NMR solvent for Metabolomics

D_2O and KH_2PO_4 buffer

1. 0.01% or 0.1% TSP in D_2O (w/v)
    1) If you do not want to use buffer solvent you can use it.
    2) It is not common solvent for metabolomics.
    3) 100 mL is enough for one year.
    4) Prepare 100 mL of D_2O (ask NMR department), Trimethylsilylpropionic acid sodium salt (TSP, ask NMR department).
    5) Add 10 mg (for 0.01%) or 100 mg (for 0.1%) of TSP to 100 mL D_2O.
    6) Keep it at room temperature.

2. KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.01% or 0.1% TSP (w/w)
    1) Prepare 100 or 500 mL of D_2O (500 mL is preferable, ask NMR department), Trimethylsilylpropionic acid sodium salt (TSP, ask NMR department), KH_2PO_4 (non deuterated reagent, stored in the cupboard of room HB038).
    2) In some case 0.01% TSP solution is used but for general metabolomics 0.1% TSP solution is more commonly used.
    3) 500 mL is better for 0.1% TSP solution and 100 mL for 0.01% TSP solution.
    4) Add 10 mg (for 0.01%) or 100 mg (for 0.1%) of TSP to 100 mL of D_2O.
    5) Add 1.232 (1.2 – 1.3) g of KH_2PO_4 to 100 mL of D_2O.
    6) Shake and mix well till there is no precipitant in the solution.
    7) Adjust pH (6.0) using NaOD (1.0 M) and pH meter in NMR department.

    <u>Manual of pH meter</u>

    - Open cap from electrode.
    - Press ‘Exit’ button when pH meter is turned off. Then, you can see ‘stabilizing’ on the screen. You can read the pH after 1-5 minutes.
    - You don’t have to press ‘Exit’ button when it is already turned on.
    - Wash carefully electrode with deionized water.
    - For calibration use pH 7.0 buffer.
    - Press ‘Cal’ for calibration.
    - You can see ‘1?’ for the first calibration buffer on the screen.
    - Type ‘7.0’ and enter. It will take 1-5 minutes for stabilizing and you can see ‘2?’ for the second buffer solution.
    - Wash electrode with deionized water.
    - Use pH 4.0 buffer or pH 11.0 depending on your target pH (generally we are using pH 4.0) buffer.
    - Type 4.0 and enter. It will take 1-5 minutes for stabilizing.
    - Press ‘Exit’ button to make it ready for measuring.
    - Wash electrode with deionized water and clean it with paper tissue.
    - Measure pH of your KH_2PO_4 solution. Initial pH of the solution might be around 5.0.
    - Add 1 M NaOD solution to adjust pH 6.0. About 4.0 mL of 1 M NaOD will be needed for pH 6.0 for 500 mL solution.
    - When it is finished wash electrode with deionized water and close cap.

    8) Label the description in the NMR solvent bottle (e. g. 0.1% TSP, D2O-KH2PO4 buffer, pH6, Date, Name).

* TSP:

- 3-(trimethylsilyl)propionic acid-d_4 sodium salt
- $(CH_3)_3SiCD_2CD_2COONa$
- FW; 172.28 (in case of D)
- $D^{20} = 1.3$

$CDCl_3$ and CH_3OH-*d_4*

3. 0.01% HMDSO in $CDCl_3$ (v/v)
    1) Generally you do not need any internal standard. If you need internal standard for general quantitation, Hexamethyldisiloxane (HMDSO) is better than Tetramethylsilane (TMS). However, chemical shift of HMDSO is not exactly 0.0 ppm (around 0.06 ppm). If you need internal standard for chemical shift calibration TMS (small addition) is better than HMDSO because you can calibrate chemical shift 0.0 ppm for TMS.
    2) Prepare 500 mL $CDCl_3$ (ask NMR department), Hexamethyldisiloxane (HMDSO, ask NMR department).
    3) Add 50 μL of HMDSO to 500 mL $CDCl_3$ using autopipette.
    4) Keep in refrigerator (+ 4 ^oC). Before using, keep it at room temperature at least for 1 hour.
    5) If $CDCl_3$ does not contain internal standard keep it at room temperature.
    6) If $CDCl_3$ does not contain internal standard you can calibrate 7.26 ppm for $CDCl_3$.

