**SUPPLEMENTARY MATERIALS**

**FOR**

**Enabling adoption of 2D-NMR for the higher order structure assessment**

**of monoclonal antibody therapeutics**

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**Figure S1**. Representative 1H,15N gHSQC spectral fingerprints for the SSS at 900 MHz and 500 MHz showing the peaks with arbitrary numbering used for CCSD precision analyses. (**A**) D1A-8495-009 spectrum at 900 MHz; (**B**) D1A-9966-001 spectrum at 500 MHz. Both representative spectra were collected at 37 °C. Contour levels were chosen for best visualization of the picked peaks. The arbitrarily numbered peak lists were initially defined from a 500 MHz gHSQC spectrum for the SSS and copied to all other spectra. All 1H,15N spectra were aligned to arbitrary cross peak #68 (see arrow). The peaks in red were subsequently removed from the list due to poor resolution or S/N in a subset of spectra. Arbitrary cross peaks 146 and 147 were removed due to a subset of spectra recorded with reduced 15N spectral width, leading to folding of these two cross peaks (experimental code: E2). Due to the relatively few number of spectra measured on the NIST-Fab, no attempt was made to define a common peak list between the NIST-Fab and SSS. For spectra of SSS collected at different temperatures, many cross peaks shifted significantly due to the temperature difference, resulting in peak tracking error. As a result, only 1H,15N spectra collected at 37 °C were included in the peak list tables. See Materials and Methods section for more details.



**Figure S2**. Representative 1H,13C gHSQC spectral fingerprints for the SSS at 900 MHz and 500 MHz showing the peaks with arbitrary numbering used in CCSD and chemometric analyses. (**A**) D2C-8495-012 spectrum at 900 MHz; (**B**) D2C-3897-012 spectrum at 500 MHz. Both representative spectra were collected at 37 °C. Contour levels were chosen for best visualization of the picked peaks. A common arbitrarily numbered peak list was initially defined from a 500 MHz gHSQC spectrum for both the SSS and NIST-Fab at 37 °C and copied to all other 1H,13C spectra at all temperatures. All 1H,13C spectra were aligned to arbitrary cross peak #29, shown in bold. The peaks in red were subsequently removed from the list due to poor resolution or S/N in a subset of spectra. The peaks in green were not resolved in the SOFAST-HMQC spectra (spectral code: E2C). See Materials and Methods section for more details.



**Figure S3.** Comparative analysis of the common peak list.(**A**) Overlays of D2C-7425-012 of SSS and D3A-7425-015 of the NIST-Fab representative 1H,13C gHSQC spectral fingerprints at 900 MHz with the common peak list from **Figure S2**. These common peaks are marked with an ‘X.’ For clarity, all excluded peaks not used for further analysis are removed from this overlay (see **Figure S2** caption for more details.) (B) CCSD plot of D3A-7425-015 spectrum of the NIST-Fab at 900 MHz benchmarked against the average uniformly sampled D2A/D2C reference peak positions. As can be seen in **Figures 2E, F** and **S3**, the 1H,13C spectrum contained additional peaks due to the four extra amino acids on the N-termini of both the heavy and light chains of the SSS. However, since these extra cross peaks were not common to both samples, they were not used in the analyses. The differences between the SSS and NIST-Fab spectra highlighted by PCA can therefore be explained by the shifting of a number of resonances by up to 40 ppb.



**Figure S4**. Breakdown of 1H,13C CCSD analysis of the SSS recorded at 37 °C. In general, all spectra were collected with a gHSQC, with the exception of E1C spectra, which used the SOFAST-HMQC pulse sequence. The red bars represent average CCSD values for D2A/D2C US spectra and D2B/D2D/D2E NUS spectra, respectively. The bolded number above each bar represents the total number of spectra included in the analysis for each respective experimental type. Custom NUS E1A-type experiments tended to have slightly less peak precision, highlighting the need for careful selection of NUS schedule. Error bars represent 95% confidence intervals for standard error of the mean (SEM). For detailed breakdown of experimental codes, see **Table 1** in the main text.



**Figure S5**. Highlight of PC4 outlier from PCA of all peak lists from 354 1H,13C spectra. The only substantial PC4 outlier, spectrum E1A-5479-103, is labeled. The inner and outer ellipsoids represent 95% and 99% confidence regions, respectively, based on chi-square probabilities. See the caption of **Figure S6** for more details.



**Figure S6**. Analysis of a representative outlier 1H,13C spectrum E1A-5479-103. (**A**) Overlay of E1A-5479-103 NUS spectrum recorded at 500 MHz in red, with uniform sampled D2C-7425-012 spectrum collected at 900 MHz in black. (**B**) CCSD plot of E1A-5479-103 versus D2C-7425-012, both benchmarked against the average uniformly sampled D2A/D2C reference peak positions. E1A-5479-103 was collected with a custom NUS schedule that was generated with the manufacturer’s software, TopSpin 3. Unlike the NIST NUS schedules that were generated with the Poisson-gap algorithm, the custom NUS schedule for 5479-103 contained many holes at the beginning of the schedule, leading to the observed artifacts. As can be seen in the CCSD plot in panel **B**, a subset of resonances were also shifted from the average uniformly sampled D2A/D2C reference peak positions. However, despite these shifts, the majority of resonances still were under 10 ppb from the reference. The effect of sampling schedule is beyond the scope of this manuscript.



