Supporting Information

Measuring Solvent Hydrogen Exchange Rates by Multifrequency Excitation ^{15}N CEST: Application to Protein Phase Separation

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Additional Discussion

Calibration of the Weak B_1 Fields for ^{15}N D-CEST Experiments. As described in the text, the calibration of the weak B_1 field in D-CEST experiments is based on the method of Guenneugues et al.[1] In this approach a series of FIDs with different T_{Ex} values is recorded and the size of the B_1 field obtained from the separation of the resulting peak doublets in the Fourier transformed 2D spectrum. The calibration of very weak B_1 fields ($<$ ~5 Hz) via this approach can be error prone, as the doublet components overlap. We have therefore used the approach illustrated in Figure 1B where a single peak is obtained, displaced from the center of the spectrum by the magnitude of the weak B_1 field. For very weak B_1 fields it is also important to ensure that small residual J-couplings are fully suppressed, as these otherwise introduce systematic errors to the calibration results. For example, consider the calibration of a B_1 = 3.0 Hz field and a pulse sequence in which ^{15}N–^{1}H scalar couplings are suppressed with a 4 kHz $90_x240_y90_x$ ^{1}H decoupling scheme. Simulations show that a calibration value of 3.23 Hz is obtained when the ^{15}N spin is coupled to a proton with a 5 Hz scalar coupling that, in turn, is offset by -4000 Hz from the center of the ^{1}H carrier (as an H$^{\alpha}$ proton might be). A similarly large calibrated B_1 value is obtained due to incomplete decoupling of the one-bond ^{15}N–^{1}H scalar interaction when the amide proton is 1000 Hz offset from the ^{1}H decoupling field. When any of the broadband ^{1}H decoupling schemes (*i.e.*, DIPSI-2, DIPSI-3, WALTZ-16) are used, instead of the $90_x240_y90_x$ element, the correct input B_1 value is obtained from the simulations. Further, these schemes restore full intensities to the CEST dips, as illustrated in Figure S1.

Choice of ^{1}H and ^{2}H Decoupling Schemes in ^{15}N D-CEST with Weak ^{15}N B_1. In ^{15}N CEST experiments where the weak ^{15}N B_1 field is greater than ~10 Hz it is recommended that a $90_x240_y90_x$ ^{1}H decoupling scheme be used during the CEST element since decoupling sidebands are weak and generally lie outside the region of interest (*i.e.*, where ^{15}N spins resonate).[2] In contrast, we have shown previously that broadband decoupling

schemes typically generate multiple decoupling sidebands that are intense, complicating the analysis of CEST profiles.[2] However, since the size of the decoupling sidebands decreases as the B_1 field becomes smaller, we reasoned that for very weak ^{15}N B_1 fields (< 10 Hz) it might nevertheless be possible to achieve good results with broadband ^{1}H decoupling schemes. Simulations indicate that schemes such as WALTZ or DIPSI, Figure S2, perform well so long as ^{15}N B_1 fields are under 10 Hz. In experimental tests with a 3 mM deuterated protein L sample the sizes of the decoupling sidebands never exceeded 2% for DIPSI-3 (or ~5% for WALTZ-16) using a weak ^{15}N B_1 field of 10 Hz and T_{Ex} = 1 s, in agreement with numerical simulations.

As illustrated in Figure S1 the DIPSI-3 scheme is particularly effective in removing $^2J_{HN}$ and $^3J_{HN}$ couplings that are a concern in the case of weak B_1 fields. We have compared experimental CEST profiles recorded on a sample of a fully protonated A39G FF domain using a weak B_1 field of 5 Hz and ^{1}H decoupling achieved with either $90_x240_y90_x$ or DIPSI-3 decoupling schemes. For most residues the D-CEST profiles obtained with the two ^{1}H decoupling schemes are nearly identical, with the $90_x240_y90_x$ element generating slightly broadened dips, as expected from simulations. Therefore, we recommend that for $B_1 \leq 10$ Hz the DIPSI-3 scheme be used, with the more common $90_x240_y90_x$ train used for larger B_1 fields.