4. 0.01% HMDSO in CH_3OH-*d_4* (v/v)
    1) Generally you do not need any internal standard. If you need internal standard for general quantitation, Hexamethyldisiloxane (HMDSO) is better than Tetramethylsilane (TMS). However,chemical shift of HMDSO is not exactly 0.0 (around 0.06). If you need internal standard for chemical shift calibration TMS (small addition) is better than HMDSO because you can calibrate chemical shift 0.0 ppm for TMS.
    2) Prepare 100 mL CH_3OH-*d_4* (deposit in NMR department), Hexamethyldisiloxane (HMDSO, deposit in NMR department).
    3) Add 50 μL of HMDSO to 450 μL CH_3OH-*d_4* in 1.5 mL-ependorff tube using micropipette (Solution A).
    4) Add 100 μL of solution A to 100 mL of CH_3OH-*d_4*.
    5) Before using, keep it at room temperature at least for 1 hour.
    6) If $CDCl_3$ does not contain internal standard keep it at room temperature.
    7) If $CDCl_3$ does not contain internal standard you can calibrate 3.30 ppm for $CDCl_3$.

* HMDSO:
- Hexamethyldisiloxane
- $(CH_3)_3SiCOSi(CH_3)_3$
- FW; 162.38 (in case of H)
- $D^{20} = 0.764$

* TMS:
- Tetramethylsilane
- $Si(CH_3)_4$
- FW; 88.22 (in case of H)
- $D^{20} = 0.648$

Extraction Method for Metabolomics

1. Direct Extraction Method for General Metabolomics
    1) Prepare CH_3OH-d_4 (without any internal standard), KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% (w/w) TSP, 2 mL-ependorff tube, 1.5 mL-ependorff tube.
    2) Weigh 50 mg of freezing dried plant material. If you want to extract 100 mg, the plant material should be very fine powder.
    3) Add plant material to 2 mL-ependorff tube.
    4) Add 0.75 mL of CH_3OH-d_4 and 0.75 mL of KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% (w/w) TSP.
    5) Vortex for 1 min at room temperature.
    6) Ultrasonication for 5-20 min at room temperature.
    7) Centrifuge at room temperature for 5 – 20 min using microtube centrifugator (13000 rpm, room temperature).
    8) Transfer supernatant (more than 1 mL) to 1.5 mL-ependorff tube.
    9) If more centrifugation is necessary centrifugator using microtube centrifuator (13000 rpm, 1 min, room temperature).
    10) Transfer 800 μL of supernatant to 5 mm NMR tube

    * If you have more than 150 mg of plant material use 10 mL-glass cap test tube and centrifuge for glass ware (3000 rpm).

2. Indirect Fractionation Method for Large-scale extraction, separation of non polar and polar metabolites
    1) Prepare $CHCl_3$, MeOH, Deionized Water, 10 mL-glass cap-Test tube, 25 mL-evaporating flask, Pasteur pipette.
    2) Make the mixture of MeOH and Water (1:1, Solution A).
    11) Weigh 150 mg – 1 g of plant material.
    12) Add plant material to 10 mL-glass cap-Test tube.
    13) Add 4 mL of Solution A and 4 mL of $CHCl_3$ to plant material.
    14) Vortex for 1 min at room temperature
    15) Ultrasonicate for 5-20 min at room temperature
    16) Centrifuge for 20 min at 4 ^oC (3000 rpm).
    17) Separate lower ($CHCl_3$ fraction) and upper phase (MeOH-Water fraction) using Pasteur pipette and transfer to evaporating flasks.
    18) Repeat steps of 13) – 16) steps and combine the second extract to the first one for each fraction.
    19) Evaporate each flasks using rotary evaporator
    20) Make filter using Pasteur pipette and paper tissue.
    21) Load the filter on 5 mm-NMR tube
    22) Add 1 mL of $CDCl_3$ to $CHCl_3$ fraction and vortex for 2-3 min.
    23) Load $CDCl_3$ solution on the filter. Then sample will be filtered in NMR tube (wait for 1-3 min).
    24) Add 1 mL of KH_2PO_4 - D_2O buffer (pH 6.0, 0.1% TSP) or the mixture of CH_3OH-d_4 and KH_2PO_4 - D_2O buffer (pH 6.0, 0.1% TSP) to MeOH-Water fraction.
    25) Filter D_2O fraction using the same method as $CDCl_3$ fraction.

    * If there is no precipitant you don't have to follow filtering steps.