**Figure S7**. Annotated PCA plot of temperature deviant outlier 1H,13C spectra from **Figure 4A** of the main text. Several SSS spectra were determined to be deviant from the reported temperature. For the NIST-Fab outlier spectra labeled on the plot, these are likely temperature deviant as well due to the high spectral similarity of the NIST-Fab to the SSS even though no calibration curve was made. The inner and outer ellipsoids represent 95% and 99% confidence regions, respectively, based on chi-square probabilities. **Figure S8** shows a representative example of a 1H,13C spectrum with shifted peaks due to temperature, and **Table S2** lists the calculated temperatures from calibration curves using selected 1H,13C chemical shift positions.



**Figure S8**. Overlay of SSS temperature outlier at 600 MHz. The outlier 1H,13C gHSQC spectrum of D2E-8822-065 is in red, D2C-8822-036 at 37 °C in black, and E1B-8822-051 at 45 °C in green. (**A**) Complete methyl fingerprint region; (**B**) Expansion showing the four cross peaks that exhibited temperature sensitivity. Relevant temperature-sensitive arbitrarily-numbered cross peaks used to generate temperature calibration curves are labeled. Laboratory 8822 recorded these spectra on two different 600 MHz spectrometers.

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**Figure S9**. *k*-Medoids clustered PCA plots of peak tables from 1H,13C spectra recorded at 37 °C. All peak tables were clustered with the *k*-medoids clustering algorithm. (**A**) 3D plot of two clusters (*k* = 2); (**B**) 2D plot of the same two clusters (*k* = 2) clusters; (**C**) 2D plot of four clusters (*k* = 4). (**D**) 2D plot of PC2 versus PC3. To simplify panel **D**, all clusters (*k* = 4) were colored the same with the exception of the PC3 outlier in magenta. The magenta point in panels **C** and **D** is spectrum E1A-5479-103, which the *k*-medoids algorithm clustered as its own individual group and outside all confidence intervals. The inner and outer ellipsoids represent 95% and 99% confidence regions, respectively, based on chi-square probabilities. For more discussion of panels **B** and **C**, see **Figure S10**, for which the data is annotated differently to highlight the likely relevant differences between *k* = 2 and *k* = 4 clusters. See **Figure S6** for more discussion of this outlier.



**Figure S10**. Re-annotated *k*-medoids clustered PCA plots of peak tables from 1H,13C spectra recorded at 37 °C. As in **Figure S9**, all peak tables were clustered with the *k*-medoids clustering algorithm. (**A**) Two clusters (*k* = 2); (**B**) Four clusters (*k* = 4). The inner and outer ellipsoids represent 95% and 99% confidence regions, respectively, based on chi-square probabilities. For both panels, red diamonds represent a subset of spectra collected with a known pulse program (pp) error. The pulse program error arose on some NUS spectra that were recorded on Bruker spectrometers that used the software TopSpin 2. On this version of TopSpin, NUS acquisition needed to be hard-coded into the pulse program, whereas TopSpin 3, used on all other Bruker spectrometers, allowed facile implementation of NUS within the software. For the TopSpin 2 version of the pulse sequence, an uncompensated 15N decoupling pulse was present during 13C evolution, resulting in spectra that could not be appropriately phased in the 13C dimension. In panel **B** this subset of NUS spectra collected on TopSpin 2 pulse sequence error appears to have been loosely clustered by the *k*-medoids clustering algorithm. The spectra with this systematic error are denoted in the center column of **Table S10**.

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**Figure S11**. Data clustered with the *k*-medoids algorithm using two principal components (PCs). (**A**) *k* = 7; (**B**) *k* = 8; (**C**) *k* = 9. The clustering results clearly show that the *k*-medoids algorithm is less effective at analyzing this data set, since it divides the 37 °CSSS spectra into two or three distinct clusters. Furthermore, the *k*-medoids algorithm groups the NIST-Fab spectra (*k* = 7 and 8) collected at 45 °C with the NIST-Fab spectra collected at 50 °C. When 9 clusters were explicitly specified, the algorithm divided these two sets of spectra separate into their appropriate clusters.

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**Figure S12**. Data clustered using the min-max group pair algorithm using two principal components. (**A**) *k* = 7; (**B**) *k* = 9. The inner and outer ellipses depict 95% and 99% confidence regions, respectively, based on chi-square probabilities. For panel **A**, it is seen that this algorithm detected an outlier and placed this spectrum in its own cluster. This singleton cluster (dark green) represents a single spectrum E1B-8179-312 that is a known temperature outlier (**Table S2**). Additionally, the placement of spectrum E1B-8179-312 in its own cluster had the effect of grouping the SSS spectra collected at 45 °Cwith the SSS spectra collected at 50 °C. For *k* = 8, the incorrect temperature cluster was appropriately separated out into distinct groups (**Figure 4B**, main text). In panel **B**, two known NIST-Fab spectral outliers, D3A-6272-013 and D3B-6272-014, due to temperature are separated into a distinct cluster (in orange). It should be noted that UPGMA produces identical clusters to the min-max group pair algorithm when *k* = 8 and *k* = 9.