The decoupling sidebands of the sort described in the context of ^{1}H decoupling during the CEST element are not an issue for ^{2}H decoupling for a number of reasons. First, amide nitrogens of the major state are coupled to protons and the resulting ^{15}N–^{1}H spin-pairs are not affected by ^{2}H pulses. In contrast, spin-pairs reporting on the minor state are of the ^{15}N–^{2}H variety and while ^{2}H decoupling modulation sidebands can in principle result, leading to spurious dips in CEST profiles, simulations establish that for weak B_1 fields on the order of 10 Hz and $p_E \sim 10\%$ these are not expected and indeed they are not observed experimentally. Even in cases where $p_E = 50\%$ and for a weak B_1 of 10 Hz, simulations that take into account ^{2}H relaxation establish that the spurious dips are negligible. Nu-

merical simulations also indicate that ^{2}H decoupling sidebands generated with DIPSI-3 are typically smaller than for WALTZ-16; therefore DIPSI-3 is preferred. Note that both decoupling waveforms have similar bandwidth coverage, while ^{2}H decoupling sidebands are negligible under most situations with either scheme.

Finally, we prefer to carry out measurements on samples that are not ^{13}C-labeled as the ^{13}C decoupling schemes used to suppress ^{13}C–^{15}N J-couplings (both ^{13}CO–^{15}N and $^{13}C^{\alpha}$–^{15}N) result in sidebands as well.

Measurement of Solvent Exchange via CLEANEX-PM. Values of k_{ex} were obtained from CLEANEX-based experiments according to[3]

$$\frac{I}{I_0} = \frac{f k_{ex}}{R_{H_N} + k_{ex} - R_w}\left[e^{-R_w \tau_{mix}} - e^{-(R_{H_N}+k_{ex})\tau_{mix}}\right] \quad \text{(S1)}$$

where I is the intensity of an amide correlation as a function of mixing time τ_{mix}, R_w is the water relaxation rate during the CLEANEX-PM element and I_0 is the intensity of the corresponding peak in a fully relaxed spectrum. The water preservation factor, f, defined as the fraction of the equilibrium water magnetization at the start of the mixing period was calculated by measuring the water intensity immediately prior to mixing using a small flip angle pulse ($\theta < 10^{\circ}$) and comparing this signal with that obtained when equilibrium water magnetization is excited with the corresponding small flip angle pulse. The high Q-value of a cryoprobe prevents accurate measurements to be made using larger θ values, although this is not an issue on room-temperature probes.

We have found it necessary to accurately measure R_w values, in particular for applications involving highly concentrated phase-separated Ddx4. Although measured water R_1 rates of 0.35 s^{-1} (Ddx4$_{RtoK}$) and 0.57 s^{-1} (Ddx4$_{cond}$) are small, R_w values of 1.03 s^{-1} and 4.31 s^{-1}, respectively, are obtained. This significant increase reflects a contribution from exchange with amide protons of Ddx4, especially for the Ddx4$_{cond}$ sample where the protein concentration is ~400 mg/mL. In this regard both Ddx4 samples, but particularly Ddx4$_{cond}$, have significant water ^{1}H $R_{1\rho}$ dispersion.