Manual Measurement of NMR Spectra

1. Find your NMR folder by type ‘s’ for searching.
2. Find the last exp No. in your folder.
3. Close search window.
4. Type ‘i’ to increase exp No ready for measurement.
5. In the new exp No., type ‘h’ or ‘c’ to choose appropriate NMR experiments. *‘h’ is for ^{1}H- and related NMR such as ^{1}H-NMR, COSY, NOESY, J-resolved…*’c’ is for ^{13}C-NMR related NMR experiments such as ^{13}C-NMR, APT, HMQC, HSQC, HMBC…
6. Select your experiment and you will see ‘RPAR’ window.
7. Choose ‘Copy All’.
8. Type ‘t’ for changing title and you will see title window.
9. Click ‘File’ in title window and click ‘Exit’. Then you will see a message, ‘save title before closing’. Choose ‘Yes’.
10. Type ‘ns’ to choose appropriate number of scans’. Type number of scans and enter. Number of scans should be 4^{n}. 64-256 scans for ^{1}H-NMR, 6k (1024 × 6) scans are necessary for ^{13}C-NMR experiment.
11. If you want to see measuring time expected, type ‘expt’.
12. Click ‘Lift on’ in the control box. Then you can hear the sound of air.
13. Load the NMR tube on NMR instrument.
14. Unclick ‘Lift on’ in the control box. Then the NMR tube is loaded in the instrument.
15. Type ‘atma’ for matching and tunning. To see the process of matching and tuning, type’a’ in 400 MHz NMR (when it is finished, click ‘Return’.) In 500 MHz NMR the matching window automatically appears and disappears when it is finished.
16. Type lock. Then you can see the list of solvent. Select appropriate solvent.
    * For the mixture of CH_3OH-d_4-D_2O, choose CH_3OH-d_4 (MeOD) for the lock of the mixture. For alternative locking you can press ‘Autolock’ in the control box.
17. For shimming, Click ‘Z_1’ in the control box. Then find maximum in lock window adjusting handle in the control box. If the line reach top click ‘Lock Gain’ in the control box and reduce the value by handle (You can see the value in the display of control box). Click again ‘StdBy’ and continue shimming from Z_1 (by clicking Z_1). Then continue Z_2, Z_3, X, and Y continuously. For selecting X or Y, first press X or Y, and Z_0.
18. Click ‘OnAxis’ in the control box.
19. Click ‘AutoShim’ in the control box.
20. Type ‘rga’ for receiving.
21. Type ‘zg’ for measuring the spectra.
22. The procedure of ^{13}C-NMR including APT is the same to ^{1}H-NMR experiment. For measuring use only ‘zg’ without ‘rga’. In case of ^{13}C-labled experiment use both ‘rga’ and ‘zg’.

* If you want to do ‘rga’ and ‘zg’ at the same time, type ‘rz’.
* When you want to do watersupression, o1p = center of water signal, pl9 = instensity of dB.
* If you have a problem with shimming, type ‘tune’ and select ‘$Z_1Z_2Z_3XY$’. The NMR machine will find optimized shim value.

*Useful NMR parameters

TD : total number of data points for FID, NS : number of scans, DS : dummy scans (extra pulse, normally for presaturated water suppression or 2D experiments), SWH: Spectra width in Hertz, FIDRes: FID resolution, AQ : acquisition time, DW: dwell time (time for 1 data point), DE: dead time, D1: additional relaxation delay time, RD (Relaxation Dealy) = AQ + D1

Measurement of 2D NMR Experiments

1. Find your NMR folder by type ‘s’ for searching.
2. Find the last exp No. in your folder.
3. Close searching window.
4. Type ‘i’ to increase exp No ready for measurement.
5. In the new exp No., type ‘h’ or ‘c’ to choose appropriate NMR experiments. *‘h’ is for ^{1}H- and related NMR such as ^{1}H-NMR, COSY, NOESY, J-resolved…*’c’ is for ^{13}C-NMR related NMR experiments such as ^{13}C-NMR, APT, HMQC, HSQC, HMBC…
6. Select your experiment and you will see ‘RPAR’ window.
7. Choose ‘Copy All’.
8. Type ‘t’ for changing title and you will see title window.
9. Click ‘File’ in title window and click ‘Exit’. Then you will see a message, ‘save title before closing’. Choose ‘Yes’.
10. Type ‘ns’ to choose appropriate number of scans’. Type number of scans and enter. Number of scans should be 4^{n}.
11. * 8 or 16 scans for J-Resolved, 2 or 4 scans for COSY, 16 or 32 scans for HMQC or HMBC. If you want to see measuring time expected, type ‘expt’.
12. Click ‘Lift on’ in the control box. Then you can hear the sound of air.
13. Load the NMR tube on NMR instrument.
14. Unclick ‘Lift on’ in the control box. Then the NMR tube is loaded in the instrument.
15. Type ‘atma’ for matching and tunning. To see the process of matching and tuning, type’a’ in 400 MHz NMR (when it is finished, click ‘Return’.) In 500 MHz NMR the matching window automatically appears and disappears when it is finished.
16. Type lock. Then you can see the list of solvent. Select appropriate solvent.
    * For the mixture of CH_3OH-d_4-D_2O, choose CH_3OH-d_4 (MeOD) for the lock of the mixture. For alternative locking you can press ‘Autolock’ in the control box.
17. For shimming, Click ‘Z_1’ in the control box. Then find maximum in lock window adjusting handle in the control box. Then continue Z2, Z3, X, and Y continuously. For seleting X or Y, first press Z0 and X or Y.
18. Click ‘OnAxis’ in the control box.
19. Click ‘StdBy’ in the control box.
20. Click ‘AutoShim’ in the control box.
21. Type ‘rga’ for receiving.
22. Type ‘zg’ for measuring the spectra.
23. Generally default range of each 2D-experiment is enough. However, if you want to change the range go to ^{1}H- or ^{13}C-NMR spectra of the sample which you want to measure.
24. Expand range of the spectra which you want to measure.
25. Click two times ‘SW-SFo1’ on the bottom. Then you will see ‘SW’ and ‘O1P’ values. SW value is whole range for measurement and O1P is the ppm value of center in the whole range.
26. Go to the Exp No. for your 2D measurement.
27. Type ‘eda’ for putting in the ‘SW’ and ‘O1’ value. Then you will see the window of ‘eda’.
28. Type ‘sw’ and ‘o1p’ on the bottom of ‘eda’ window. Then you can find ‘sw’ and ‘o1p’ value. Change the value to what you want.
29. For COSY, NOESY, and TOCSY experiments, F1 and F2 axis should be the same to each other (both for ^{1}H axis). For HMQC, HSQC, and HMBC, F1 axis is for ^{13}C and F2 axis for ^{1}H.