**Figure S13**. Data clustered using the UPGMA algorithm using two principal components (**A**) *k* = 7; (**B**) *k* = 9. The inner and outer ellipses depict 95% and 99% confidence regions, respectively, based on chi-square probabilities. For panel **A**, it is seen that this algorithm correctly clustered the spectra, including the addition of D2E-8822-065 into the SSS 45 °C cluster. For *k* = 8, spectrum E1B-8179-312 is then separated out into its own singleton cluster (**Figure 4B**, dark green point, **Table S2**). In panel **B**, two known NIST-Fab spectral outliers, D3A-6272-013 and D3B-6272-014, due to temperature are separated into a distinct cluster (in orange). It should be noted that UPGMA produces identical clusters to the min-max group pair algorithm when *k*=8 and *k*=9.



**Figure S14**. Representative 1H, 15N gHSQC spectral fingerprint outliers not included in the peak analysis. (**A**) D1A-7244-014 spectrum of SSS at 800 MHz; (**B**) E2-3655-101 of SSS at 800 MHz. All representative spectra were collected at 37 °C. Many cross peaks in these two representative spectra are missing or have very low S/N. Compare to **Figure 1** in the main text. All excluded spectra are listed in **Table S10**. These excluded spectra suggest the need for care in experimental set-up rather than a problem with 2D-NMR method. Unfortunately, they also represent a weakness in peak analysis. If a spectral perturbation occurs in the fingerprint region that does not impact one of the defined peaks, such a perturbation would be missed. A total point-by-point analysis of the entire spectral region will ‘catch’ any such spectral change, although this type of analysis is also sensitive to field strength, pulse sequence, and sampling schedule. A total point-by-point analysis could also help evaluate the severity of possible experimental errors and how these may adversely affect the analysis of the spectral fingerprint.



**Figure S15**. Representative 1H, 13C gHSQC spectral fingerprint outliers not included in the peak analysis. (**A**) E1A-1894-103 spectrum of SSS at 600 MHz; (**B**) D3B-2461-107 of the NIST-Fab at 800 MHz. All representative spectra were collected at 37 °C. Positive contours are in black and negative contours in gray. Both spectra are plotted just above the noise threshold. Many cross peaks in these two representative spectra are missing or have very low S/N. In addition, the arbitrarily numbered cross peak used for alignment (peak #29, see **Figure S2**) is missing or has extremely weak intensity, precluding accurate spectral alignment. Compare to **Figure 2** in the main text. All excluded spectra are listed in **Table S10**. See Materials and Methods in main text and **Figure S14** caption for more details.

**Table S1**. Detailed summary of the experiments performed by each institution. Institutions who submitted more than one data package received multiple institutional identifiers. See **Table 1** in main text for the description of each experimental code.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Institutional Identifier | Total by Institution | D1A | D2A | D2B | D2C | D2D | D2E | D3A | D3B | E1 | E1A | E1B | E1C | E2 | E2N | E2A | E2B | E2C |
| 1247 | **12** | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 5 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| 1894 | **8** | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 4 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 2146 | **8** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2461 | **8** | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 2974 | **8** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3655 | **4** | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| 3676 | **6** | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| 3897 | **24** | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4233 | **88** | 2 | 3 | 2 | 3 | 2 | 2 | 4 | 4 | 0 | 15 | 32 | 12 | 0 | 0 | 4 | 0 | 3 |
| 4533 | **3** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 1 | 0 |
| 5417 | **20** | 2 | 2 | 2 | 2 | 2 | 2 | 4 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5422 | **8** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5479 | **10** | 0 | 1 | 0 | 1 | 1 | 1 | 2 | 2 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 5487 | **8** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6211 | **12** | 0 | 1 | 4 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 6272 | **21** | 0 | 3 | 2 | 3 | 3 | 1 | 2 | 2 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 1 | 1 |
| 6324 | **23** | 1 | 2 | 1 | 2 | 1 | 1 | 2 | 0 | 0 | 5 | 3 | 0 | 0 | 1 | 3 | 1 | 0 |
| 7244 | **16** | 2 | 2 | 2 | 3 | 2 | 1 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7425 | **16** | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8179 | **13** | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 5 | 1 | 0 | 0 | 0 | 1 | 1 | 1 |
| 8473 | **8** | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| 8495 | **21** | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8543 | **9** | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8822 | **65** | 4 | 3 | 3 | 3 | 3 | 3 | 9 | 4 | 0 | 0 | 10 | 7 | 0 | 1 | 6 | 3 | 6 |
| 9516 | **8** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9936 | **10** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |
| 9963 | **7** | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9966 | **7** | 1 | 1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**Table S2**. Corrected temperatures for outlier 1H,13C spectra of SSS in the PCA plot. Temperature calibration curves were separately generated for 1H and 13C resonance positions for arbitrarily numbered cross peaks, 6, 30, 34, and 35 and averaged. For peak 30, only the 1H chemical shift was used due to negligible perturbation of the 13C chemical shift. See **Figure S8**.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Experimental Type‡ | Experimental Code | Field  (MHz) | Reported Temperature | Corrected Temperature |
| D2A | 8822-061 | 600 | 37 °C | 39.1 °C |
| D2B | 8822-062 | 600 | 37 °C | 39.6 °C |
| D2C | 8822-063 | 600 | 37 °C | 39.5 °C |
| D2D | 8822-064 | 600 | 37 °C | 39.0 °C |
| D2E | 8822-065 | 600 | 37 °C | 42.9 °C |
|  | | | | |
| D2A | 8179-302 | 500 | 37 °C | 35.0 °C |
| E1A | 8179-303 | 500 | 37 °C | 34.6 °C |
| D2C | 8179-304 | 500 | 37 °C | 34.6 °C |
| E1A | 8179-305 | 500 | 37 °C | 34.6 °C |
| E1A | 8179-306 | 500 | 37 °C | 35.4 °C |
| E1B | 8179-312 | 500 | 25 °C | 22.7 °C |
|  | | | | |
| E1B | 4533-004 | 600 | 50 °C | 48.3 °C |

‡See **Table 1** in main text for complete description of experimental types.