Supplementary Figures

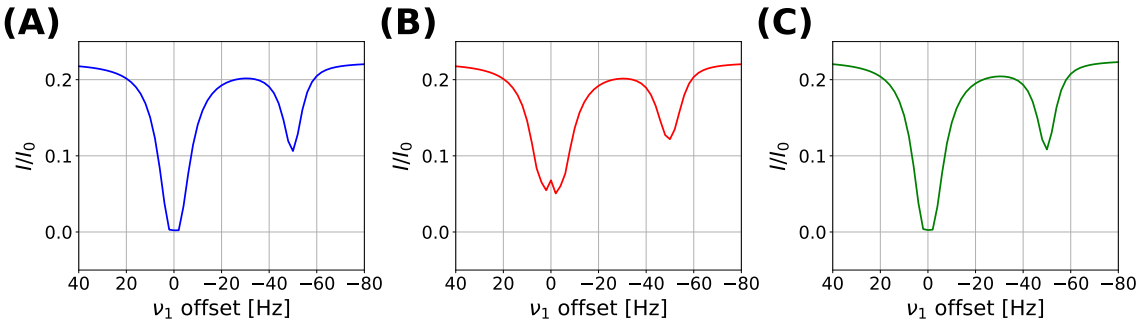

Figure S1. Comparison of the performance of $90_x240_y90_x$ [4] and DIPSI-3 [5] ^{1}H decoupling schemes during T_{Ex}. Simulations of ^{15}N CEST profiles with B_1 = 3 Hz, $R_{1,G} = R_{1,E} = 1.5$ s^{-1}, $R_{2,G} = R_{2,E} =$ 5 s^{-1}, T_{Ex} = 1 s, (p_E, k_{ex}) = (10%, 10 s^{-1}), $\Delta\nu_{GE} = -50$ Hz. The profile in (A) has been simulated with all J values set to 0, while profiles in (B, C) are generated by including a pair of ^{1}H spins: an amide proton at a 500 Hz offset with respect to the ^{1}H carrier ($^1J_{HN} = -93$ Hz) and a second proton -4000 Hz offset from the carrier with a coupling to the ^{15}N spin of 5 Hz. Both ^{1}H spins are, in turn, coupled (5 Hz coupling). Profiles in Panels (B) and (C) are simulated with 4 kHz $90_x240_y90_x$ and DIPSI-3 ^{1}H decoupling schemes, respectively.

