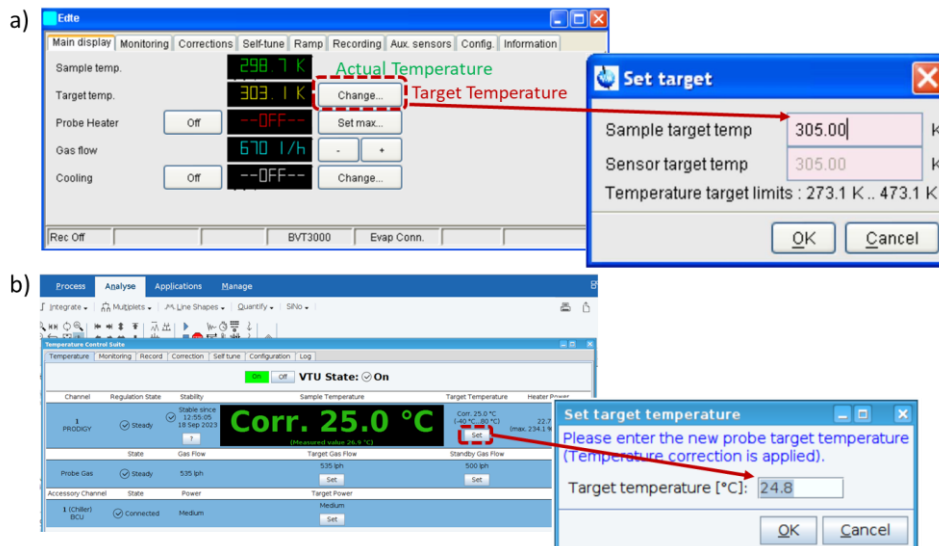
> **edte** <ENTER>

Figure 4: Temperature Control suite interface in Topspin. A) Topspin 3.X. b) Topspin 4.X.

Click **Change** (Topspin 3.x) or **Set** (Topspin 4.x) to change the temperature. **You should only heat in 5ºC increments.** Increasing the temperature too fast can put too much power into the probe heating component which could damage the probe.

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Insert sample

> **ej** <ENTER>

To turn ON the eject air flow and eject sample.

<u>*NOTE:*</u> always clean the tube before inserting it, otherwise contaminants may transfer to the NMR probe, potentially affecting its sensitivity and performance while increasing the risk of maintenance issues.

The sample can also be ejected and injected using the BSMS window – Bruker Smart Matching/Shimming system, which is open by double-clicking the BSMS status message in the Status bar.

> **ij** <ENTER>

To lower the sample into the magnet

The sample can also be ejected and injected using the BSMS window – Bruker Smart Matching/Shimming system, which is open by double-clicking the BSMS status message in the Status bar.

Go to any data set (it is required that some experiment is opened), preferably in your own directory and open an experiment.

> **edc** <ENTER>

This command will create a new experiment using the same parameters as the ones in the experiment you currently are.

Figure 5: New experiment display. Topspin will not acquire data before some information has been entered in this window: **NAME** - the name of the folder that your data will be created, it is the identifier of your sample. **EXPNO** - experiment number; each type of

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experiment (^{1}H, ^{13}C, NOESY.etc..) must have a unique number. 1-99999 are allowed. Use Current Parameters: if you have an experiment loaded that you wish to repeat, check this. Experiment: Here you select the experiment you want. If you have selected "Use current parameters" then this cannot be selected. **Set Solvent:** Sets the sample solvent. **DIR:** This is where your data is stored. Make sure to set this to /opt/data/your UserID otherwise you won't find your data.

Select the pulse program

Since the experiment you just created may not be the one you want to execute, we need to define it:

> **rpar** <ENTER>

The command *rpar* reads a parameter set (experiment) to the current dataset. This opens a dialog box with a list of the available parameter sets (more than 600 are available).

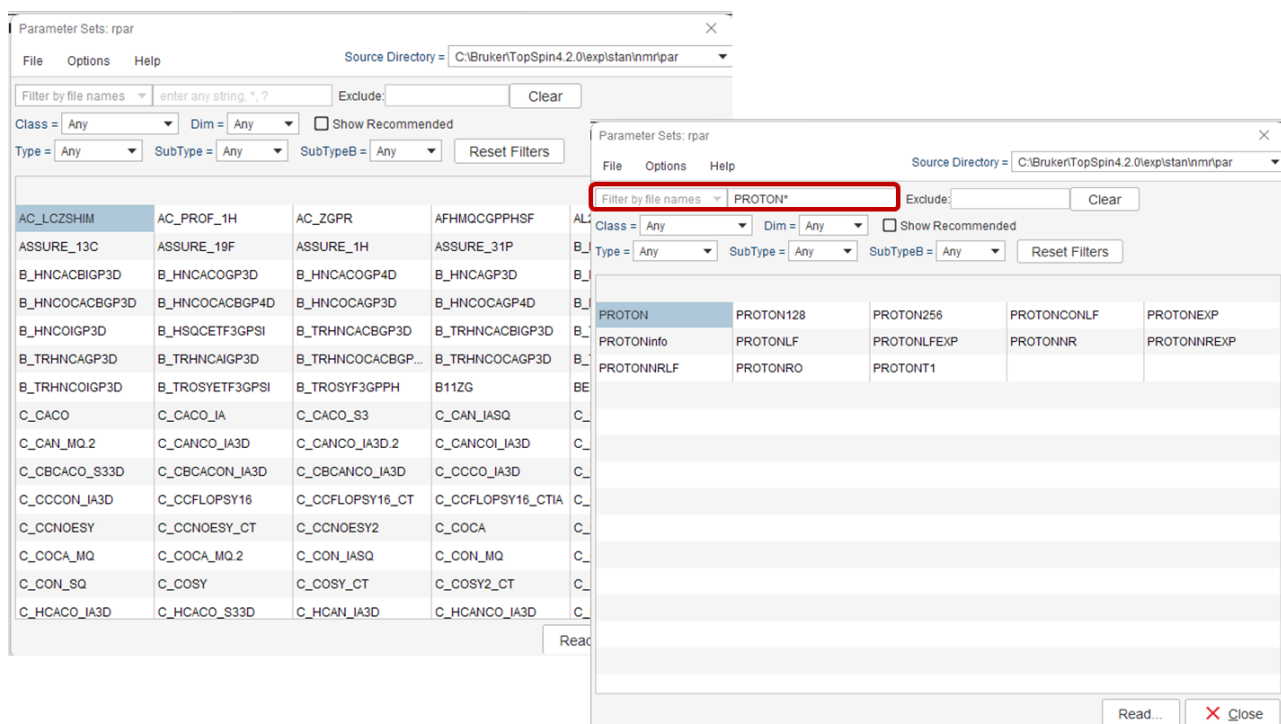**Figure 6:** Parameter set display box.

Select: **PROTON**

In this case it will read the pulse program ZG30, which corresponds to a 1D ^{1}H NMR experiment. You can filter the list by writing in the filter section the name or partial name of the required experiment.