**Table S3**. Percent of resolved 1H,13C methyl cross peaks for the unlabeled NIST-Fab sample at 37 °C. The SSS data were not used in this analysis. This analysis of the NIST-Fab spectra was performed on the D3A-type experiments with 25 ms acquisition time in the 13C dimension. A total of 232 cross peaks were expected to be observed from 25 alanines, 11 isoleucines (2 cross peaks each), 32 leucines (2 cross peaks each), 5 methionines, 44 threonines, and 36 valines (2 cross peaks each). Lower percent coverage is observed at 500 MHz for the common peak list between the NIST-Fab and the SSS (**Table S12**) because this list covers all acquisition strategies and temperatures explored in this study. Using a peak table for a single molecule (*e.g.*, the NIST-Fab) with a single field strength and experimental set-up affords much higher spectral coverage even at 500 MHz.

|  |  |  |
| --- | --- | --- |
| Field (MHz) | Number of Picked Peaks | % Coverage |
| 900 | 212 | 91 |
| 850 | 209 | 90 |
| 800 | 207 | 89 |
| 750 | 201 | 87 |
| 700 | 194 | 85 |
| 600 | 180 | 78 |
| 500 | 155 | 67 |

**Table S4.** Complete instrument list. The institutional identifier and equipment list were anonymized by NAPT to further reduce potential data traceability. Rows in blue, QCI cold probes; row in gray, BBO cold probe; rows in white, TCI or other cold probe; rows in orange, room temperature probe; rows in green, Agilent/Varian equipment.

|  |  |  |  |
| --- | --- | --- | --- |
| **1H Frequency** | **Vendor** | **Console** | **Probe** |
| 500 MHz | Bruker BioSpin | Avance III | CP QCI H/F-C/C-D CryoProbe, Z-gradients |
| 500 MHz | Bruker BioSpin | Avance II+ | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 500 MHz | Bruker BioSpin | Avance III-HD | CP BBO 500S2 BBF-H-D-05 Z-gradients |
| 600 MHz | Agilent/Varian | INOVA | 5mm Triple Resonance (1H,13C,15N,2H)  cold probe, Z-gradients |
| 600 MHz | Agilent/Varian | DD2 | Triple resonance PFG cold probe |
| 600 MHz | Bruker BioSpin | Avance III HD | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III HD | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III | PA BBO 600S3 BBB-H-D-05 Z SP |
| 600 MHz | Bruker BioSpin | Avance III | CP QCI 600S3 H/F-C/N-D-05 Z-gradients |
| 600 MHz | Bruker (Oxford) | Avance II+ | 5mm QCI 1H-13C/15N/31P/D CryoProbe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III HD | 5 mm CP TCI 1H/19F-13C/15N/D Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III | 5mm DCH 1H/13C/D CryoProbe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III | 5mm TXI 1H-13C/15N/D triple-resonance probe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance | Cold, 13C/15N/31P tunable 1H/19F |
| 600 MHz | Bruker BioSpin | Avance III HD | CP TCI 600S3 H&F-C/N-D-05 Z-gradients |
| 600 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 600 MHz | Bruker BioSpin | Avance | 5mm TXI 1H-13C/15N/D RT probe, z-gradients |
| 700 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 700 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 700 MHz | Bruker BioSpin | Avance III | 5mm TCI CryoProbe, xyz-gradients |
| 700 MHz | Bruker BioSpin | Avance III HD | 5mm H{C/N} Cryo ( 13C enhanced), Z-gradients |
| 700 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 700 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 750 MHz | Bruker BioSpin | Avance III | 5mm TXI 1H-13C/15N/D RT probe, Z-gradients |
| 750 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 800 MHz | Agilent/Varian | VNMRS | 5mm ColdProbe gen 2, 1H-13C/15N/D, Z-gradients |
| 800 MHz | Agilent/Varian | VNMRS | Triple resonance PFG cold probe |
| 800 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 800 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 800 MHz | Bruker BioSpin | Avance III | 5mm TXI 1H-13C/15N/D probe, Z-gradients |
| 800 MHz | Bruker BioSpin | Avance III HD | 5mm TCI CryoProbe |
| 850 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 850 MHz | Bruker BioSpin | Avance III | TCI, Z-gradients |
| 850 MHz | Bruker BioSpin | Avance III | 5mm QCI 1H-13C/15N/31P/D CryoProbe, Z-gradients |
| 900 MHz | Bruker BioSpin | Avance III HD | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |
| 900 MHz | Bruker (Oxford) | Avance III HD | 5mm TCI CryoProbe |
| 900 MHz | Bruker BioSpin | Avance III | 5mm TCI 1H-13C/15N/D CryoProbe, Z-gradients |

