HYNOE and HYCOSY
Running Hypercomplex Phase-Sensitive Experiments On The GN500

Phase sensitive experiments, also called "pure phase" or "pure absorption" mode experiments, are a way to get higher resolution and more information out of a 2D-dataset. By generating quadrature phase detection in the second dimension, you can not only distinguish positive cross peaks, negative cross peaks, and diagonal peaks, but you can get better resolution and (indirectly) better signal-to-noise. [By avoiding the magnitude calculations used in absolute-value/magnitude mode COSY's and NOESY's, you get narrower linewidths (better resolution) and, since you don't need to use sine-bell apodizations to compensate for magnitude-calculation-induced-linebroadening, you get correspondingly better signal-to-noise.] The tradeoffs for all this "wonderfulness" (of course there's a tradeoff!) are: acquisition times are longer (because of the longer required phase cycling), datasets are larger (which means more storage space is needed and processing takes longer), a more involved and complex data work up is required, and the pulse sequences are more demanding upon the instrument.

The characteristic feature of phase-sensitive COSY data is that the cross peaks are absorptive peaks (both positive and negative) while the diagonal peaks are dispersive. Phase-sensitive NOE and double-quantum COSY experiments, however, are phased totally absorptive. For phase-sensitive 2D-NOE (HYNOE), the absorptive diagonal peaks are in phase with the negative-NOE cross peaks, while the cross peaks from positive NOEs are 180° out of phase with the diagonal peaks.

An additional reason for running phase sensitive experiments is that they allow determination of the relative signs of coupling constants. Not only is this of interest of itself, but it means that the cross peak patterns of complex multiplets indicate which coupling constants belong to which nuclei (like 1D-selective irradiation experiments). This cannot be done by absolute value (magnitude) mode COSY.

There are two basic methods of generating phase sensitive datasets: the RSH (also called the RuSH or States method - illustrated below), and the Time Proportional Phase Incrementation (TPPI) methods (discussed in another handout). Follow the instructions below to obtain an RSH dataset: you should assume that an HYNOE will take at least 18-36 hours while an HYCOSY may only take 12-18 hours. (Don't try to make solutions too concentrated in an effort to speed up the experiment: the increased viscosity of saturated solutions will give broader lines (and a shorter FID) and produce poorer spectra.) The instructions are written to acquire a 512 x 2K x 2 dataset on the GN500; you may need to modify the instructions for your application.

BASIC SET-UP

1. Lock and shim on your sample and stabilize the spin rate. Make sure default parameters are set (i.e. XM macro #1 or #2).
2. (OPTIONAL) find out if the probe needs to be tuned. Pulse widths become longer the more out-of-tune a probe is, but an exactly tuned probe can produce very broad lines if the solvent resonance is strong (like H2O).
3. (OPTIONAL) Turn on temperature controller and let it stabilize.
4. (OPTIONAL) Find the decoupler offset of the solvent if solvent suppression by pre-saturation is needed.
5. (semi-OPTIONAL) Set IF-3 (Butterworth OFF) and CR = 0.45. Acquire a spectrum, phase it CAREFULLY, change CR and repeat until the "B" phase correction needed is as close to zero as possible.
6. Run a routine $^1$H experiment in which you have optimized the spectral window ($\text{SF}$ and $\text{SW}$ - e.g. use the "W" subcommand of $\text{ZO}$). Try to set $\text{SF}$ so that the carrier frequency (the exact center of the spectrum) is on a region of flat baseline and not a peak of interest (unless you are using aqueous solutions or other spectra needing selective excitation solvent suppression techniques. In these cases, set $\text{SF}$ on the solvent to remove quadrature images (use $\text{TH T}$ to set the $\text{SF}$ directly). Try to adjust $\text{SW}$ so that there is flat baseline on each side of the spectrum - ideally 5-10\% of the entire $\text{SW}$ on each side. Note the value of $\text{DW}$, then SAVE THE FID FROM YOUR BEST SPECTRUM (as an example, we'll call it $1\text{PULS.ØØ1}$). This file should have the latest values of $\text{SF}$, $\text{SW}$, $\text{OF}$, and $\text{DW}$, since these data will be used by the $\text{PG}$ command later. This spectrum should be good enough to overlay on the 2D plot to assign resonances.