Load probe and solvent-dependent parameters
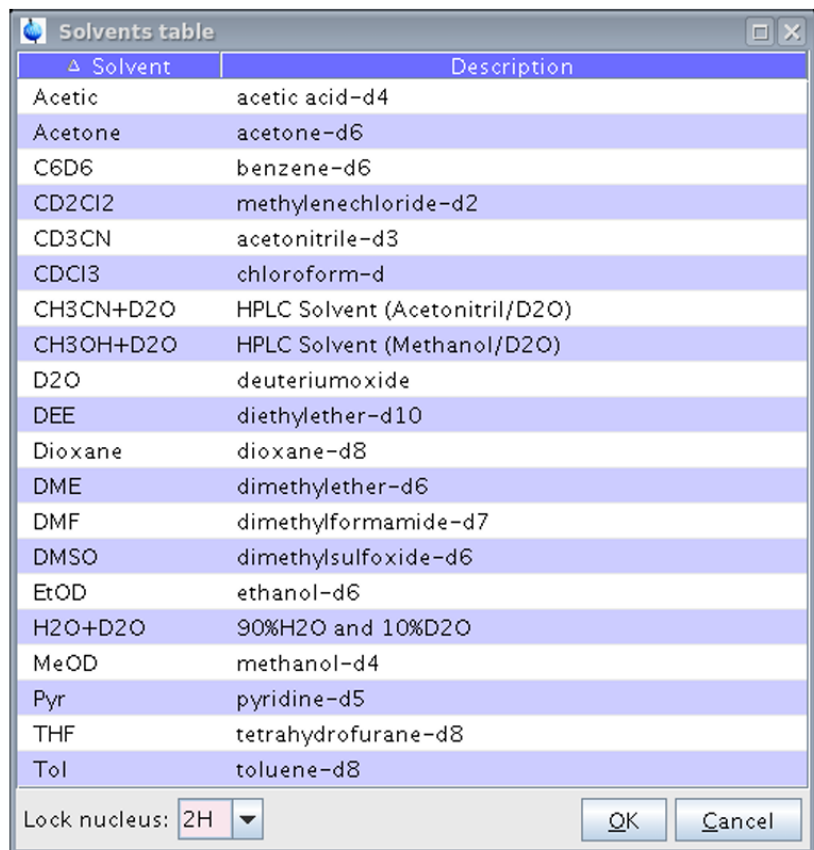
> **getprosol** <ENTER>

This reads the probe and solvent dependent parameters.

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Lock

To stabilize the field/frequency using a reference signal (^{2}H). Since it uses the ^{2}H signal to correct the drifting of the NMR magnet, the values of the 2H transmitter output power in the spectrometer needs to be adjusted for each deuterated solvent, due to the different relaxation behavior and degree of deuteration of the solvent.

> lock <ENTER>

This will open a display box with a table of solvents. Choose the solvent for the lock (e.g. **H2O + D2O** /or **D2O**)

△ Solvent	Description
Acetic	acetic acid-d4
Acetone	acetone-d6
C6D6	benzene-d6
CD2Cl2	methylenechloride-d2
CD3CN	acetonitrile-d3
CDCl3	chloroform-d
CH3CN+D2O	HPLC Solvent (Acetonitril/D2O)
CH3OH+D2O	HPLC Solvent (Methanol/D2O)
D2O	deuteriumoxide
DEE	diethylether-d10
Dioxane	dioxane-d8
DME	dimethylether-d6
DMF	dimethylformamide-d7
DMSO	dimethylsulfoxide-d6
EtOD	ethanol-d6
H2O+D2O	90%H2O and 10%D2O
MeOD	methanol-d4
Pyr	pyridine-d5
THF	tetrahydrofurane-d8
Tol	toluene-d8

Lock nucleus: 2H ▼   OK   Cancel

Figure 7: Solvent lock selection.

Tuning and Matching

To *tune* the probe to the radiofrequency of the desired nucleus; and to *match* the impedance of the combination coil/sample with that of the transmitter and receiver so that the maximum possible r.f. can pass from the transmitter to the sample and subsequently from the sample to the receiver.

Matching involves adjusting the probe so that the minimum wobble curve is at the base of the display. This represents minimum reflection of the transmitted signal.

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Tuning involves ensuring that this occurs at the frequency of transmission which will be at the center of the screen's horizontal scale. However, the tune and match adjustments interact with each other and must be adjusted in an iterative process.

> **atma** <ENTER>

This will automatically tune and match the probe to whatever nuclei specified in the active experiment. To tune and match other nuclei, the command needs to be done in an experiment that uses that nucleus. Once atma is completed the user will see a "atma finished" message will appear.

This can also be performed manually using the command **atmm** and adjusting the wobble curve of the lock signal manually using the tune and matching controls and saving the final position.
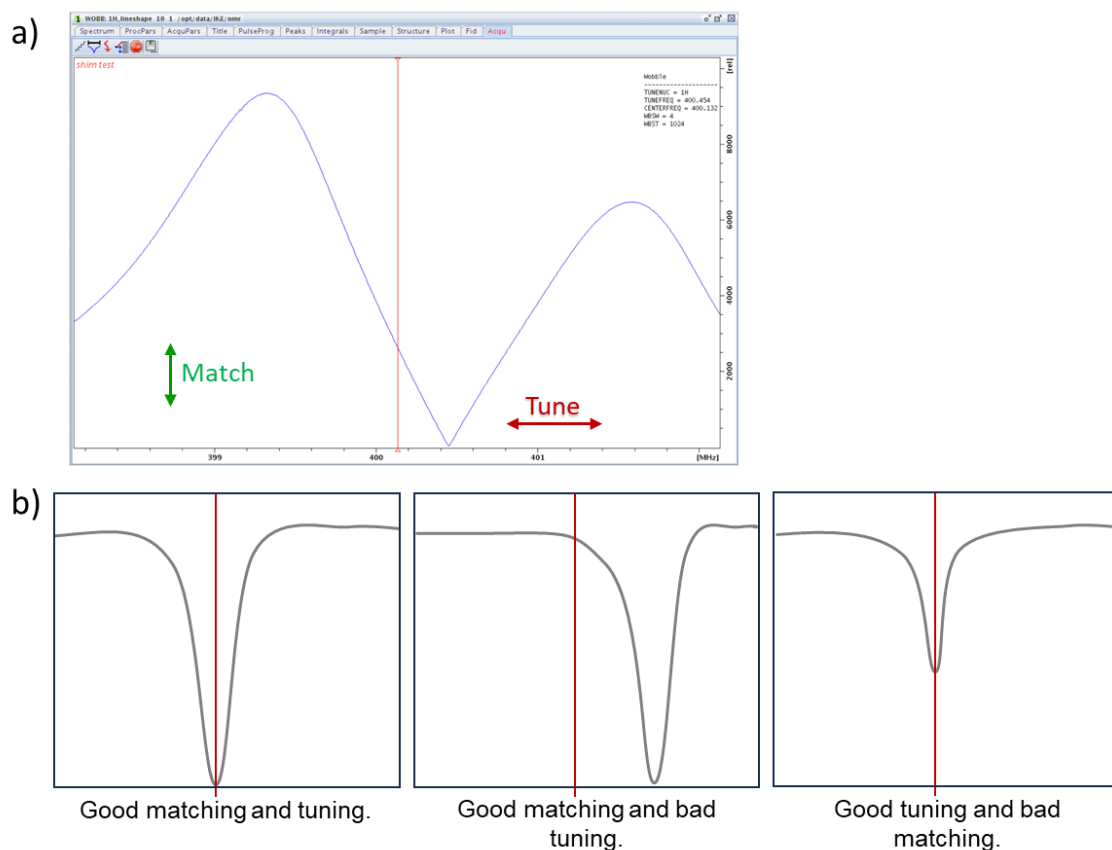

Figure 8: a) Wobble curve window showing the atma process. For atmm this also appears but it will also show a control display for the tuning and matching adjustments. b) Examples of wobble Curves with different tuning and matching.

Shimming

To optimize the magnetic field homogeneity. Improving the homogeneity will result in better spectral resolution. In Topspin this is achieved using a gradient shimming process. Gradient shimming involves making precise adjustments to

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specialized coils known as 'shim coils' surrounding the primary NMR magnet. These coils allow for the fine-tuning of the magnetic field strength at various locations within the sample, addressing any irregularities or variations in the field.

> **topshim gui** <ENTER>

This will open the Shim Panel, which is divided into three sections (Shim, Tune and Status). Our major focus will be on the shim section. In the *Optimisation* option and choosing the preferable option.