**Table S5.** Experimental parameters for the required D1A experiment: 1H,15N gHSQC

|  |  |
| --- | --- |
| **Scans per Increment** | Enough scans per increment collected to achieve an average minimum S/N of 10:1 |
| **Recycling Delay** | *Discretion of individual laboratory; typically 1.0 s – 1.5 s* |
| **J1NH** | 93 Hz |
| **1H Carrier Position** | Water resonance |
| **1H Sweep Width** | 20 ppm |
| **1H Acquisition time** | 100 ms |
| **15N Carrier Position** | 117 ppm |
| **15N Sweep Width** | 40 ppm |
| **15N Acquisition Time** | 20 ms (See **Table S6** for number of total points) |
| **Sampling Schedule** | Uniform |
| **Temperature** | 37 ± 0.1 °C |

**Table S6.** Varied experimental parameters for the required D1A experiment: 1H,15N gHSQC

|  |  |  |
| --- | --- | --- |
| **Field**  **(MHz)** | **15N Acquisition**  **Time** | **15N Total**  **Points** |
| **500** | 20 ms | 82 |
| **600** | 20 ms | 98 |
| **700** | 20 ms | 114 |
| **750** | 20 ms | 122 |
| **800** | 20 ms | 132 |
| **850** | 20 ms | 138 |
| **900** | 20 ms | 146 |

**Table S7.** Experimental parameters for the required 1H,13C gHSQC experiments. The overall goal was to achieve an average S/N of at least 10:1.

|  |  |
| --- | --- |
| **Scans per increment** | **System Suitability Ssample**  **Experiments D2A - D2D**: Same number of scans per increment  **Experiment D2E**: Twice the scans per increment of Experiment D2C  **Unlabeled NIST-Fab**  **Experiment D3A:** Scans per increment set to achieve S/N of at least 10:1  **Experiment D3B:** Same number of scans per increment as Exp. D3A |
| **Recycling Delay** | *Discretion of individual laboratory; typically 1.0 s – 1.5 s* |
| **J1CH** | 145 Hz |
| **1H Carrier Position** | Water resonance |
| **1H Sweep Width** | 14 ppm |
| **1H Acquisition time** | 100 ms |
| **13C Carrier Position** | 20 ppm |
| **13C Sweep Width** | 30 ppm |
| **13C Acquisition Time** | Varied, see **Table S8** |
| **Temperature** | 37 ± 0.1 °C |

**Table S8.** Varied experimental parameters for the required 1H,13C gHSQC experiments

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Experiments D2A, D2B** | | **Exp. D2C, D2D, D2E,**  **D3A, D3B** | |
| **Field**  **(MHz)** | **13C Acquisition**  **Time** | **13C Total**  **Points‡** | **13C Acquisition**  **Time** | **13C Total**  **Points‡** |
| **500** | 17.0 ms | 128 | 25 ms | 188 |
| **600** | 14.1 ms | 128 | 25 ms | 226 |
| **700** | 12.1 ms | 128 | 25 ms | 264 |
| **750** | 11.3 ms | 128 | 25 ms | 282 |
| **800** | 10.6 ms | 128 | 25 ms | 302 |
| **850** | 10.0 ms | 128 | 25 ms | 320 |
| **900** | 9.4 ms | 128 | 25 ms | 338 |

**‡**For NUS experiments, sampling schedule was 50%.

**Table S9**. Recommended parameters for the optional SOFAST-HMQC E1C- and E2C-type experiments. Some laboratories performed these experiments with different shape pulses.

|  |  |  |
| --- | --- | --- |
|  | **1H,15N Experiment** | **1H,13C Experiment** |
| **Acquisition time, *t2*** | 50 ms | 50 ms |
| **Excitation Pulse** | Pc.9.90 | Pc.9.90 |
| **Refocusing Pulse** | Reburp | Reburp |
| **Excitation Window** | 5.55 ppm | 4.44 ppm |
| **Center of Shape Pulses** | 8.25 ppm | 0.0 ppm |

**Table S10.** Spectra excluded from peak analysis due to poor S/N or poor resolution. An average S/N of at least 10:1 was required for each peak list. For the spectra with the known pulse program error (see **Figure S10**), these were not included in the CCSD analysis (center column) but were included in the PCA plots. In addition, only peak lists of the SSS recorded at 37 °C were used to benchmark the peak position precision using CCSD. Establishing arbitrary common peak lists for all 1H,15N spectra recorded at different temperatures were impractical due to difficulty in tracking the shifting of the cross peaks. All 1H,15Nspectra of the NIST-Fab and two NUS spectra of the SSS were excluded due to the limited number collected. See **Table 1** in main text for descriptions of experiment type.