Measurement of Serial NMR Experiment

1. When you want to do NMR experiment continuously for one sample, e. g. ^{1}H-NMR, COSY, HMQC, and HMBC, make new exp No in your file for each NMR experiment.
2. Choose appropriate NMR experiment in each exp. No.
3. Do ‘atma’ in the first exp No. If you want to do both of ^{1}H- and ^{13}C- related NMR experiment (e. g. APT, HMQC, HSQC, or HMBC), do ‘atma’ in both exp No. of NMR experiments.
4. Locking and shimming are the same to that of single measurement.
5. Type ‘rga’ in the first exp No.
6. Type ‘multizg’ in the first exp No. Then you will see the message for asking No. of experiments. Then type number of experiments and press ‘enter’.
7. You will see total experimental time.

1D-NMR Data Processing Using XWIN-NMR (Bruker)

1. Change title if necessary.
    1) Type ‘t’.
    2) Change the title.
    3) Click ‘file’ in the title window.
    4) Click ‘Exit’. Then you will see a message, ‘save title before closing’.
    5) Choose ‘Yes’.
2. Type ‘pscal’.
    - Select ‘global’.
3. Type ‘efp’ to see spectrum.
4. Click ‘phase’ for phasing
    1) Increase signal intensity for fine phasing using options of intensity (*2, *8, or arrow key) on top-left of XWIN-NMR window.
    2) Click ‘biggest’. Then you will see green dot line.
    3) Focusing on the green dot line adjust phase to make symmetry with ‘PH0’.
    4) For the other range use ‘PH1’.
    5) Click ‘return’. Then you will see a message.
    6) Click ‘save & return’
2. Type ‘b’ for base line correction.
    1) Correct base line using ‘A’ or ‘B’. Keeping left click of mouse and adjust base line. If you make a mistake click ‘reset’.
    2) If you do phasing well only ‘A’ will be enough (sometimes ‘A’ and ‘B’).
    3) If you measure standard ^{1}H-NMR spectrum you can type ‘abs’ for automatic base line correction. But don’t use ‘abs’ for presaturated-watersuppressed ^{1}H-NMR.
    4) Click ‘return’. Then you will see a message.
    5) Click ‘save & return’
3. Calibration
    1) Expand spectrum for the signal of internal standard or solvent.
    2) Left click of mouse on the spectra.
    3) Middle click of mouse for starting point.
    4) Dragging to ending point.
    5) Middle click of mouse for ending point.
    6) Click ‘calibrate’.
    7) Drag cursor to find a top position.
    8) Middle click of mouse.
    9) Type chemical shift.

    * e. g. TSP (^{1}H : 0.00, ^{13}C: 0.00), CH_3OH-d_4 (^{1}H : 3.30, ^{13}C: 49.00), $CDCl_3$ (^{1}H : 7.26, ^{13}C: 78.00), DMSO (^{1}H : 2.49, ^{13}C: 39.50).
4. Integration
    1) Interesting signal can be integrated. But it is not recommended for plant extract because of complexity.
    2) Make expansion for interesting part of spectra by clicking arrows on the top-left.
    3) Click ‘integrate’.
    4) Use mouse for integration.

    * Left click: starting point of integration (cursor will be changed to be in black).
    * Middle click : end point of integration.

5) For another signal do left click of mouse to make cursor in red. You can use also arrows on the top-left for other region.
6) If you normalize all integral intensity to that of a signal make cursor on the top of target integration. Click ‘calibrate’ and type ‘1’ or ‘10’. Then all integral will be normalized to this value.
7) Click ‘return’.
8) Middle click of mouse.