The next parameters can be modified freely, and each NMR user has their own preferences, depending on their experience:

- TopShim process there is some flexibility for the user to adjust the process aiming specific needs of an NMR experiment and/or probe. That is achieved using the optimisation criteria in the shim panel which presents options, such as such as a narrow line width or hump, or good solvent suppression.
- The role of Z6 shim can vary significantly. Optimal shim settings may differ noticeably for slightly different situations, but this often doesn't significantly impact spectrum quality. Generally, for low fields (300-500 MHz) or when using certain probes like BBO or QNP, S/N levels in the field profile may not warrant Z6 shimming. However, evidence suggests that cryoprobes benefit from using Z6 for improved performance.
- Sometimes it may be helpful to remove residual shim gradients after shimming. This can be done using the TUNE option in Topshim. This feature will just maximize the lock level using a subset of the shims. By default, TUNE switched off. It can be done before the gradient shimming (to prepare the best off-axis shims before running a 1D TopShim); or after the gradient shimming to ensure the absolute best shims when some residual shim gradients might remain. However, Bruker's language in this context is somewhat unclear. The term "atma" refers to an electronic tuning and matching process specific to the probe's circuitry, and it should not be confused with the "TUNE" operation in TopShim. A more suitable description for the TopShim "TUNE" operation might be "lock-based shimming (LBS)" or a similar term.
- The parameter section can be used to define additional parameters for the Topshim process to consider. One of these parameters is convcomp, which activates a convection compensation into the topshim process.

In this case, we'll select:

o   Use *Z6*
o   TUNE after *Z-X-Y-XZ-YZ-Z*
o   *Start*
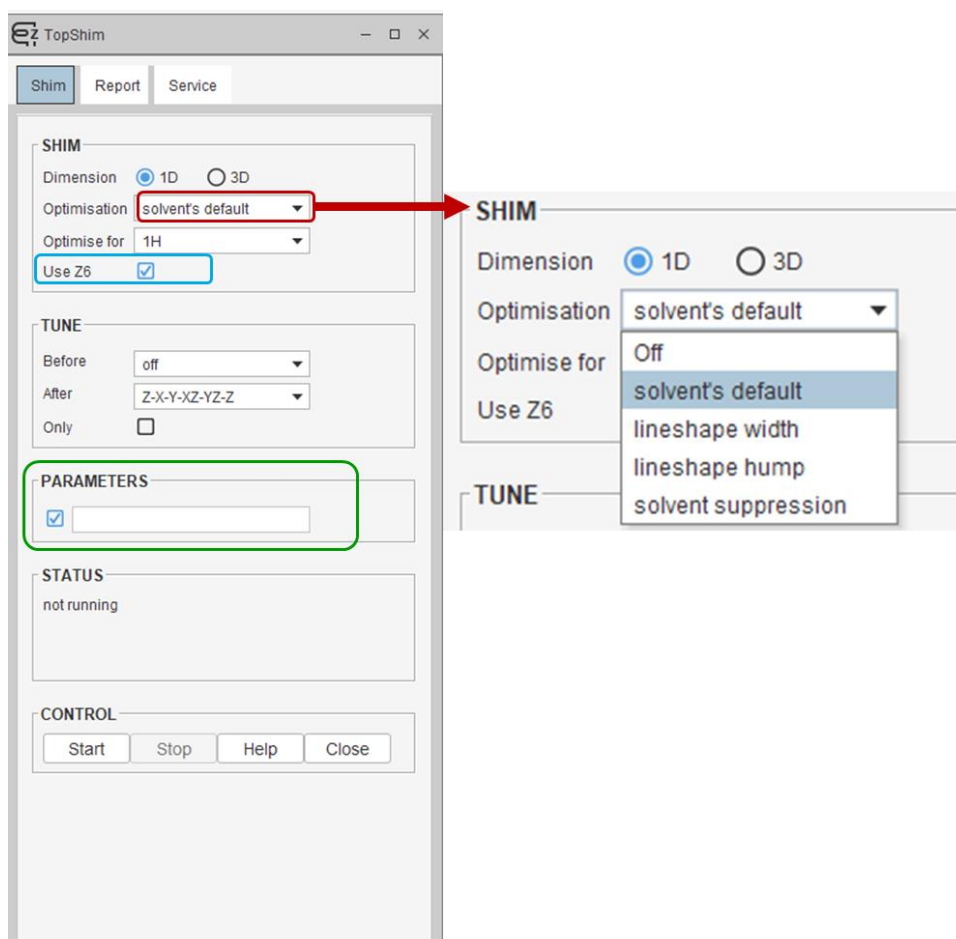
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Figure 9: Topshim graphic user interface.

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Determination of the ^{1}H 90^0 pulse

Select the correct pulse program

In the same experiment

Because we want to calibrate a 90^0 pulse (and the pulse program associated to PROTON defined using the **rpar** command is a 30^0 pulse – ZG30), we need to change the pulse program:

> **eda** <ENTER>
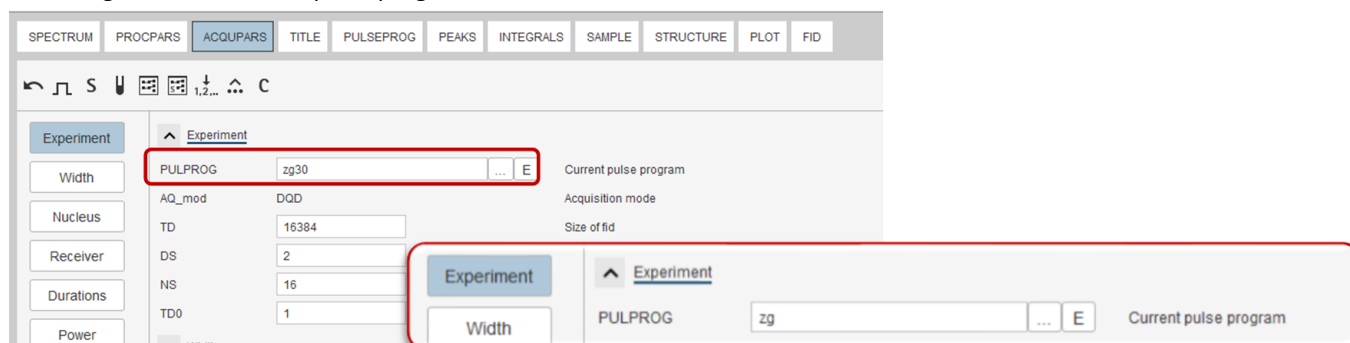
Change the name of the pulse program to **ZG**

Figure 10 – Acquisition tab in topspin, enlighten the change in the pulse program.

Set the acquisition parameters

Make the following changes (you can change directly in the acqupars tab or write "**parameter new value**" in the command line (e.g. **TD 14k** <ENTER>)

- o **TD**: 14k
- o **DS**: 0
- o **NS**: 1
- o **RG**: 1

Set p1: > **p1 2** <ENTER>

Acquire the data: > **zg** <ENTER>

Process the data: > **ef** <ENTER>

Correct the phase: > **apks** <ENTER>

    Or type > **.ph** <ENTER> (to manually correct the phase)

Prior to acquiring an NMR spectrum, it is essential to adjust the transmitted pulse frequency to align with the central resonance frequency of interest. In the case of biomolecules dissolved in aqueous solutions, this typically corresponds to the water proton frequency, which is approximately 4.7 ppm.

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Determination of the transmitter offset (O1)

Zoom-in in the solvent peak and type: **> pp** <ENTER>

*R*ead the value and type: > ***O1p xxxxx*** <ENTER> *(in ppm)*

OR

Expand the peak at 4.7 ppm. In the tool bar click Set RF from cursor bottom.

Move the cursor line to the center of the peak and press the mouse button.
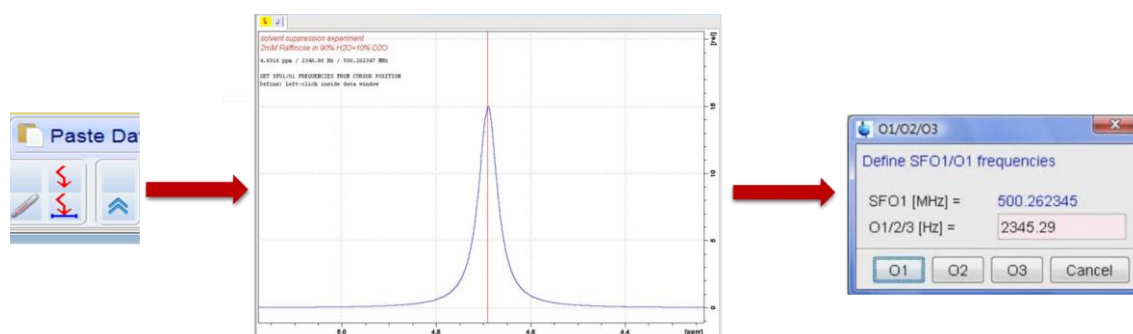
In the O1/O2/O3 window, click **O1** *(in Hz).*

Figure 11: Illustration of the workflow to determine the o1 in Hz.