|  |  |  |
| --- | --- | --- |
| Excluded 1H,15N spectra | Excluded 1H,13C spectra from CCSD† only | Excluded 1H,13C Spectra from CCSD and PCA‡ |
| E2-1894-101 | D2B-2146-003 | E1A-1247-002 |
| D1A-2461-101 | D2D-2146-005 | E1A-1247-004 |
| E2B-2461-108\* | D2E-2146-006 | E1A-1247-005 |
| E2-3655-101 | D2B-6211-405 | D2A-1894-102 |
| E2-3655-103\* | D2B-6211-406 | E1A-1894-103 |
| E2-3676-103\* | E1A-6211-408 | D2C-1894-104 |
| E2-3676-107\* | D2B-6211-409 | E1A-1894-105 |
| E2A-4233-117 | D2D-6211-410 | E1A-1894-106 |
| E2A-4233-123 | D2E-6211-411 | D3B-2461-107 |
| E2A-4233-142 | D3B-6211-412 | D2A-3676-102 |
| E2A-4233-148 | D2B-6272-005 | E1-3676-104 |
| E2B-4533-003 | D2D-6272-008 | D3A-3676-105 |
| E2-6211-401 | D2E-6272-009 | E1A-3676-106 |
| E2N-6324-002 | D2D-6272-010 | E1A-4233-180 |
| E2A-6324-011 | D3B-6272-014 | D2B-6211-403 |
| E2A-6324-012 | D2B-6272-017 | E1B-6324-016 |
| E2A-6324-013 | D2D-6272-019 | D2B-7244-015 |
| D1A-7244-014 | D3B-6272-021 | E1A-8543-003 |
| E2A-8179-309 |  | E1B-8822-024 |
| E2B-8179-310 |  | E1B-8822-025 |
| E2C-8179-311 |  | E1C-8822-049 |
| E2A-8822-020 |  |  |
| E2A-8822-023 |  |  |
| E2A-8822-026 |  |  |
| E2A-8822-027 |  |  |
| E2C-8822-030\* |  |  |
| E2A-8822-043 |  |  |
| E2B-8822-044 |  |  |
| E2C-8822-047 |  |  |
| E2A-8822-050 |  |  |
| E2C-8822-053 |  |  |
| E2C-8822-057\* |  |  |
| E2N-8822-071 |  |  |