7. Using the same parameters, find the exact $^1$H 90° flip angle and leave $\text{P2}$ set at that value. (Ask lab personnel if the current $\text{OC}$ level is suitable.) With $\text{NA} = 1$, properly set the gain ($\text{GN}$; don't use $\text{SG}$).

8. (Semi-OPTIONAL: most useful for HYNHOE) Do a quick T1 study (using the null condition). Use the conditions set above and follow the instructions in handout 031A.

9. For HYNHOE, set the parameters as indicated in section 9A (page 3). For HYCOSY, set the parameters as indicated in section 9B (page 4).

9A. For HYNHOE, set the following parameters:

$$
\begin{align*}
\text{EX} &= \text{HYNHOE} \\
\text{P2} &= \text{(the 90° pulse length from above)} \\
\text{D5} &= \text{(1.5-3 (or more) times the longest T1 in your sample)} \\
\text{D8} &= 1\text{U} \ \text{(msec)} \\
\text{D10} &= 200\text{M} \ \text{(msec)} \ 1/2 \text{T}_m \ (\text{T}_m = \text{mixing time; typically 50-500 msec. The value of this parameter is related to T1s [which regulate the rate of NOE buildup].}) \\
\text{D11} &= 0 \ \text{(saturation time - zero unless pre-saturating - see below)} \\
\text{I8} &= (1/4 \text{ of the } 1\text{H DW time}) \\
\text{L1} &= 0 \ \text{(pre-saturation power zero unless pre-saturing - see below)} \\
\text{PG} &= 1\text{PULS.ØØ1} \ \text{(use your 1D-filename from step 2)} \\
\text{CB} &= 2\text{K} \ \text{(variable - depends on the storage space available, the required resolution, and the experimental time available)} \\
\text{QP+Ø} \\
\text{AB-} \\
\text{MB} &= 2 \\
\text{DG} &= 2 \\
\text{NA} &= 32 \ \text{(some multiple of 32)}
\end{align*}
$$

$\text{TT}$ (it asks for the $\text{GS}$ number) will calculate an approximate experimental time. Adjust $\text{GS}$, $\text{CB}$, $\text{D5}$, (D11 if used), and $\text{NA}$ to fit the experiment to the instrument time available. $\text{TT}$ does not account for $\text{MB} = 2$ (so double the $\text{TT}$ value to get the experiment time).

Before starting, check the spin rate (if spinning), the temperature, and the lock level; do a $^\text{D}$ and check that all parameters are set correctly ($\text{F1SF=F2SF, F1SW=F2SW, F1DW=F2DW, OF=VOF, 18=1/4F1DW}$).

To start the experiment, type $\text{GS} = 512$ (or the number of blocks needed - this doesn't need to be a power of two) using your filename WITH A .DAT EXTENSION! (If you use a .ØØ1 extension, the hard disk will run for a LONG time and your experiment won't work - if you make this mistake, exit with $^\text{O}$).

Once acquisition has started, make sure that the first FID is bigger than the second FID (only in your first block of data), and run through $^\text{D}$ to check your parameters again.
If you need to include pre-saturation in your HYNOE experiment, use the above setup, except:

D5 = Ø (set to zero since relaxation will occur during D11 instead)
D11 = 1S (seconds) (approximate - try to set to the same value that D5 would have been)
L1 = 35 (larger values for broader peaks; <60 dB in all cases)

L1 and D11 work together - just like L2 and D10 do in PRESAT. L1 must be below 60dB to avoid heating, and D11 must be short enough to not take forever on a run with many scans. (D11 and D5 work together to allow relaxation of all spins, but during D5, the saturated peak recovers, so D11 must be longer to compensate. In most conditions, it would seem that the use of D5 would be self-defeating.) I suggest eliminating D5 and using only D11.