Determine the 360° pulse. The selection of 360° is preferable since it is easier to find the pulse at which the signal is null.

> p1 32 <ENTER> *(for instance, if the default p1 is ~8μs)*

> zg <ENTER>

Process with > **efp** <ENTER>

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Optimize the p1 value

- Observe the signal of the peak and correct the duration of the pulse accordingly (**Figure 12**)
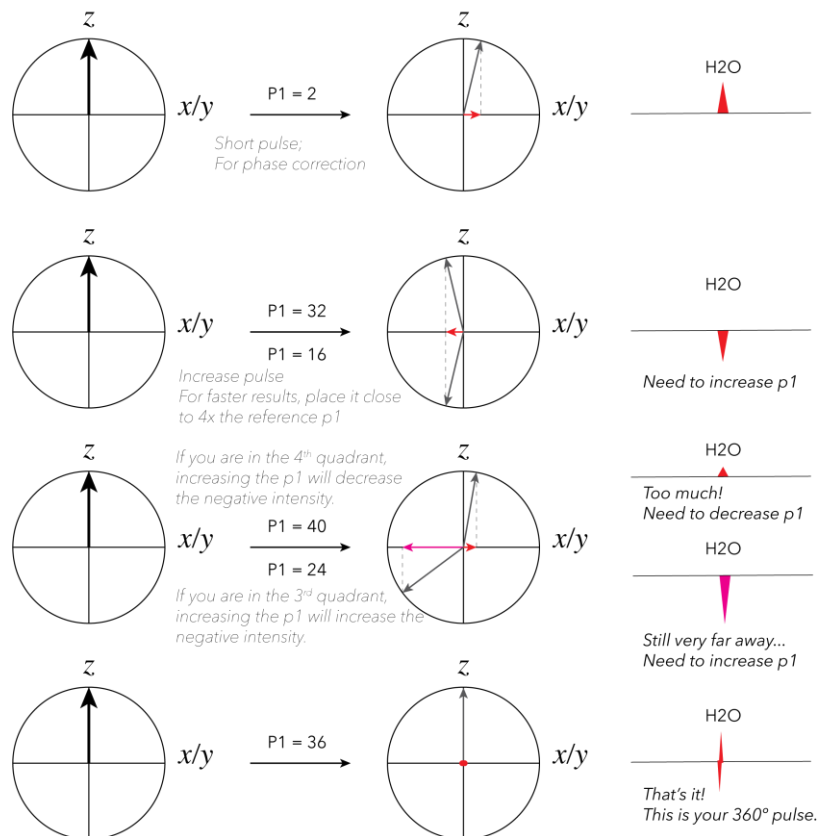

Figure 12: Effect of the p1 value in the signal near the 360⁰ pulse value, for the pulse determination.

Once you have the 360⁰ pulse, divide it by 4 to obtain the 90⁰ pulse.

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Structural Analysis of carbohydrates/glycans by 1D and 2D experiments

^{1}H 1D-NMR (zg)

Create new experiment

> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>

Figure 13: zg pulse sequence.

Select: **PROTON**

As described previously this will read a zg30 experience that corresponds to a proton with a 30º pulse.

> **eda** <ENTER>

Change the name of the pulse program in the window to **ZG** (see above)

Define probe and solvent-dependent parameters according to the determined p1

The command ***getprosol*** will read the reference probe and solvent-dependent parameters and copies them to the acquisition parameters. However, these are dependent on the p1 value (e.g. all the pulses' durations). Thus, we need to recalibrate them according to the newly determined p1 value. This is done in the command line:

> **getprosol 1H** *p1 pl1* <ENTER>

Where "p1" and "pl1" above are the values of the determined p1 and the reference power level (in DBs, it is probe dependent), respectively *(e.g. for p1=8.2 in a probe with pl1= - 7.24 db; write: getprosol 1H 8.2 -7.24)*

Set the acquisition parameters

> **ased** <ENTER>

Change: **d1**, **SW**, **TD**, **DS**, **NS**, **O1**, etc, depending on your experiment and on your sample. (see parameters/commands document)

Define the Receiver Gain (rg)
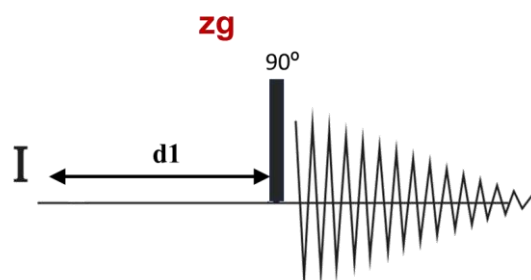
> **rga** <ENTER>

Start acquisition

> **zg** <ENTER>

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Samples in D_2O or H_2O/D_2O have protonated signal (HOD and H_2O) which masks the sample signals. To overcome this limitation, we can perform water suppression methods (e.g., zgpr or zgesgp – see below).

^1H 1D NMR with water suppression using pre-saturation (ZGPR)

A low power pulse (**pl9**) at the solvent frequency (**O1**) is applied during the preparation delay (**d1**). This low power pulse excites the water proton signal such that no signal can fully accumulate and be measured.
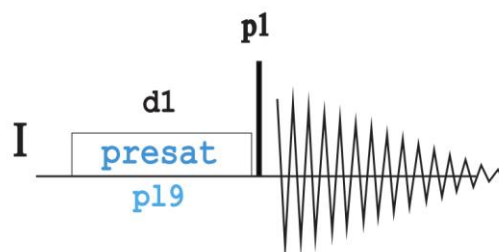

Figure 14: zgpr pulse sequence.

Create new experiment

> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>

Select: **ZGPR** from the pulse program display box

Define probe and solvent-dependent parameters according to the determined p1

> **getprosol 1H** *p1 pl1* <ENTER>

Set the acquisition parameters

> **ased** <ENTER>

Change your sample dependent parameters **d1**, **SW**, **TD**, **DS**, **NS**, **O1**, etc.(see parameters/commands document)

Define the Receiver Gain

> **rga** <ENTER>

Start acquisition

> **zg** <ENTER>

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1H 1D NMR with water suppression using excitation sculpting with gradients (ZGESGP)

Create new experiment

> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>

Select: **ZGESGP** from the pulse program display.

Figure 15: zgespgp pulse sequence.

Define probe and solvent-dependent parameters according to the determined p1

> **getprosol 1H** *p1 pl1* <ENTER>

Set the acquisition parameters

> **ased** <ENTER>

Change: **d1**, **SW**, **TD**, **DS**, **NS**, **O1**, etc.

Set the 180º shaped pulse (sp1)

This is automatically set when you do the *rpar* and *getprosol*.
p12 – 2ms
Sp1 - Squa100.1000

Define the Receiver Gain
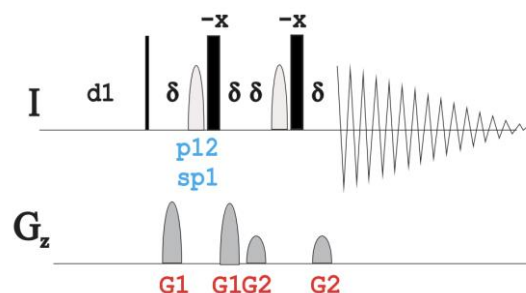
> **rga** <ENTER>

Start acquisition

> **zg** <ENTER>

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2D NMR Experiment

TOCSY - Total Correlation Spectroscopy

TOCSY (Total Correlation Spectroscopy) provides information about the through-bond connectivity of protons within a given spin system. TOCSY spectra display correlations between all protons in spin system which is particularly valuable when pinpointing protons within sugar rings or amino acids. Specifically, all protons residing on the same sugar ring exhibit correlations with each other and not with protons in another ring. In this experiment, the magnetization is transferred successively over 5 or 6 bonds, interrupted by negligible proton-proton couplings, especially when hetero-atoms like oxygen are present. Adjusting the spin-lock time (d9) modifies the number of transfer steps. A short time (e.g., 20ms) results in a single-step transfer like a COSY spectrum, while a longer time (e.g., 80ms or 120ms) allows up to 5 or 6-step transfers, depending on specific coupling details.
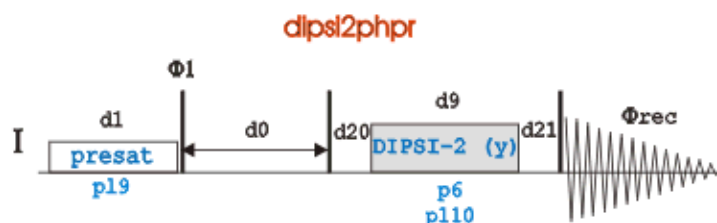

Figure 16: TOCSY DIPSI2PHPR pulse sequence.