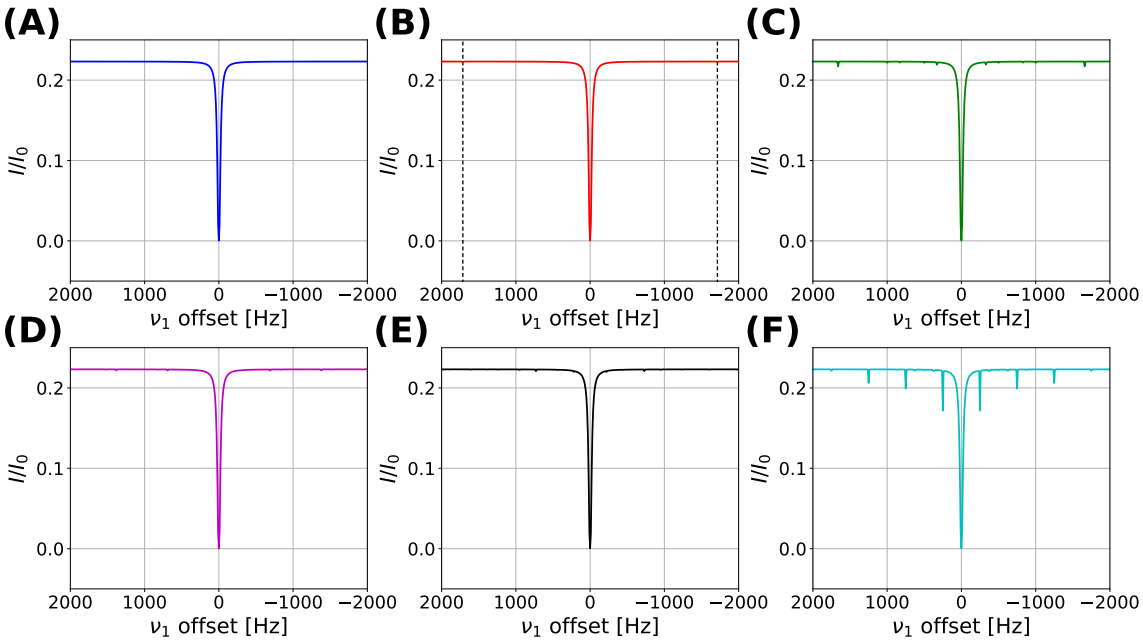

Figure S2. Comparison of decoupling sideband intensities from a number of different decoupling waveforms. Simulation of ^{15}N CEST profiles (no exchange) with B_1 = 10 Hz, R_1 = 1.5 s^{-1}, R_2 = 5 s^{-1} and T_{Ex} = 1 s. The profile in Panel (A) is simulated without J-couplings, while those in Panels (B–F) are simulated with $^1J_{HN}$ = −93 Hz and 4 kHz ^{1}H decoupling applied on-resonance using (B) $90_x240_y90_x$,[4] (C) WALTZ-16,[6] (D) DIPSI-2,[5] (E) DIPSI-3[5] and (F) GARP-1.[7] In Panel (B) the dashed lines indicate the positions of the first decoupling sidebands generated by the $90_x240_y90_x$ decoupling element, located at $3\nu_1/7$ from the main peak, where ν_1 is the strength of the ^{1}H decoupling field in Hz.[2] In all of the simulations B_1 inhomogeneity has been included, as described previously.[2]

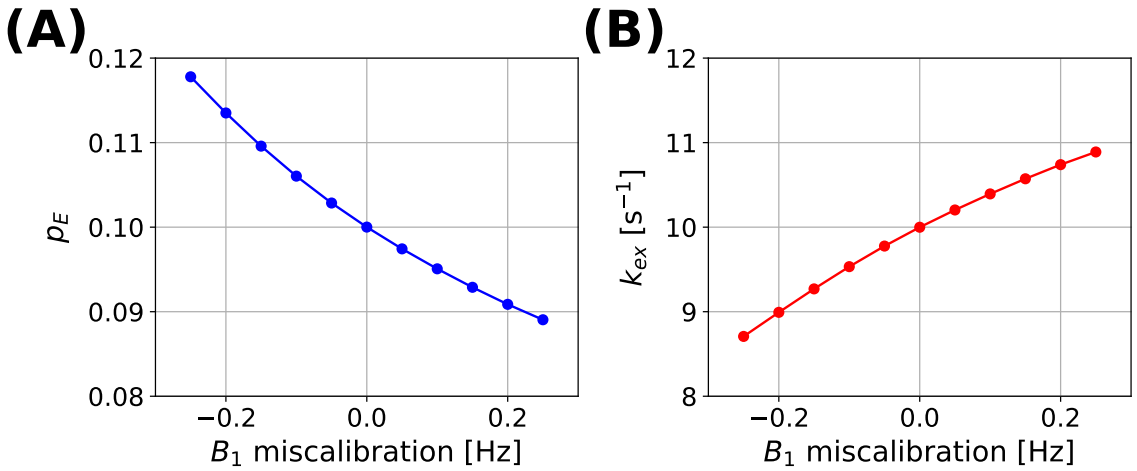

Figure S3. The effect of B_1 miscalibration on extracted p_E (A) and k_{ex} (B) parameters obtained from fits of simulated D-CEST profiles. Simulations were performed with $R_{1,G} = R_{1,E} = 1.5$ s^{-1}, $R_{2,G} = R_{2,E} = 5$ s^{-1}, T_{Ex} = 1 s and (p_E, k_{ex}) = (10%, 10 s^{-1}) to generate a pair of profiles with B_1 = 3 and 5 Hz. For simplicity, it is assumed that the B_1 miscalibration error is the same for both weak fields. The resultant profiles were fit in an identical fashion as the experimental data and the errors in p_E and k_{ex} tabulated. It is noteworthy that even in the presence of calibration errors the product $p_E k_{ex}$ remains reasonably constant.