9B. For HYCOSY, set the following parameters:

EX = HYCOSY
P2 = (the 90° pulse length from above)
D5 = 1S (sec) (full relaxation isn't required)
D8 = 1U (µsec)
D11 = Ø (saturation time - zero unless presaturating - see below)
I8 = 1H DW time
L1 = Ø (pre-saturation power - zero unless presaturating - see below)
PG = 1PULS.ØØ1 (use your 1D-filename from step 2)
CB = 2K (variable - depends on the storage space available, the required resolution, and the experimental time available)

QP+Ø
AB-
MB = 2
DG = 2
NA = 8* (some multiple of 8)

TT (it asks for the GS number) will calculate an approximate experimental time. Adjust GS, CB, D5, (D11 if used), and NA to fit the experiment to the instrument time available. TT does not account for MB = 2 (so double the TT value to get the experiment time).

Before starting, check the spin rate (if spinning), the temperature, and the lock level; do a ^D and check that all parameters are set correctly (F1SF=F2SF, F1SW=F2SW, F1DW=F2DW, OF=VOF, I8=F1DW).

To start the experiment, type GS = 512 (or the number of blocks needed - this doesn't need to be a power of two) using your filename WITH A .DAT EXTENSION! (If you use a .ØØ1 extension, the hard disk will run for a LONG time and your experiment won't work - if you make this mistake, exit with ^O).

ONCE acquisition has started, make sure that the second FID is bigger than the first FID (only in the first block of data), and run through ^D to check your parameters again.

If you need to include pre-saturation in your HYCOSY experiment, use the above setup, except:

D5 = Ø (set to zero since relaxation will occur during D11 instead)
D11 = 500M (msec) (approximate - try to set to the same value that D5 would have been)
L1 = 35 (larger values for broader peaks; <60 dB in all cases)
L1 and D11 work together - just like L2 and D10 do in PRESAT. L1 must be below 60db to avoid heating, and D11 must be short enough to not take forever on a run with many
scans. (D11 and D5 work together to allow relaxation of all spins, but during D5, the saturated peak recovers, so D11 must be longer to compensate. In most conditions, it would seem that the use of D5 would be self-defeating.) I suggest eliminating D5 and using only D11.

**PROCESSING:**

NOTE: All processing needs to be performed on a hard disk or you'll run out of room (disk error #34). A 512 x 2K x 2 dataset requires 7% of an 88 MB hard disk (installed in the GN500, GN300NB, and the GE-SDS).

(Lets assume you stored the data as PAK.DAT):

Set up the double transform:

```
DT
BASELINE CORRECT? N
APODIZATION ROUTINE: GM <return>
# OF TM TO DO = Ø <return>
# OF ZEROFILLS = Ø <return>
BASELINE FIX? Y
```

Set up LB to go with the apodization

(For GM, use 0.2-1.0 like you would for EM).

**GC** PAK.DAT (call up block #1); process this first block with the same parameters you set up in DT:

```
GM (ZF if needed)
FT
AK = S (sets AI parameter to Same as the value needed for the first block - the biggest block)
PS or PE as needed (phase to get a flat baseline, with the "B" phase correction as close to zero as possible)
```

**IT IS IMPORTANT TO PHASE YOUR SPECTRUM VERY WELL AT THIS POINT.**

Do a ^F; you should see four spectra. For HYNOE, the 1st spectrum is to be phased absorption, the 2nd dispersion, and the 3rd and 4th are to be ignored (they should be small). Phasing of the first spectrum is straightforward. For HYCOSY, the 1st two spectra are ignored and the 3rd is to be phased absorptive (which makes the 4th spectrum dispersive). To phase the 3rd spectrum, type MB = Ø <return>, then change to (the computer's) block 1 (type 1). The "3rd" spectrum will fill the screen and now it can be phased as usual. (Change to block zero when finished.)