Create new experiment

> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>

Select: **DIPSI2PHPR** from the pulse program list. This pulse sequence is a TOCSY pulse sequence with solvent suppression with presaturation.

Define probe and solvent-dependent parameters according to the determined p1

> **getprosol 1H** p1 pl1 <ENTER> (eg. for p1=8.2 in a probe with pl1= - 7.24 db; write: getprosol 1H 8.2 -7.24)

Set the acquisition parameters

> **ased** <ENTER>

Change: **d1**, **SW**, **TD**, **DS**, **NS**, **O1**, etc.

Critical parameters for TOCSY that should be checked are:

o   **NS** – should be a multiple of 8

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- **DS** - 16
- **TD1** – number of points in the F1 dimension, must be na even number, preferably at least 256
- **TD2** – number of points in the F2 dimension, usually set to 2K

Since this is a homonuclear experiment the offset and spectral width will be the same in both dimensions. Set **o1/o2** and **sw1/sw2** (ppm) to the values from the conventional ^{1}H spectrum. Type in the command line:
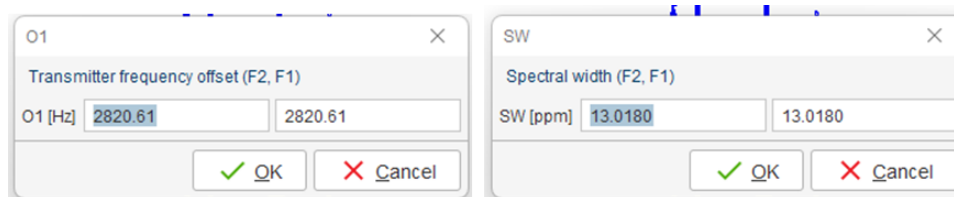
> **o1** <ENTER>

> **sw** <ENTER>

Figure 17: Change of the offset and spectral width for a 2D experiment.

Set the pre-saturation pulse (pl9)

This is automatically set when you do the **rpar** and **getprosol.**

Set the mixing time (d9)

D9 = spinlock mixing time; it is common to acquire 2 or 3 spectra with different mix:

- = 15ms will observe 2-3 bond couplings only (1st shell)
- = 30ms start to see next shell (i.e., protons 2-3 bonds from 2nd)
- = 50ms 2 shells, with some hints of 3
- = 80ms 3 shells, with hints of 4
- 120ms is the maximum spinlock that should be used; shows all shells

Define the Receiver Gain

> **rga** <ENTER>

Start acquisition

> **zg** <ENTER>

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25th—28th September '23
Scientific Hands–on course
in protein/glycan interactions:
a structural perspective.

^1H-^1H NOESY - Nuclear Overhauser Effect Spectroscopy

2D NOESY (Nuclear Overhauser Effect Spectroscopy) is a critical technique in nuclear magnetic resonance (NMR) spectroscopy. It reveals spatial relationships between atomic nuclei in molecules and does not depend upon through-bond J couplings. In a 2D NOESY experiment, close nuclei influence each other's magnetization, creating cross-peaks in the spectrum. These peaks indicate proximity between them and orientation information. The strength of the NOE gives information on how close two protons are. To notice that chemical exchange correlations are also observed in the NOESY spectra.
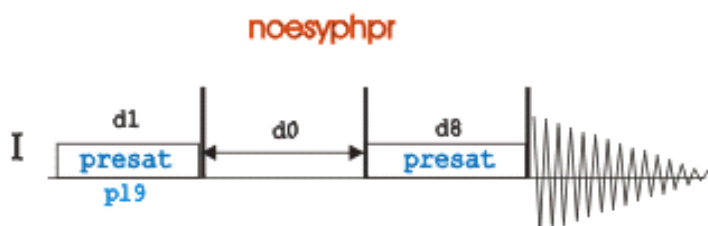

Figure 18: NOESYPHPR pulse sequence.

Create new experiment
> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>

Select: **NOESYPHPR**. Like the previous TOCSY experiment, this pulse sequence also performs solvent suppression using presaturation.

Define probe and solvent-dependent parameters according to the determined p1

> **getprosol 1H** p1 pl1 <ENTER> (eg. for p1=8.2 in a probe with pl1= - 7.24 db; write: getprosol 1H 8.2 -7.24)

Set the acquisition parameters

> **ased** <ENTER>
Change: **d1**, **SW**, **TD**, **DS**, **NS**, **O1**, etc.
o   **NS** – should be a multiple of 8
o   **DS** - 16
o   **TD1** – number of points in the F1 dimension, must be an even number, preferably at least 256
o   **TD2** – number of points in the F2 dimension, usually set to 2K

Since this is a homonuclear experiment the offset and spectral width will be the same in both dimensions. Set **o1/o2** and **sw1/sw2** (ppm) to the values from the conventional ^{1}H spectrum. Type in the command line:

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> o1 <ENTER>

> sw <ENTER>

Set the pre-saturation pulse (pl9)

This is automatically set when you do the **rpar** and **getprosol.**

Set the mixing time (d8)

The mixing time in a 2D NOESY experiment depends on the size and molecular weight (Mw) of the molecules studied, and on the frequency of the magnet and temperature you are operating.

A general guideline for the mixing times and molecular weight limits for small, medium, and large molecules in 2D NOESY experiments:

- For small molecules, (Mw <500) short mixing times are used (300ms – 1s)
- For intermediate molecules (500<Mw<1200), depending on the frequency of your magnet you will be probably at NOE zero. In this case a ROESY type experiment is preferable. A roesyphpr.2 version is available on Bruker's library.
- For large molecules (molecular weight >1200) typically require small mixing times (50 – 200ms)

Define the Receiver Gain

> rga <ENTER>

Start acquisition

> zg <ENTER>

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^1H-^{13}C HSQC – Heteronuclear Single Quantum Coherence

While the previous experiments were homonuclear experiments, in the 1H,13C-HSQC we observe heteronuclear correlations (different nuclei). The ^1H-^{13}C HSQC (Heteronuclear Single Quantum Coherence) NMR spectroscopy is an experiment that shows which hydrogens are directly bonded to which carbon atoms. Hence, in the spectrum, you observe a set of cross-peaks, corresponding to the single bond correlation (1H- 13C). There are several pulse sequences, however, we present the edited pulse sequence that not only gives the connectivity information but also the multiplicity, like a DEPT-135 experiment, where the CH and CH3 signals are phased up and the CH2 are phased down.
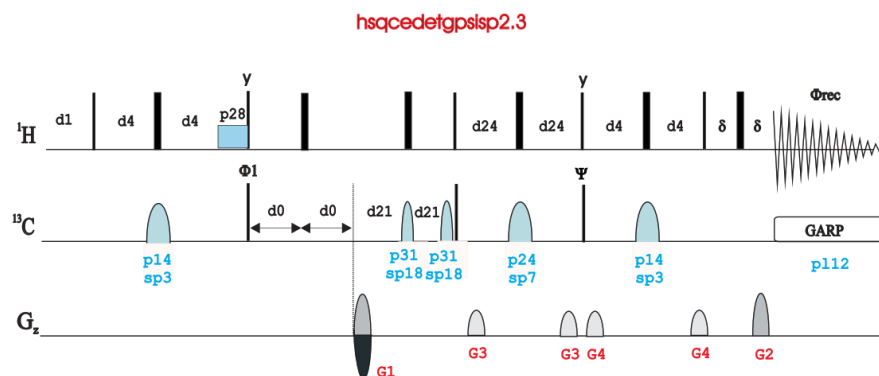

Figure 19: hsqcedetgpsisp2.3 pulse sequence.