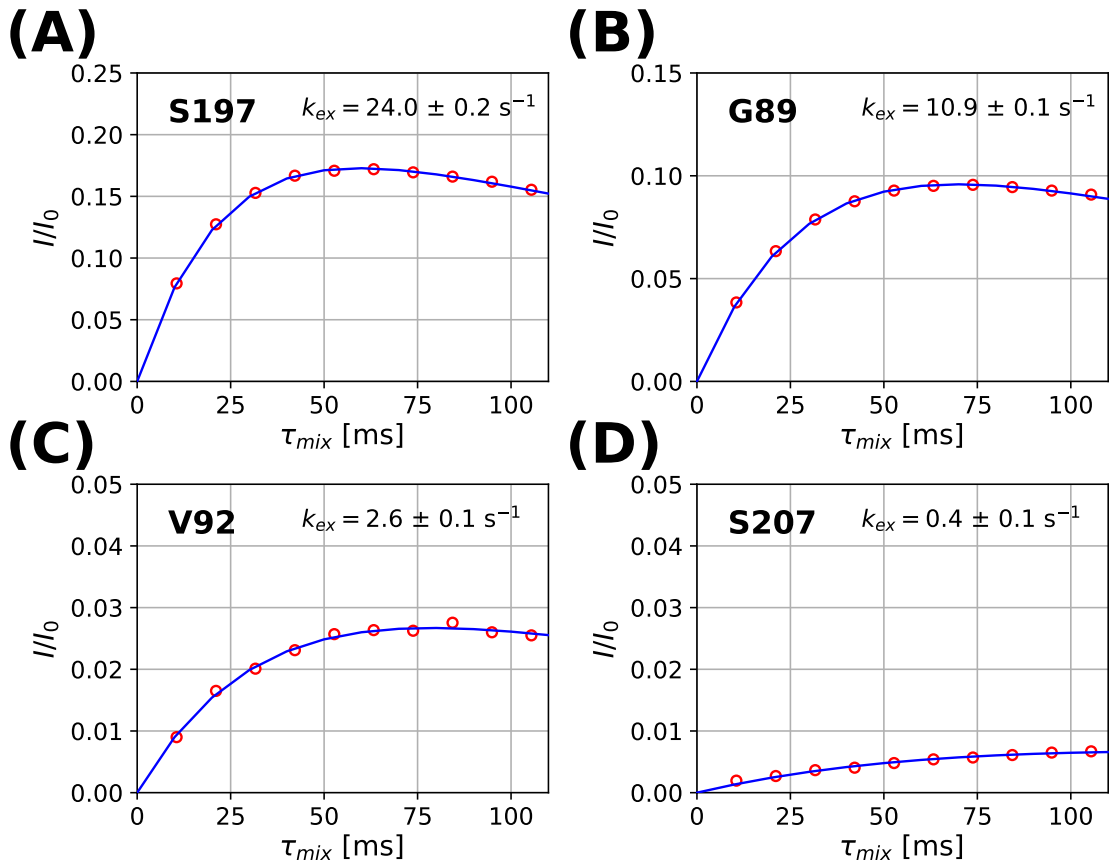

Figure S4. Buildup of amide proton magnetization in CLEANEX-PM experiments as a function of mixing time, τ_{mix} for four residues in Ddx4$_{cond}$ (the corresponding CEST profiles are in Figure 4). The amide ^{1}H relaxation rates during the CLEANEX-PM element for S197, G89, V92 and S207 are 19.0, 22.9, 25.6 and 11.8 s^{-1} respectively (30 °C, 800 MHz).