Your processing of this first block of data must duplicate your DT parameters exactly (#ZF, apodization, etc.). When finished phasing:

```
LI = GCDT
AU = 1
```

(dataset C = PAK.DAT; dataset D = PAK1.DAT)

Check the spectrum to be sure the phasing, baseline flatness, and apodization are what you want. Modify DT parameters and repeat until acceptable. Then:

```
LI = GCDTSD
AU = 512 (# of blocks in PAK.DAT)
```

(dataset C = PAK.DAT; dataset D = PAK1.DAT)

(Correct the baselines of PAK1.DAT with IC if needed - get help from lab personnel.)

```
MB = Ø, ^F, TD (with dataset C = PAK1.DAT; dataset D = PAK2.DAT)
```
LI = GCMSZFFTSDNO  (this is the second link, called the t1 link)
MS = 5  (you may need to experiment with this setting for your dataset - for HYNOE try MS = 10 to 20, or try DM = 3-4 for GS = 300 [where TD forces 512 blocks and a discontinuity])
AU = 1K  (with dataset C = PAK2.DAT; dataset D = PAK3.DAT)
   (1/2 the CB value of PAK.DAT x (1 + [# of ZF in DT command])
   = 1/2(2K) x (1 + 0) = 1K or 1024

For HYCOSY datasets, skip now directly to plotting (XY, CM, PR...) described on page 5.

For HYNOE datasets, you might want to check the dataset's phasing in the f1 direction before plotting. Instructions are given below. While tricky, you can tweak the phases to eliminate all dispersive character from the displayed dataset. The idea is to select two blocks from the dataset, each representing a peak from different ends of the spectrum, add them together, phase this additive spectrum, and apply the phase correction to the entire dataset. The phases should not need much correction.

To tweak up the f1 phasing in an HYNOE dataset:

VW PAK3.DAT - (the f1 x f2 dataset)
   (in VW: N displays the next block; R = previous block)

Find two blocks; one block having a phaseable peak on the left and another block having a phaseable peak on the right. Note their block numbers. Say, for example, you decide to use blocks #157 and #914:

Then: GC = PAK3.DAT - call up block #157 and SA this block as FILE.157 and: GC =
   PAK3.DAT - call up block #914 and SA this block as FILE.914
   Use AS (add and subtract) to add the two block's spectra together:
   AS DATASETB = FILE.914 <return>
   ADD FULL DATASETS? Y
   DATASETA = FILE.157 <return>
   K = 1 <return>
   <return> (exits AS and retains the summed dataset)
   SA = SUM.ØØ1 <return>, (add a comment) - this saves the added spectrum as SUM.ØØ1.
   PE - use PE and phase SUM.ØØ1. Negative NOEs and the diagonal peaks should be phased up.
   Then: LI = GCPSSD and AU = 1K

With this AU, you GC the file PAK3.DAT, PS it, and save it as PAK4.DAT. Now all plotting will occur with PAK4.DAT unless the dataset is further symmetrized (which is acceptable, but not always recommended).

PLOTTING:

Set XY = Ø
Set CM = 20 <return>, 20 <return>, <return>, <return>
PR = PAK3.DAT  (or PAK4.DAT for an HYNOE spectrum; use your filename.DAT)
   do a "projection" at Ø° (type "P", then "Ø")
Type YS
Enter CV (for your filename.DAT, try 5 contours)
   (All plotting takes place within CV; use the following subcommands)
   Y - re-defines YF scaling factor
   L - re-defines contour number for display/plot
   X - re-defines XY orientation of display/plot
(and resets the "CM" or "D" values)