Create new experiment

> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>
Select: **hsqcedetgpsisp2.3** from the pulse library.

Define probe and solvent-dependent parameters according to the determined p1

> **getprosol 1H** p1 pl1 <ENTER> (eg. for p1=8.2 in a probe with pl1= - 7.24 db; write: getprosol 1H 8.2 -7.24)

Tune the probe for ^1H and ^{13}C

> **atma** <ENTER>

The automatic tuning and matching will take longer since it will tune and match two different nuclei, first the ^{13}C and then the ^1H.

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Set the acquisition parameters

> ased <ENTER>

Change the parameters as desired (**d1**, **SW**, **TD**, **DS**, **NS**, **O1**, etc.) The F2 column refers to the ^{1}H dimension (direct) and the F1 column to the ^{13}C dimension (indirect). The typical carbon spectral width range for a HSQC is from 0 to -165 ppm, as carbonyl groups generally do not have directly bonded protons.

- o **NS** – should be a multiple of 2
- o **DS** – ≥16
- o **TD1** – number of points in the F1 dimension (^{13}C), must be an even number, preferably at least 128
- o **TD2** – number of points in the F2 dimension (^{1}H), usually set to 2K
- o Since this is a heteronuclear experiment the offset and spectral width will be different for each dimension.
- o **o1** – center of the ^{1}H spectrum (same as in the ^{1}H 1D spectrum)
- o **o2p** – center of the ^{13}C spectrum in ppm (default: 85 ppm)
- o **sw**– F2 :^{1}H spectral width in ppm: F1: ^{13}C spectral width (default: 180 ppm)

Define the Receiver Gain

> rga <ENTER>

Start acquisition

> zg <ENTER>

Glycan-protein interactions investigated by NMR

Glycan-based NMR binding experiments

Saturation-Transfer Difference NMR (STD-NMR)

STD NMR (Saturation Transfer Difference NMR) is a powerful and versatile technique used to investigate glycan-protein interactions. The STD-NMR experiment is based on the transfer of the saturation from a protein (e.g. lectin) to a binding ligand (e.g. small glycan). **STD-NMR has its major application for glycan/protein complexes in fast exchange regime in the NMR relaxation time scale**. Thus, STD-NMR has its major potential for medium to weak binders.

In STD NMR, a selective irradiation pulse of a restricted region of the protein 1H spectrum is applied to exclusively saturate the protein resonances and not affect the ligand. Then, the spin diffusion occurs (process highly efficient in large receptors), meaning that there is a rapid transference of the saturation through the network of protons in the protein, via the NOE effect. In this sense, any ligand that interacts with the protein for a relevant time will receive saturation by this process from the protein. Lastly, when the ligand goes back into the solution, it will carry the transferred saturation, which is translated into a decrease of its resonance intensities in the 1H spectrum. For the STD-NMR experiment at least two different spectra are acquired. One is the off-resonance spectrum, since it is recorded by irradiating a region far from the protein or ligand resonances, usually > ± 40 ppm, giving rise to a 1H spectrum of the ligand which is used as the reference. The other is the on-resonance spectrum, where the selective irradiation of the protein occurs, often targeting the aliphatic (0.5 ppm) or aromatic regions (7 ppm). The region at <1 ppm is also a good option to avoid direct saturation of certain ligands. The STD-NMR spectrum is then obtained as the difference between these two spectra. Subtraction of the on-resonance spectrum from the off-resonance spectrum allows deducing the resonances of the ligand that have received saturation from the protein. The comparison of the STD-NMR spectrum and the off-resonance spectrum allows extracting STD-NMR enhancements for each signal and consequently building an epitope map on the ligand structure, to identify which ligand's protons are in close contact with the protein.

Requirements/Conditions:
o   No size limitation for the protein since it is a ligand-based analysis approach.
o   Requires just small amounts of receptor.
o   No need for isotope labeling.
o   Use a buffer solution condition suitable for your protein and ligand. Ensure it is properly pH-adjusted and matches the conditions of your experiment. The use of deuterated buffer solutions is also indicated since it reduces the relaxation of the protons from the protein and the ligand through interactions with water, which helps the retainment of the magnetization of the ligand in the free state, increasing the sensitivity of the experiment. Furthermore, the use of proton-free or deuterated buffers is advisable. Notice that in deuterated water only the non-exchangeable protons of the protein will be responsible for transferring the saturation to the ligand.
o   STD control experiments should be performed. A STD experiment using the same experimental conditions and STD setup should be performed with a sample only containing the glycan (ligand) and only containing the protein.

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Sample Preparation:

Prepare an NMR tube with protein (~20-100uM) and an excess of ligand (Protein/ligand ratio – usually 1:20 to 1:100 ratio). The concentration of the protein depends on protein M_W and K_D (efficient saturation transfer for KD 100 nM to 10 mM).
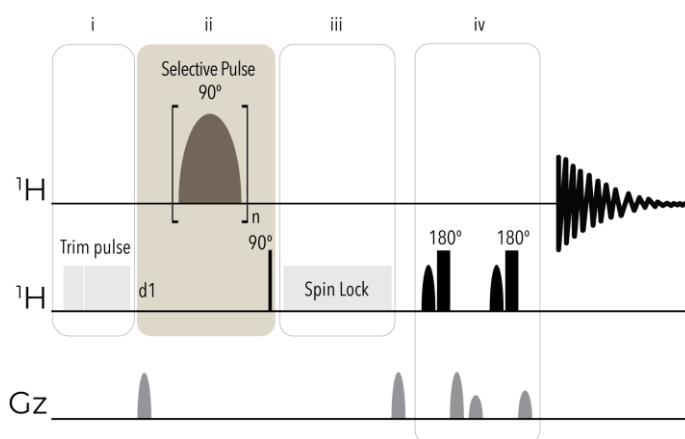

Figure 20: STDDIFFESGP pulse sequence.

Create new experiment

> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>

Select: **STDDIFFESGP**

NOTE: there are several STD pulse schemes. In the figure above you can see a schematic representation of an STD-NMR pulse program (adapted from Bruker's stddiffesgp.3), which is the most "complicated" one.

This pulse sequence can be roughly divided into four sections:

i) Trim pulse - this section is optional and corresponds to a spoil sequence to destroy unwanted magnetization – OPTIONAL!

ii) The basic saturation loop for STD – this section corresponds to the simplest STD-NMR experiment and contains the relaxation delay (d1), the selective shaped pulse (sp9, 90°) which will saturate the target protein, and the reading 90° pulse (p1). Importantly, the STD element can be incorporated just prior the read 90° pulse in any nD experiments. However, the shape of the pulse, its duration and its power can all be modified and optimized for maximum saturation efficiency - **MANDATORY!**

iii) Spin lock – this section is optional and serves to eliminate the broad resonance signal of the protein by using a spin-lock filter ($T_{1\rho}$) for approximately 10–20 ms with a strength of 5 kHz - OPTIONAL!

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iv)   Solvent suppression – this section contains the solvent suppression scheme. However, it is optional and/or can be replaced other schemes - OPTIONAL!

Define probe and solvent-dependent parameters according to the determined p1

> getprosol 1H p1 pl1 <ENTER> (eg. for p1=8.2 in a probe with pl1= - 7.24 db; write: getprosol 1H 8.2 -7.24)

Set the acquisition parameters

> ased <ENTER>

Set the following parameters:
o   **O1** to value of solvent.
o   Set the **saturation time (d20)** (by default is 2s)
   (e.g. 2 seconds: **> d20 2** <ENTER>)

o   Set the **relaxation delay (d1)**
   Make sure it is ≥ d20 or the experiment will not run.