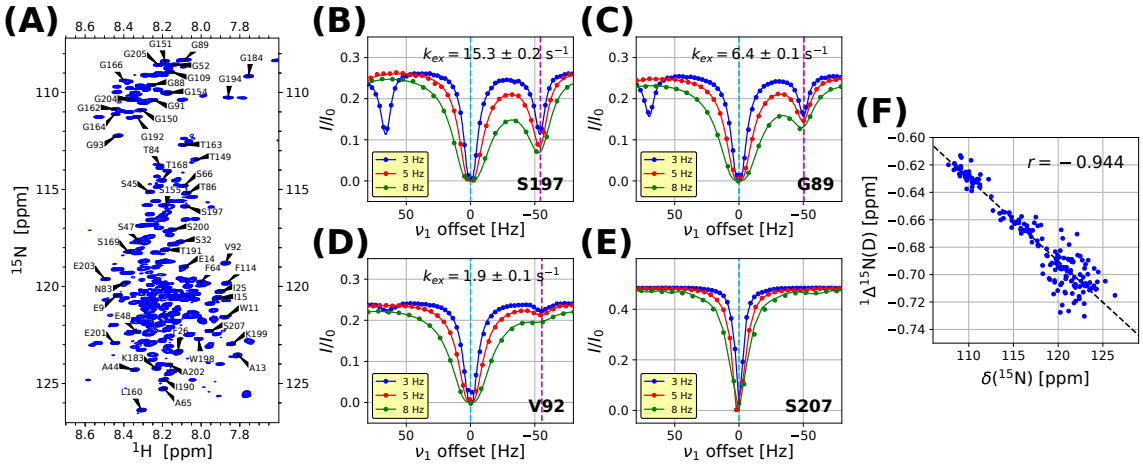

Figure S5. As Figure 4 except that the presented data is based on measurements on a sample of $\text{Ddx4}_{\text{RtoK}}$, 30 °C, 800 MHz. See Figure 4 legend for details. The slope of the best-fitting line in (F) is −0.0060 based on 178 residues.

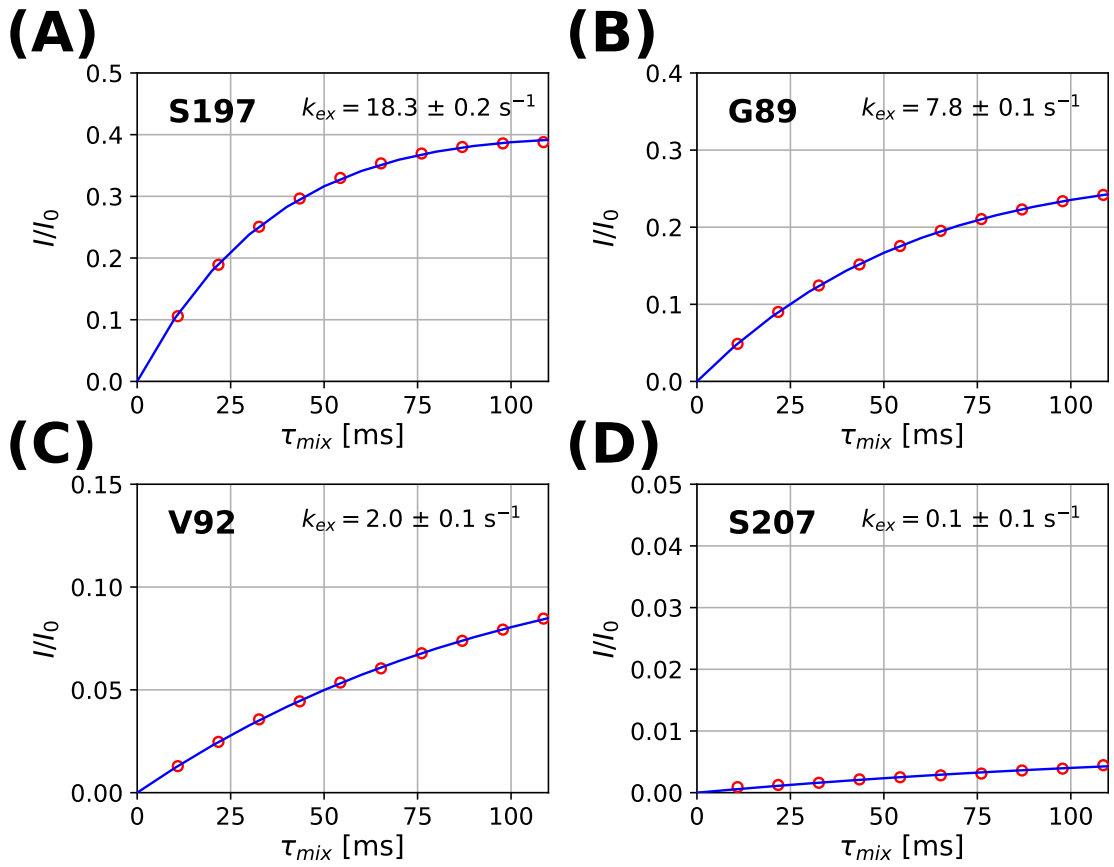

Figure S6. CLEANEX-PM buildup profiles, as in Figure S4, for selected residues from $Ddx4_{RtoK}$ (30 °C, 800 MHz).