Click ‘Save as ‘intrng’ & return’.

5. Adjust signal intensity
   1) Click ‘utilities’.
   2) Click ‘minimum’ to choose the lowest signal detected.
   3) If you see the message ‘set CY: does not work’ click ‘YU’.
6. Printing the spectra
   1) Type ‘xw’. Then you can see the window of XWIN-Plot Editor.
   2) There are three parts on the window such as ‘title’, ‘parameter’, and ‘spectra. There are two green dot buttons on the left of the window of XWIN-Plot Editor. Click left one (Mark Object).
   3) Click spectra and you can see ‘green dot’ line surrounding the spectra.
   4) Delete spectra, parameter, and title.
   5) Click ‘Data’ on the top-left of the window of XWIN-Plot Editor. Then you can see ‘Data Set Selector’.
   6) Click ‘Edit’ in ‘Data Set Selector’. Then, you can see ‘Porfolio Editor’.
   7) Remove present files of spectra in the list of ‘portfolio’ of ‘Portfolio Editor’ by clicking ‘remove’.
   8) Find your spectra in ‘User’ (e. g. AV032006, ‘Name’ (e. g. YoungRe032006), and ‘Expno’ (e. g. 10) by left click of mouse.
   9) Click ‘Append’ and ‘Apply’.
   10) Click ‘Close’ on ‘Porfolio Editor’.
   11) Click ‘Apply’ and ‘OK’ on ‘‘Data Set Selector’.
   12) Click ‘create 1D spectrum’ (drawing of 1D spectrum of the left).
   13) Keeping left click of mouse drag on the window of XWIN-Plot Editor for appropriate size.
   14) You can see gray and red dot lines surrounding spectra. The red dot line should be inside of gray dot line.
   15) Click left green dot button (Mark Object) and spectra.
   16) Click ‘Edit’ on the top-right of the window of XWIN-Plot Editor.
   17) You can see ‘’Basic Object Editor for position and dimension in ‘Edit Display Object’.

   * Suggested position: 0.7 × 1.7, Suggested dimension : 20.0 × 13.0

   18) Type numbers in ‘Xmin, Xmax in ‘Graph Object Editor’ of ‘Edit Display Object’ for the range of chemical shift which you want to see.
   19) Click ‘Axes’ in Attributes of ‘Graph Object Editor’.
   20) Choose Font type (e. g. Courier is recommended.), type 16 for fontsize, and click ‘Apply’ and ‘OK’.
   21) If you want to see chemical shift or integral of each signal click ‘show peaks’ or ‘show integral label’in ‘1D Spectrum Object Editor’ in ‘Edit Display Object’, respectively. But it is not recommended for whole range because the number of chemical shift and integral are too crowded. For

interesting range the chemical shift can be applied after adjusting range of chemical shift in Xmin, Xmax.

22) Click ‘Apply’ and ‘OK’ in ‘Edit Display Object’.
23) Click ‘1D/2D-Edit’ for changing intensity of signal and position of base line.
24) Click ‘*2’ (2 times) or ‘*8’ (8 times) to increase intensity. Click ‘/2’ (2 times) or ‘/8’ (8 times) to decrease intensity. You can also adjust intensity by using arrows.
25) Clicking ‘↕’ and keeping left click of mouse you can adjust position of baseline.
26) Click ‘create parameter object’ (drawing of NMR near to 1D NMR drawing).
27) Keeping left click of mouse drag on the window of XWIN-Plot Editor for appropriate size.
28) Click ‘Edit’. You can see ‘Edit Display Object’.
29) Unclick ‘Recalculate object dimension’ on the bottom.
30) Type position (suggested : 21.3 × 4.7) and dimension (4 × 12) in ‘Basic Object Editor’ of ‘Edit Display Object’.
31) Click ‘Title’ on the left and click on the top of spectra (inside of green dot line of spectra).
32) Put the title on the appropriate position of the top of spectra.
33) Click ‘Print’ in ‘File’ of XWIN-Plot Editor. Then you can see the window of Print.
34) Click ‘Print’ in the print window.

* Dimension and Position can be also adjusted by mouse.
- Make ‘spectra’, ‘Parameter’, or ‘Title’ green.
- Keeping left click of mouse and moving : adjust size.
- Keeping middle click of mouse and moving : adjust position.