D - re-defines dimensions of display/plot
U - updates display after above changes (after Y, L, X, and D)
+ - displays positive contours (default - gold colored display)
- - displays negative contours (blue display)
Z - enters zoom mode (see manual for description)
C - enters XY cursor mode (see manual)
P - plot displayed contour map
^X - plots X-axis displayed by PR command
^Y - plots the corresponding Y-axis
F - new page feed (like NP command)
R - return plotter to zero point (like RZ command)
B - draws a box around the contour map
Q - quit CV

A typical plotting procedure would be:

Set the plotter up correctly (so NP/RZ works properly). After setting XY, CM, PR, and YS, go into CV and choose a reasonable contour level display (varying Y and L), then type P, ^X, ^Y, R, B, Q. Call up the 1D spectrum (process it), set CM (to do a 20 x 5 cm plot), then PL.

If you want a parameter listing on the right side of the plot, this is best done with the LC command. The only good way to do that is to set WP so that the spectrum and axes are plotted with a nonexistent pen (i.e. #2-8) and the labels are plotted with pen #1. Call up the raw dataset, YS [on the FID; don't bother to process the data], set XY = Ø and CM = 20 <return>, .1 <return>, <return>, <return>. Return the pen carriage to the zero point with RZ if needed, then plot the "ZL-like" list with PL LC. Any other method probably won't work correctly.

MISC. NOTES on Phase-Sensitive 2D-NMR

Total experiment time:
With 512 blocks (CB=2K, MB=2, DG=2; therefore a 512 x 2K x 2 dataset):
  if D5 = 5 sec and NA = 32: ca. 50 hours
  if D5 = 3 sec and NA = 32: ca. 31 hours

Resolution: The number of blocks determines the resolution in the f1 domain; the block size determines the resolution in the f2 domain.

Zero filling: Don't bother to use any more than two zero fills in any one direction. Be aware that zero fills dramatically increase the processing time and required disk space. Zero filling will greatly decrease the "blockiness" or "stairstep" quality of your contour lines.

You should do a quick T1 study before an HYNOE to properly determine an appropriate D5 delay. Any time spent doing the T1 study is recouped many times over by not overestimating T1 values and hence having a needlessly long D5 delay. (Underestimating - having D5 too short - will produce poor spectra.) The D5 delay is really the only easily adjustable variable in HYNOE (aside from the matrix size) which affects the total running time - it is the rate-limiting step.

Since digital resolution for these experiments is only moderate when using the smaller block sizes, you can acquire data with the spinner off; this will lessen t1 noise. If you attempt to get good resolution by using a narrow spectral width or a large block size, however, you might want the spinner on. Spinning modulates the Q value of the probe and can give varying 90° pulses.
To help eliminate t1 noise, try setting the lock to "LOCK SLOW" and narrow the lock's sweep window (longer dwell time).

Suggested dataset sizes are 256-to-1K (number of blocks) x 512-to-4K (block size), however, a 368 x 512 x 2 dataset is the biggest dataset that can fit on a totally empty floppy disk (368 x 512 x 2 = 368 blocks, 512 block size, and MB = 2). Datasets bigger than a floppy can take a LONG time to work up. A 512 x 2K x 2 dataset requires 7% of an 88 MB hard disk (like the ones on the GN500, GN300NB, and the GE-SDS).

The length of the dispersion "tails" in the f1 direction of an HYCOSY experiment is related to the apodization. A smaller phase shift (MS = 3˚) makes shorter tails. If the S/N is too low, go for MS = 10-20˚ (which makes longer tails) or try GM instead.

Symmetrization is certainly possible for an HYNDE or a PS2QCOSY experiment, but you really need to look at both the symmetrical and non-symmetrical datasets to assure yourself that the symmetrization isn't losing weak NOE data. Note that HYCOSY datasets CANNOT be symmetrized.

If any 2D link executes very quickly and comes back with the message ":=1", it didn't work. Just try AU again.