\*1H,15N spectral maps of NIST-Fab

†CCSD = Combined Chemical Shift Deviation

‡PCA = Principal Component Analysis

**Table S11**. Average 1H,15N chemical shift values for arbitrary peak list from D1A/E2 gHSQC experiments at 37 °C. A 2D 1H, 15N gHSQC with all peaks plotted is given in **Figure S1**. All values are referenced to the methyl resonance of 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), which was determined by spiking DSS into a NIST-Fab sample and recording a gHSQC spectrum at 900 MHz. All other spectra were then aligned to peak #68 in bold, whose position was determined to be the same at all temperatures. See Materials and Methods in main text for more details.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Peak** | **D1A/E2 15N ppm)** | **D1A/E2 1H (ppm)** | **Peak** | **D1A/E2 15N ppm)** | **D1A/E2 1H (ppm)** | **Peak** | **D1A/E2 15N (ppm)** | **D1A/E2 1H (ppm)** |
| 1 | 101.233 | 7.814 | 67 | 120.097 | 7.537 | 133 | 118.489 | 8.763 |
| 2 | 105.149 | 8.923 | **68** | **118.978** | **7.546** | 134 | 119.893 | 8.650 |
| 3 | 107.930 | 9.086 | 69 | 118.764 | 6.084 | 135 | 119.996 | 8.892 |
| 4 | 108.891 | 9.096 | 70 | 121.571 | 7.647 | 136 | 119.815 | 8.978 |
| 5 | 110.773 | 9.135 | 71 | N/A | | 137 | 121.808 | 9.045 |
| 6 | 109.710 | 10.461 | 72 | 120.887 | 7.743 | 138 | 120.906 | 8.502 |
| 7 | 111.847 | 9.736 | 73 | 121.818 | 7.753 | 139 | 120.782 | 8.462 |
| 8 | 111.653 | 9.637 | 74 | 123.598 | 7.793 | 140 | N/A | |
| 9 | 112.045 | 8.957 | 75 | 124.558 | 7.769 | 141 | 123.042 | 8.546 |
| 10 | N/A | | 76 | 125.081 | 7.973 | 142 | 123.878 | 8.776 |
| 11 | 109.991 | 8.852 | 77 | 125.390 | 7.901 | 143 | 125.486 | 8.789 |
| 12 | 106.763 | 8.721 | 78 | 123.297 | 7.928 | 144 | 129.486 | 8.567 |
| 13 | 108.604 | 8.633 | 79 | 125.566 | 7.998 | 145 | 130.643 | 8.444 |
| 14 | 111.552 | 8.516 | 80 | 125.349 | 8.057 | 146 | N/A | |
| 15 | 111.214 | 8.448 | 81 | 125.074 | 8.134 | 147 | N/A | |
| 16 | 111.379 | 8.368 | 82 | 125.956 | 8.182 | 148 | 130.886 | 9.163 |
| 17 | 111.311 | 8.188 | 83 | 127.394 | 8.098 | 149 | N/A | |
| 18 | 110.077 | 8.348 | 84 | 128.247 | 8.214 | 150 | 129.193 | 8.846 |
| 19 | 109.599 | 8.243 | 85 | 126.809 | 8.350 | 151 | 129.392 | 8.914 |
| 20 | 109.692 | 8.137 | 86 | 128.459 | 8.418 | 152 | 129.086 | 9.011 |
| 21 | 109.755 | 7.994 | 87 | 126.646 | 8.459 | 153 | 128.180 | 8.819 |
| 22 | 110.632 | 7.757 | 88 | 124.634 | 8.222 | 154 | 127.284 | 8.850 |
| 23 | 109.054 | 7.906 | 89 | 124.648 | 8.506 | 155 | 126.583 | 8.884 |
| 24 | 107.613 | 7.963 | 90 | 125.233 | 8.330 | 156 | N/A | |
| 25 | 105.592 | 7.859 | 91 | 122.255 | 8.363 | 157 | 123.870 | 9.124 |
| 26 | 106.997 | 7.646 | 92 | 122.208 | 8.445 | 158 | 123.035 | 9.104 |
| 27 | 107.613 | 7.547 | 93 | 121.409 | 8.276 | 159 | 122.280 | 9.285 |
| 28 | 108.777 | 7.684 | 94 | 121.553 | 8.082 | 160 | 123.535 | 9.293 |
| 29 | 109.503 | 7.508 | 95 | 121.169 | 8.118 | 161 | 124.928 | 9.319 |
| 30 | 110.900 | 7.426 | 96 | 121.602 | 7.861 | 162 | 125.144 | 9.224 |
| 31 | N/A | | 97 | 119.680 | 7.897 | 163 | 126.397 | 9.328 |
| 32 | 109.812 | 7.232 | 98 | 120.425 | 8.216 | 164 | 127.362 | 9.236 |
| 33 | 109.607 | 7.135 | 99 | 119.641 | 8.539 | 165 | 127.463 | 9.352 |
| 34 | 106.215 | 7.154 | 100 | 118.690 | 8.525 | 166 | 130.142 | 9.643 |
| 35 | 108.478 | 6.978 | 101 | 118.071 | 8.317 | 167 | 131.210 | 9.606 |
| 36 | 106.127 | 6.500 | 102 | 118.158 | 8.009 | 168 | 131.160 | 9.909 |
| 37 | 104.963 | 6.158 | 103 | 117.883 | 7.799 | 169 | 130.192 | 10.281 |
| 38 | 110.169 | 6.406 | 104 | 117.681 | 7.683 | 170 | 131.784 | 10.752 |
| 39 | 109.838 | 6.621 | 105 | 116.673 | 7.594 | 171 | 128.884 | 10.126 |
| 40 | 110.596 | 6.658 | 106 | 116.839 | 7.465 | 172 | 127.794 | 10.166 |
| 41 | 110.904 | 6.766 | 107 | 115.903 | 7.758 | 173 | 125.565 | 10.465 |
| 42 | 112.066 | 6.940 | 108 | 114.746 | 7.596 | 174 | 127.704 | 9.779 |
| 43 | 112.758 | 6.910 | 109 | 114.373 | 7.420 | 175 | 126.810 | 9.888 |
| 44 | 112.980 | 6.840 | 110 | 114.016 | 7.368 | 176 | 125.661 | 9.889 |
| 45 | 112.349 | 6.837 | 111 | 112.836 | 7.382 | 177 | 125.231 | 9.760 |
| 46 | 112.966 | 7.299 | 112 | 112.983 | 7.855 | 178 | 124.912 | 9.693 |
| 47 | 112.395 | 6.744 | 113 | 112.381 | 7.526 | 179 | 125.393 | 9.522 |
| 48 | 112.901 | 6.779 | 114 | 112.056 | 7.717 | 180 | 127.169 | 9.470 |
| 49 | 111.988 | 6.581 | 115 | 113.189 | 8.237 | 181 | 122.517 | 9.496 |
| 50 | 113.316 | 6.550 | 116 | 112.962 | 8.404 | 182 | 122.334 | 9.402 |
| 51 | 114.783 | 6.776 | 117 | 114.583 | 8.326 | 183 | 121.662 | 9.375 |
| 52 | N/A | | 118 | 114.736 | 8.191 | 184 | 120.014 | 9.397 |
| 53 | 114.431 | 6.918 | 119 | 115.255 | 8.278 | 185 | 120.736 | 9.455 |
| 54 | 114.815 | 7.022 | 120 | 115.374 | 8.366 | 186 | N/A | |
| 55 | 114.434 | 7.240 | 121 | 116.920 | 8.413 | 187 | 121.632 | 9.622 |
| 56 | 116.129 | 6.982 | 122 | 116.813 | 8.489 | 188 | 121.638 | 9.710 |
| 57 | 117.004 | 7.2578 | 123 | 116.544 | 8.591 | 189 | 119.758 | 9.623 |
| 58 | 118.952 | 7.317 | 124 | 115.773 | 8.531 | 190 | 119.452 | 9.768 |
| 59 | 119.293 | 7.194 | 125 | 114.412 | 8.651 | 191 | 118.144 | 10.104 |
| 60 | 119.134 | 6.950 | 126 | 113.978 | 8.715 | 192 | 116.842 | 10.043 |
| 61 | 121.014 | 7.093 | 127 | 114.124 | 8.824 | 193 | 115.706 | 9.509 |
| 62 | 121.751 | 7.208 | 128 | 115.669 | 9.132 | 194 | 116.759 | 9.256 |
| 63 | 122.158 | 7.424 | 129 | 116.226 | 9.077 | 195 | N/A | |
| 64 | 120.702 | 7.319 | 130 | 115.469 | 8.953 | 196 | 116.051 | 8.196 |
| 65 | 120.864 | 7.405 | 131 | 116.395 | 8.951 | 197 | 116.980 | 8.168 |
| 66 | 120.481 | 7.475 | 132 | 117.113 | 9.004 | 198 | 117.085 | 8.068 |

N/A = not applicable. The peak was removed from the reference peak list due to its absence or lack of resolution in a subset of spectra.