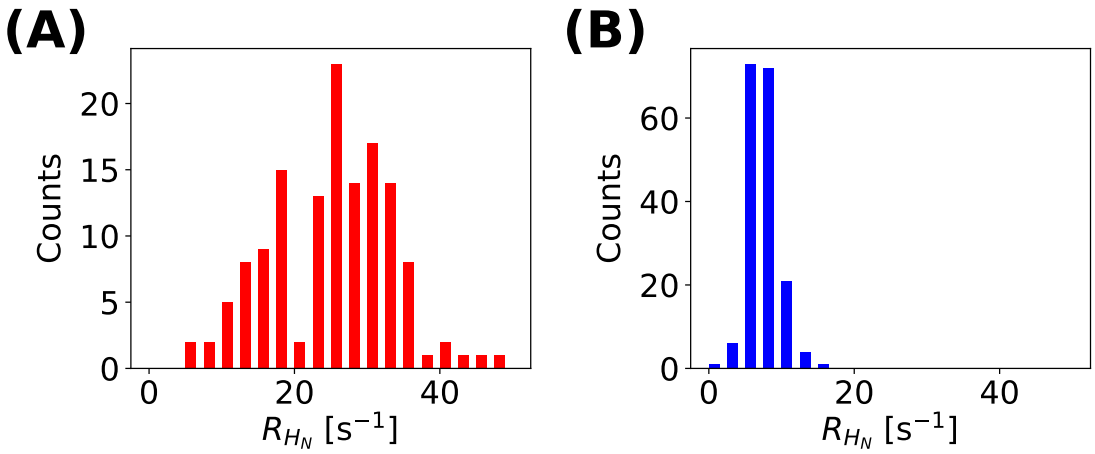

Figure S7. Histograms showing the distribution of R_{H_N} rates obtained from fitting CLEANEX-PM datasets recorded on $Ddx4_{cond}$ (A) and $Ddx4_{RtoK}$ (B) samples.

References

(1) Guenneugues, M.; Berthault, P.; Desvaux, H. A Method for Determining B_1 Field Inhomogeneity. Are the Biases Assumed in Heteronuclear Relaxation Experiments Usually Underestimated? *J. Magn. Reson.* **1999**, *136*, 118–126.

(2) Vallurupalli, P.; Bouvignies, G.; Kay, L. E. Studying "Invisible" Excited Protein States in Slow Exchange with a Major State Conformation. *J. Am. Chem. Soc.* **2012**, *134*, 8148–8161.

(3) Hwang, T. L.; van Zijl, P. C. M.; Mori, S. Accurate Quantitation of Water–Amide Proton Exchange Rates Using the Phase-Modulated CLEAN Chemical EXchange (CLEANEX-PM) Approach with a Fast-HSQC (FHSQC) Detection Scheme. *J. Biomol. NMR* **1998**, *11*, 221–226.

(4) Levitt, M. H. Symmetrical Composite Pulse Sequences for NMR Population Inversion. II. Compensation of Resonance Offset. *J. Magn. Reson.* **1982**, *50*, 95–110.

(5) Shaka, A. J.; Lee, C. J.; Pines, A. Iterative Schemes for Bilinear Operators; Application to Spin Decoupling. *J. Magn. Reson.* **1988**, *77*, 274–293.

(6) Shaka, A. J.; Keeler, J.; Frenkiel, T.; Freeman, R. An Improved Sequence for Broadband Decoupling: WALTZ-16. *J. Magn. Reson.* **1983**, *52*, 335–338.