2D-NMR Data Processing Using XWIN-NMR (Bruker)

1. Change title if necessary.
    1) Type ‘t’.
    2) Change the title.
    3) Click ‘file’ in the title window.
    4) Click ‘Exit’. Then you will see a message, ‘save title before closing’.
    5) Choose ‘Yes’.
2. Type ‘xfb’ to see spectrum.
    1) Type ‘tilt’ and ‘symj’ for J-resolved after ‘xfb’.
    2) Type ‘sym’ for COSY after ‘xfb’.
    3) Click ‘phase’ for phasing.
2. Phasing is not necessary except for phase-sensitive experiments (e. g. NOESY, TOCSY, HSQC).
3. Calibration
    1) Go to calibrated and baseline-corrected ^{1}H-NMR spectrum.
    2) Type ‘sr’ in the ^{1}H-NMR spectrum. Then you can see the sr value.
    3) Go to 2D-NMR spectrum and type ‘sr’. Put the same number to that of ^{1}H-NMR spectrum. Then ^{1}H-NMR axis will be calibrated.
    4) For ^{13}C-NMR part in HMQC, HSQC, or HMBC go to calibrated and baseline-corrected ^{13}C-NMR spectrum.
    5) Type ‘1 sr’. Then you can see the 1 sr value.
    6) Go to 2D-NMR spectrum. Put the same number to that of ^{13}C-NMR spectrum. Then ^{13}C-NMR axis will be calibrated.

    * If you don’t have ^{13}C-NMR spectrum calibrate to solvent signal or internal standard.
        - Make expansion for target signal.
        - Left click for starting point and drag to the point which you want. You can see square for the region.
        - Click ‘□’ on the top-left.
        - Click ‘calibrate’.
        - Put the cross on the target signal and enter. Then you can see the F2 frequency. Do enter once more. F1 frequency (^{13}C-NMR value) will be seen.
        - Put the number of ^{13}C-NMR chemical shift for the target signal.
        - e. g. TSP (^{1}H : 0.00, ^{13}C: 0.00), CH_3OH-d_4 (^{1}H : 3.30, ^{13}C: 49.00), $CDCl_3$ (^{1}H : 7.26, ^{13}C: 78.00), DMSO (^{1}H : 2.49, ^{13}C: 39.50).

    * For COSY click ‘calibrate’ even though you calibrated by ‘sr’ value.
        - Do enter. Then you can see the chemical shift of center of F2 axis. Enter once more. Center value of F2 axis will be seen.
        - Type the same value to F1 axis for F2 axis. Enter

    * For J-resolved click ‘calibrate’ even though you calibrated by ‘sr’ value.
        - Do enter. Then you can see the chemical shift of center of F2 axis (^{1}H-NMR part). Enter once more. Center of F2 axis (J value) will be seen.
        - Type ‘0’ for the F2 axis value. Then the spectra will be hidden.

    7) Reload the spectrum by ‘re No.’
4. Printing the spectra
    1) Type ‘xw’. Then you can see the window of XWIN-Plot Editor.

2) There are three parts on the window such as ‘title’, ‘parameter’, and ‘spectra. There are two green dot buttons on the left of the window of XWIN-Plot Editor. Click left one (Mark Object).
3) Click spectra and you can see ‘green dot’ line surrounding the spectra.
4) Delete spectra, parameter, and title.
5) Click ‘Data’ on the top-left of the window of XWIN-Plot Editor. Then you can see ‘Data Set Selector’.
6) Click ‘Edit’ in ‘Data Set Selector’. Then, you can see ‘Porfolio Editor’.
7) Remove present files of spectra in the list of ‘portfolio’ of ‘Portfolio Editor’ by clicking ‘remove’.
8) Find your 2D-spectra and 1D-spectra for projection in ‘User’ (e. g. AV032006, ‘Name’ (e. g. YoungRe032006), and ‘Expno’ (e. g. 10) by left click of mouse.
9) Click ‘Append’ and ‘Apply’.
10) Click ‘Close’ on ‘Porfolio Editor’.
11) Click ‘Apply’ and ‘OK’ on ‘’Data Set Selector’.
12) Click ‘create 2D spectrum’ (drawing of 2D spectrum of the left).
13) Keeping left click of mouse drag on the window of XWIN-Plot Editor for appropriate size.
14) You can see gray and red dot lines surrounding spectra. The red dot line should be inside of gray dot line.
15) Click left green dot button (Mark Object) and spectra.
16) Click ‘Edit’ on the top-right of the window of XWIN-Plot Editor.
17) You can see ‘’Basic Object Editor for position and dimension in ‘Edit Display Object’.
    - Suggested position: 0.8 × 1.8 (for J-resolved, HMQC, HMBC and HSQC without ^{13}C-NMR part), 3 × 1.8 (COSY, HMQC, HMBC and HSQC with ^{13}C-NMR part).
    - Suggested dimension : 19.6 × 13.2 (for J-resolved, HMQC, HMBC and HSQC without ^{13}C-NMR part), 17.5 × 12.7 (COSY, HMQC, HMBC and HSQC with ^{13}C-NMR part).
18) Type numbers in ‘Xmin, Xmax, Ymin, and Ymax in ‘Graph Object Editor’ of ‘Edit Display Object’ for the range of chemical shift which you want to see.
    - Xmin and Xmax for ^{1}H-NMR axis
    - Ymin and Ymax for J value for J-resolved, ^{1}H-NMR axis for COSY, NOESY, and TOCSY, ^{13}C-NMR axis for HMQC, HSQC, and HMBC
19) Unclick ‘Axis on left and click on Right in Graph Object Editor’ of ‘Edit Display Object’.
20) Click ‘Axes’ in Attributes of ‘Graph Object Editor’.
21) Choose Font type (e. g. Courier is recommended.), type 16 for fontsize, and click ‘Apply’ and ‘OK’.
22) In ‘2D Projection’ click ‘Top Data Set’, choose 1D spectrum for projection and click ‘on’. If necessary choose spectrum for the left projection and click ‘on’.
23) Click ‘Apply’ and ‘OK’ in ‘Edit Display Object’.