**Table S12**. Average 1H,13C chemical shift values for arbitrary peak list from D2A/D2C gHSQC experiments at 37 °C. A 2D 1H,13C gHSQC with all peaks plotted is given in **Figures S2** and **S3**. All values are referenced to the methyl resonance of DSS, which was determined by spiking DSS into a NIST-Fab sample and recording a gHSQC spectrum at 900 MHz. All other spectra were then aligned to peak #29 in bold, whose position was determined to be the same at all temperatures. See Materials and Methods in main text for more details.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Peak** | **D2A/D2C 13C (ppm)** | **D2A/D2C 1H (ppm)** | **Peak** | **D2A/D2C 13C (ppm)** | **D2A/D2C 1H (ppm)** | **Peak** | **D2A/D2C 13C (ppm)** | **D2A/D2C 1H (ppm)** |
| 1 | 11.559 | 0.600 | 38 | 18.814 | 0.443 | 75 | N/A | |
| 2 | 12.221 | 0.423 | 39 | 19.831 | 0.382 | 76 | 23.556 | 0.637 |
| 3 | 12.561 | 0.294 | 40 | 20.857 | 0.346 | 77 | 22.320 | 0.553 |
| 4 | 12.841 | 0.489 | 41 | 21.782 | 0.133 | 78 | 22.221 | 0.427 |
| 5 | 13.308 | 0.736 | 42 | 23.530 | 0.289 | 79 | 21.600 | 0.351 |
| 6 | 13.665 | 0.626 | 43 | 23.955 | 0.150 | 80 | 21.470 | 0.283 |
| 7 | 13.634 | 0.446 | 44 | 24.839 | -0.011 | 81 | 20.378 | 0.544 |
| 8 | 15.989 | 0.911 | 45 | 25.805 | -0.563 | 82 | 20.462 | 0.595 |
| 9 | 16.320 | 1.740 | 46 | N/A | | 83 | N/A | |
| 10 | 16.976 | 1.788 | 47 | N/A | | 84 | 20.581 | 1.266 |
| 11 | 17.164 | 1.748 | 48 | 25.994 | 0.194 | 85 | 20.832 | 1.399 |
| 12 | 18.207 | 2.069 | 49 | 26.848 | -0.062 | 86 | 20.971 | 1.483 |
| 13 | 18.128 | 1.505 | 50 | 26.852 | 0.278 | 87 | 21.767 | 1.559 |
| 14 | 16.752 | 1.348 | 51 | 27.242 | 0.477 | 88 | 22.039 | 1.416 |
| 15 | 18.165 | 1.357 | 52 | 27.507 | 0.638 | 89 | 23.007 | 1.372 |
| 16 | 17.691 | 1.281 | 53 | 27.361 | 0.763 | 90 | 23.625 | 1.449 |
| 17 | 18.257 | 1.165 | 54 | 28.432 | 0.901 | 91 | 23.697 | 1.377 |
| 18 | 20.629 | 1.757 | 55 | 27.571 | 0.898 | 92 | N/A | |
| 19 | N/A | | 56 | N/A | | 93 | 23.713 | 1.318 |
| 20 | N/A | | 57 | 27.230 | 0.981 | 94 | 22.802 | 1.171 |
| 21 | 19.500 | 1.321 | 58 | 26.226 | 1.045 | 95 | 22.601 | 1.227 |
| 22 | 19.013 | 1.295 | 59 | 26.007 | 0.925 | 96 | 22.609 | 1.295 |
| 23 | N/A | | 60 | 26.294 | 0.845 | 97 | 22.305 | 1.320 |
| 24 | 19.522 | 1.066 | 61 | 26.496 | 0.655 | 98 | 21.208 | 1.326 |
| 25 | 16.988 | 0.927 | 62 | 26.046 | 0.557 | 99 | 20.603 | 1.005 |
| 26 | N/A | | 63 | 25.254 | 0.632 | 100 | N/A | |
| 27 | 18.188 | 0.692 | 64 | 25.515 | 0.695 | 101 | N/A | |
| 28 | 17.358 | 0.646 | 65 | N/A | | 102 | 21.186 | 0.452 |
| **29** | **17.494** | **0.513** | 66 | 24.558 | 0.799 | 103 | 21.090 | 0.520 |
| 30 | 17.364 | 0.387 | 67 | 24.295 | 0.850 | 104 | N/A | |
| 31 | 17.348 | 0.222 | 68 | 24.915 | 0.856 | 105 | 21.819 | 0.735 |
| 32 | 17.048 | 0.100 | 69 | 24.687 | 0.939 | 106 | 21.138 | 0.766 |
| 33 | 14.821 | -0.624 | 70 | 24.441 | 0.897 | 107 | 21.854 | 0.796 |
| 34 | 18.875 | -0.355 | 71 | 25.336 | 1.021 | 108 | 22.230 | 1.020 |
| 35 | 20.238 | -0.196 | 72 | 24.167 | 1.126 | 109 | 22.697 | 1.044 |
| 36 | N/A | | 73 | 23.760 | 1.003 | 110 | 21.598 | 0.921 |
| 37 | 20.766 | -0.084 | 74 | 23.344 | 0.925 |  |  |  |

N/A = not applicable. The peak was removed from the reference peak list due to its absence or lack of resolution in a subset of spectra.