   (e.g. 3 seconds: **> d1 3.0** <ENTER>)

NOTE: the solvent suppression scheme in this experiment is the same as the one we used before, i.e. ZGESGP. When we correct the probe and solvent-dependent parameters (*getprosol*) this section already becomes calibrated, thus, no need to change it.

Set the 90⁰ shaped pulse for protein saturation

Still in **> ased** <ENTER>
Set the selective pulse shape for saturation (**SPNAM9**)
NOTE: this can be, for instance, either *Gauss1.1000* or *Eburp2.1000*. These different selective excitation pulses schemes lead to different saturation of the protein – the Gauss.1.1000 is more selective than the E.Burp2.1000, for that reason it originates less intense signals.
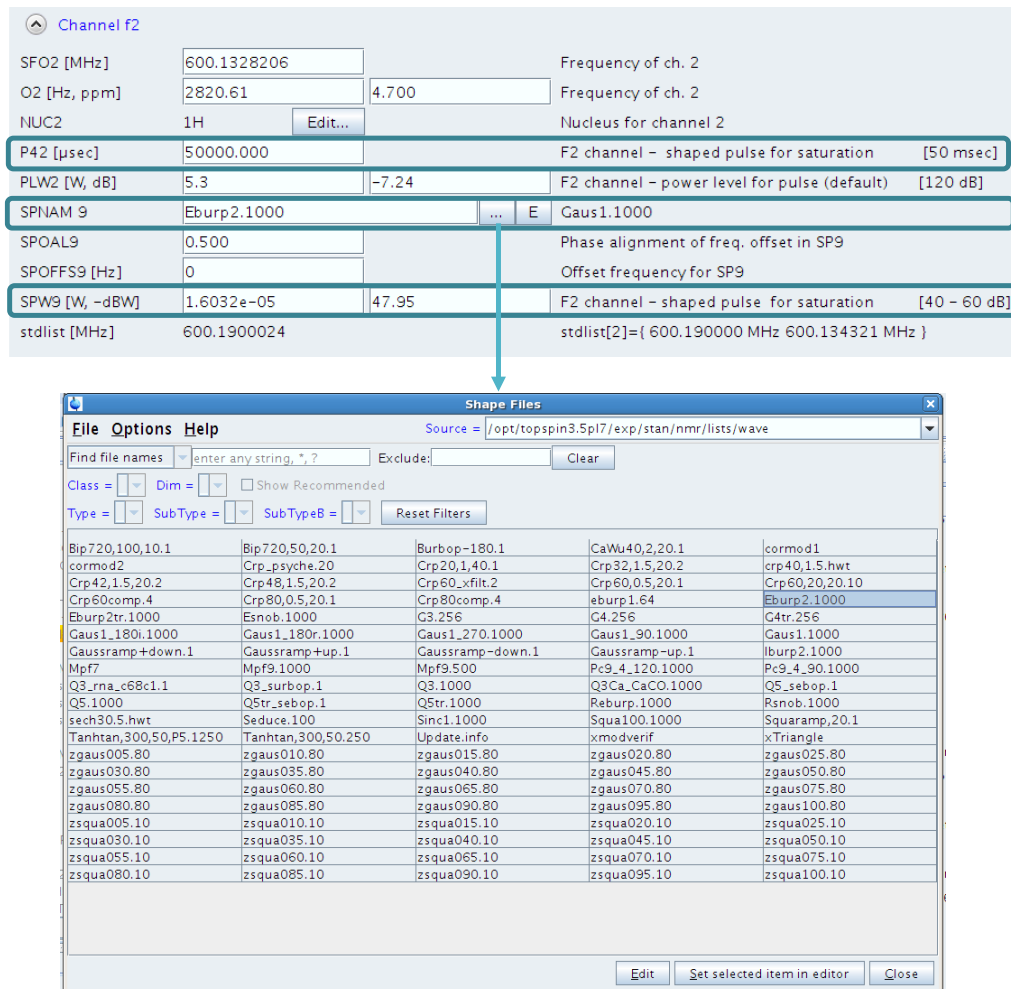
Here we will use the *Eburp2.1000* (Figure 21)

Figure 21: Location of the parameters **SPNAM 9**, **p42** and **spw9** in the acquisition display.

Calibrate the 90º shaped pulse for protein saturation

o Duration (50 ms)

> **p42** <ENTER>)

NOTE: the duration of the saturation pulse is kept constant at 50 ms. What we will calibrate next is the power of the pulse.

o Power (SP9 = 40 – 60 dB)

NOTE: Attenuation scale! The smaller the value, the higher the power of the radiofrequency.

> **stdisp** <ENTER> (or click on the *E (Edit)* button in front of the shaped pulse name) to open the Shape Tool
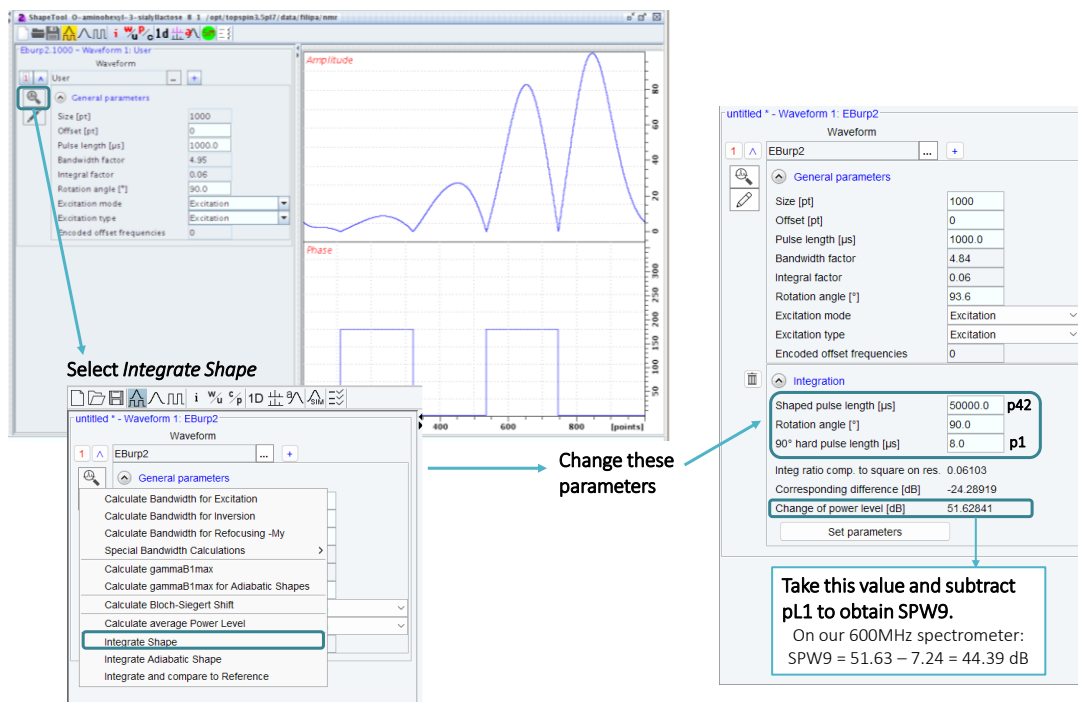
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Figure 22: Shape Tool display showing the workflow to determine the **spw9** value.

o Click on the "folder icon" to open the desired shape and select "*EBurp2.1000*".

o Click on the magnifying glass and select "*integrate shape*".

o Change the "Shaped pulse length (μs)) to **50000**

o Change the "Rotation angle [°]" to **90**

o Confirm that the "90° hard pulse length (μs)" corresponds to the **p1** value you determined earlier.

o Hit "**return**"

NOTE: in the last line you will see a value corresponding to "Change in power level [dB]"

Add your default pl1 value (e.g.: -7.28 dB, in the case of our 600MHz spectrometer) to this value:

$$SPW9 = \text{"Change in power level [dB]"} + \text{pl1}$$

e.g., in the case above: SPW9 = 51.62041+ (-7.24) = 51.62041 -7.24 = 44.34041 dB ➔ **_this is your SPW9_**

o Update the SPW9 value in *ased* (Figure 21)

Define the irradiation frequency list

o Go to **FQ2LIST** (frequency list 2) in the acquisition parameter window and click on the "**…**" icon.