24) For J-resolved spectrum choose ‘Hz’ in ‘2D spectrum Object Editor’ for units for Y-axis.
25) Click ‘1D/2D-Edit’ for changing intensity of signal and position of base line.
26) There are two parts for arrows. Using first part of arrow the intensity of 2D spectrum can be adjusted and projection spectrum can be also adjusted by the second part after click ‘Top’ or ‘’Left’. Click ‘*2’ (2 times) or ‘*8’ (8 times) to increase intensity. Click ‘/2’ (2 times) or ‘/8’ (8 times) to decrease intensity. You can also adjust intensity by using arrows. Clicking ‘↕’ and keeping left click of mouse you can adjust position of baseline.
27) Click ‘create parameter object’ (drawing of NMR near to 1D NMR drawing).
28) Keeping left click of mouse drag on the window of XWIN-Plot Editor for appropriate size.
29) Click ‘Edit’. You can see ‘Edit Display Object’.
30) Unclick ‘Recalculate object dimension’ on the bottom.
31) Type position (suggested : 23 × 4.3) and dimension (3 × 13) in ‘Basic Object Editor’ of ‘Edit Display Object’.
32) Click ‘Title’ on the left and click on the top of spectra (inside of green dot line of spectra).
33) Put the title on the appropriate position of the top of spectra.
34) Click ‘Print’ in ‘File’ of XWIN-Plot Editor. Then you can see the window of Print.
35) Click ‘Print’ in the print window.

* Dimension and Position can be also adjusted by mouse.
- Make ‘spectra’, ‘Parameter’, or ‘Title’ green.
- Keeping left click of mouse and moving : adjust size.
- Keeping middle click of mouse and moving : adjust position.

Transferring Spectra to Graphic File

1. Type ‘xw’. Then you can see the window of XWIN-Plot Editor.
2. There are three parts on the window such as ‘title’, ‘parameter’, and ‘spectra. There are two green dot buttons on the left of the window of XWIN-Plot Editor. Click left one (Mark Object).
3. Click spectra and you can see ‘green dot’ line surrounding the spectra.
4. Delete spectra, parameter, and title.
5. Click (‘File’ → ‘Print’ → ‘Setup’).
6. Choose ‘Acrobat pdf’, ‘PCX (monochrome)’ or ‘TIFF’. In general pdf or pcx format is good. For submission to journal ‘TIFF’ is recommended (file size is very big.).
7. Choose ‘portrait’ for several spectra or ‘landscape’ for one spectra or 2D-spectra.
8. Click ‘cancel’ in the window of ‘print’
9. Click ‘Data’ on the top-left of the window of XWIN-Plot Editor. Then you can see ‘Data Set Selector’.
10. Click ‘Edit’ in ‘Data Set Selector’. Then, you can see ‘Porfolio Editor’.
11. Remove present files of spectra in the list of ‘portfolio’ of ‘Portfolio Editor’ by clicking ‘remove’.
12. Find your 1D-spectra or2D-spectra with projection spectra in ‘User’ (e. g. AV032006, ‘Name’ (e. g. YoungRe032006), and ‘Expno’ (e. g. 10) by left click of mouse. You can choose multiple spectra.
13. Click ‘Append’ and ‘Apply’.
14. Click ‘Close’ on ‘Portfolio Editor’.
15. Click ‘Apply’ and ‘OK’ on ‘‘Data Set Selector’.
16. Click ‘create 1D spectrum’ or ‘create 2D spectrum’.
17. Keeping left click of mouse drag on the window of XWIN-Plot Editor for appropriate size.
18. You can see gray and red dot lines surrounding spectra. The red dot line should be inside of gray dot line.
19. Click left green dot button (Mark Object) and spectra.
20. Click ‘Edit’ on the top-right of the window of XWIN-Plot Editor.
21. You can see ‘’Basic Object Editor for position and dimension in ‘Edit Display Object’.
22. Type numbers for ‘position’ and ‘dimension’
    1) Suggested ‘position’ and ‘dimension’ for one ^{1}H-NMR spectra (in ‘landscape’).
    - 0.6 × 1.9, 25.5 × 16