  This will lead you to a table with all available lists just select one (we will edit it) or create a new one.

  If you select an existing one "**save as**" (so that you do not overwrite any data).

  The list must have at least three entries (although you can add more):

  1st line – spectrometer frequency (starting with an O)

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2nd line – one irradiation frequency (Hz)

3rd line – another irradiation frequency (Hz)

For example, for our 600 MHz spectrometer, it should be:

O 600.13

60000

4320

o Set this list in the pulse program parameters.

If you want to edit your list, click on the "*E*" icon (to edit)

Figure 23: Creation of the frequency list.

Other parameters

o **NBL** (in *ased*) and **TD** (F1) (in *eda*)

Ensure these values **match** and **correspond to the number of irradiation frequencies** (e.g.: NBL=2 and TD=2 when 1 off-resonance and 1 on-resonance frequencies are used).

o **L4** and **NS**

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Total scans = L4 * NS - The **L4** (loop counter) enables interleaved acquisition: e.g.: 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=1, then 320 scans are acquired on-resonance, then 320 scans off-resonance.

Define the Receiver Gain

> **rga** <ENTER>

Start acquisition

> **zg** <ENTER>

Protein based NMR binding experiments

Specifically, the ^{1}H-^{15}N HSQC based titration of the protein (fixed concentration) with different equivalents of the ligand (e.g. glycan) provides a powerful means to investigate glycan-protein interactions. Upon ligand binding, chemical shift perturbations (CSPs) of the protein's residues are observed. These CSPs can arise from direct interaction of ligand in the protein binding site, or due to conformational changes of the protein induced by the ligand upon binding. Therefore, analyzing CSPs helps identify binding residues and structural changes, aiding drug design and functional studies. In a protein-ligand titration, increasing ligand concentrations are added to a fixed protein sample. ^{1}H-^{15}N HSQC spectra are recorded at each step, providing insights into molecular recognition event. Below is a general protocol for conducting a protein-ligand titration by NMR spectroscopy.

Requirements/Conditions:
o   Depends on the protein size (from the initial 30 KDa limit in the 1990s, advances in NMR techniques and instrumentation now enable protein NMR for molecules up to 100 kDa, for higher NMR field spectrometers).
o   Requires large amounts of receptor. (larger amounts in comparison to STD-NMR)
o   Requires isotopic labelling of the protein.

Experimental Steps:

1.   **Sample Preparation of Protein and Ligand Solutions:**

Prepare concentrated stock solutions of the labeled protein and ligand(s). Ensure that the protein concentration and the ligand is known accurately. Prepare two NMR tubes:

Tube 1: protein free (in absence of the ligand) (**starting point**)
Tube 2: protein saturated (containing an excess of the ligand) (**end point**)

Note: These two tubes should be prepared with the same protein concentration, same buffer, same pH. The buffer should be H2O / D2O 90:10. High H2O is necessary since now you are monitoring the exchangeable NH pair in the 1H,51N-HSQC. Important also to check the pH, chemical shift of NH is sensitive to the pH and easily suffer CSP due to pH alterations.

The P:L ratio selected to prepare tube 2 is very dependent to the kinetics of the complex under study. Weak binders need large excess, while tight binders will just require lower P:L molar ratios to achieve protein saturation.

2. Initial NMR Spectrum of the Protein in absence and in presence of excess of the ligand:

Record an initial ^{1}H-,^{15}N -HSQC spectrum of the labeled protein in the absence of the ligand (tube 1) as well of the tube 2 (containing an excess of the ligand). Both spectra should be recorded **under the same experimental conditions**.

3. Ligand Titration:

Record Titration is performed by adding small volumes from tube 1 to tube 2 to achieve the interested protein/ligand ratios. Mix the contents of tube thoroughly.

4. Recording NMR Spectra:

For each sample in the titration series, acquire a ^{1}H-^{15}N HSQC spectrum **under the same experimental conditions** as the initial spectrum. This includes temperature, pH, and buffer conditions.

Ensure that the temperature and pH are maintained consistently throughout the experiment.

Also, in during the acquisition use the same 1H,15N-HSQC setup (SW, RG, NS, etc…). This is critical especially if you are in the intermediate and slow exchange regime.

5. Process NMR Spectra:

Use the same parameters and conditions for the processing of the different points in the titration. For the CSP analysis based on 1H,15N-HSQC titrations are crucial to calibrate with accuracy the spectra in both 1H- and 15N- dimensions.

HSQC – HETERO SINGLE QUANTUM COHERENCE

The HSQC is the most used heteronuclear experiment in biomolecular NMR. HSQC correlates two different types of bonded nuclei (commonly ^1H with ^{13}C or ^{15}N). It correlates the chemical shift of protons (displayed on the F2 axis) to the ^{13}C or ^{15}N chemical shift (on the "indirect," F1 axis) of their directly attached carbons via the $^1J_{CH/NH}$ coupling. In biomolecular NMR and due to low natural abundance of ^{13}C and ^{15}N; isotope enrichment of the sample is essential.
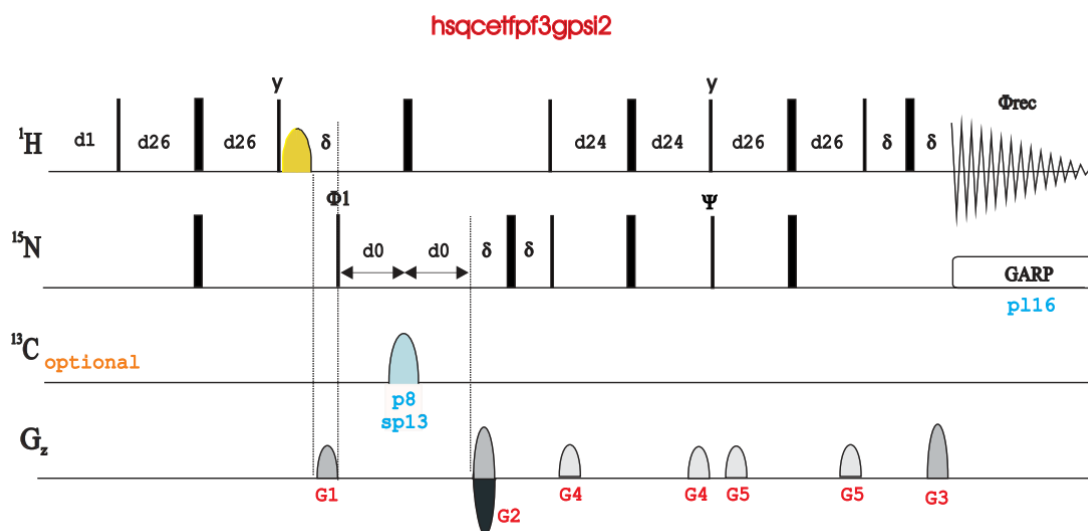
^1H- ^{15}N HSQC experiment

Figure 24: ^1H- ^{15}N HSQC pulse sequence (hsqcetfpf3gpsi2)

Create new experiment

> **edc** <ENTER>

Select the pulse program

> **rpar** <ENTER>
Select: **hsqcetfpf3gpsi2**

Define probe and solvent-dependent parameters according to the determined p1

> **getprosol 1H** *p1 pl1* <ENTER> (e.g., for p1=8.2 in a probe with pl1= - 7.24 db; write **getprosol 1H 8.2 -7.24**)

Tune the probe for ^1H and ^{13}C

> **atma** <ENTER>

The automatic tuning and matching will take longer since it will tune and match two different nuclei, first the ^{13}C and then the ^{1}H.

Set the acquisition parameters

> ased <ENTER>

Change the parameters: d1, SW (e.g. 16 ppm x 38 ppm), TD (e.g. 2048 x 128), DS, NS, O1p (4. 7 ppm), O3p (e.g. 118 ppm) etc.

Define the Receiver Gain

> rga <ENTER>

Start acquisition

> zg <ENTER>