    2) Suggested ‘position’ and ‘dimension’ for one J-resolved, HMQC, HMBC, and HSQC without ^{1}H-NMR part (in ‘landscape’).
    - 0.8 × 1.8, 23.7 × 14

    3) Suggested ‘position’ and ‘dimension’ for one COSY, HMQC, HMBC, and HSQC with ^{1}H-NMR part (in ‘landscape’).
    - 2.7× 1.8, 21.8 × 14

    4) Suggested ‘position’ and ‘dimension’ for two ^{1}H-NMR spectra (in ‘portrait’)
    - 0.7 × 14.4 (2.1), 17.1 × 11.5

    5) Suggested ‘position’ and ‘dimension’ for three ^{1}H-NMR spectra (in ‘portrait’)
    - 0.8 × 19.5 (10.8, 2.1), 17.0 × 6.3

    6) Suggested ‘position’ and ‘dimension’ for four ^{1}H-NMR spectra (in ‘portrait’)
    - 0.7 × 20.9 (14.7, 8.5, 2.3), 17.1 × 5.1

    7) Suggested ‘position’ and ‘dimension’ for five ^{1}H-NMR spectra (in ‘portrait’)

- 0.7×22.9 (17.7, 12.5, 7.3, 2.1), 17.1×3.1

23. Type numbers in 'Xmin, Xmax, Ymin, and Ymax in 'Graph Object Editor' of 'Edit Display Object' for the range of chemical shift which you want to see.
- Xmin and Xmax for ^{1}H-NMR axis
- Ymin and Ymax for J value for J-resolved, ^{1}H-NMR axis for COSY, NOESY, and TOCSY, ^{13}C-NMR axis for HMQC, HSQC, and HMBC

24. Unclick 'Axis on left and click on Right in Graph Object Editor' of 'Edit Display Object' for 2D-spectra.
25. Click 'Axes' in Attributes of 'Graph Object Editor'.
26. Choose Font type (e. g. Courier is recommended.), type 16 for fontsize, and click 'Apply' and 'OK'.
27. In '2D Projection' click 'Top Data Set', choose 1D spectrum for projection and click 'on'. If necessary choose spectrum for the left projection and click 'on'.
28. Click 'Apply' and 'OK' in 'Edit Display Object'.
29. For J-resolved spectrum choose 'Hz' in '2D spectrum Object Editor' for units for Y-axis.
30. Click '1D/2D-Edit' for changing intensity of signal and position of base line.
31. There are two parts for arrows. Using first part of arrow the intensity of 2D spectrum can be adjusted and projection spectrum can be also adjusted by the second part after click 'Top' or ''Left'. Click '*2' (2 times) or '*8' (8 times) to increase intensity. Click '/2' (2 times) or '/8' (8 times) to decrease intensity. You can also adjust intensity by using arrows. Clicking '↕' and keeping left click of mouse you can adjust position of baseline.
32. Click ('File' → 'Print' → 'To file').
33. Choose 'plot.code' in 'Diretories' and double click it.
34. In 'Selection' type your title.

To AMIX User's (new version) for data bucketing

1. Click 'AMIX'.
2. Click 'Buckets, Statistics' on 'AMIX-Tools' menu and then you will see another window.
3. Choose 'New' on 'Bucket table' menu in 'Statistics'.
4. Choose '1D NMR' and 'Simple rectangular buckets' in '1D NMR Bucket method'. Then click 'Next'.
5. Put '10.02' in 'left border', '-0.1' in 'right border'. Choose 'positive intensities' in 'Integration mode' menu and 'scale to total intensities' in 'Scaling' menu. Choose 'edit exclusion'. Click 'Next'. Then you will a table of exclusion.
6. Put the range of exclusion (e. g., water and MeOD signals) in the table. If you want to save it, click 'save exclusions'. Click 'Next'.
7. Choose target folder where you want save the bucket table. (e. g. F:\data\Young\'your folder'\'file name'. Before you choose the target folder you should have your own folder under'Young'. For file name, you can type it. Then, your bucket table will be saves as the name you type.
8. Click 'Next'.
9. Partition: Z:\,
   Data directory: data
   User name: Your folder (e. g. Fcog0507, AV012006)
   Spectroscopy: nmr
   Data set name: Name of your data folder (e. g. YHRe0503Fcog)
   Experiment #: *
   Pdata: pdata
   Processing #: *
   Click 'OK'
10. Choose NMR data and click' OK'.
11. You have a bucket table in the folder you've made before in 'the 7^{th} step'.
12. Three kinds of files are generated. Among them 'bucket_table' is the file of bucketing data. You may use this file for further PCA.