(7) Shaka, A. J.; Barker, P. B.; Freeman, R. Computer-Optimized Decoupling Scheme for Wideband Applications and Low-Level Operation. *J. Magn. Reson.* **1985**, *64*, 547–552.

```
/* N15DCEST_gd_enh_lek_800_cp

   Pulse scheme to record 15N D-CEST scheme
     Set time_T1 to typically 300-1000 ms and vary 15N saturation frequency

   Use gen_fq3list.py to generate the 15N frequency list (fq3list)
   Use gen_vclist.py to generate the vclist for B1 calibration
   Use gen_dante.py to generated cos-modulated pulses

   To calibrate 15N B1eff field: set -Dcal_NB1
   1. Use -DF2P and set FnMODE(F2) to 'States' for B1 calibration experiment
      that keeps only one quadrature component rather than a pair of doublets.
      Such scheme is useful for calibrating small B1eff (< 10 Hz), TD(F2)
      should be set as twice the number of points in 'vclist'
   2. Use -Dcp_flg to carry out selective het-cp (two-way) and excite only
      one single peak, cnst1 and cnst3 should be set to the position of 1H
      and 15N for the peak of interest respectively. The het-cp power cnst6
      should be set as 35~40 Hz to achieve good selectivity

   For -Dcp_flg (without -Dcal_NB1), het-CP total transfer time: 28.778*pw_dip*l3,
     typically set ~4 kHz power for het-cp (pw_dip ~60 us), and l3 set to ~6
     in order to achieve tau_cp ~10.8 ms (= 1/JNH)

   Use -DHdec_adj to automatically adjust 1H decoupling power, such that
     decoupling sidebands fold onto the position of major dip. cnst5 should be
     set properly reflecting the total degrees of rotation made by one unit

   Modified by TY on Oct 22, 2017 based on N15CEST_gd_enh_800_lek_v2_cp

   Modified by TY on Oct 27, 2017 to add the option of performing CaWurst
   decoupling on 13C during CEST period

*/

;prosol relations=<triple>
;aqseq 321

#include <Avance.incl>
#include <Grad.incl>
#include <Delay.incl>

/***********************/
/*   Define phases     */
/***********************/
#define zero ph=0.0
#define one ph=90.0
#define two ph=180.0
#define three ph=270.0

/***********************/
/*   Define delays     */
/***********************/
define delay hscuba      /* length of 1/2 scuba delay */
  "hscuba=30m"

#ifndef cal_NB1
  define delay time_T1
    "time_T1=d2"
  define delay time_T1_max
    "time_T1_max=d3"

  define delay time_T1_adj
  define delay time_T1_max_adj
#endif

define delay taua        /* 1 / 4J(XH)                */
  "taua=d4"              /* use JNH=105 to decreas the tauxh duration */
define delay taub        /* 1 / 4J(XH)                */
  "taub=d5"              /* use 1/4J(XH=95Hz)         */
```

```
define delay BigT1
  "BigT1=d14"

"d11=30m"
"in0=inf1/2"              /* t1/2 increment */
"TAU2=0.2u"

#ifdef ip_flg
  define delay t1_max

  define loopcounter ni
    "ni=td1/2"
#endif

/*****************************************************************/
/* f1180: Start t1 at half dwell to get -90/180 phase correction */
/*        in F1 (15N) dim - set zgoptns -Df1180                  */
/*****************************************************************/
#if defined(f1180) && !defined(cal_NB1)
  "d0=(in0/2)"
#else
  "d0=0.2u"
#endif

/***********************/
/*   Define pulses     */
/***********************/
define pulse dly_pg1      /* Messerle purge pulse*/
  "dly_pg1=5m"
define pulse dly_pg2      /* Messerle purge pulse*/
  "dly_pg2=dly_pg1/1.62"
define pulse pwh
  "pwh=p1"                /* 1H hard pulse at power level pl1 (tpwr) */
define pulse pwn
  "pwn=p3"                /* PW90 for N pulse at power level pl3 (dhpwr2)  */

#ifdef Ddec
  define pulse pwd
    "pwd=p4"              /* 2H pulse at power pl4 */
#endif

#ifdef cp_flg
  define pulse pw_dip
#ifdef cal_NB1
    "pw_dip=taub*4.0"
#else
    "pw_dip=p16"
#endif
#else
#ifdef N_sel
  define pulse pwn_sl
    "pwn_sl=p32"
#endif
#endif

#ifdef c_flg
  define pulse pwc_ad
    "pwc_ad=p22"          /* pwc_ad at sp22 (d_ad)   */
#endif

#ifndef cos_flg
  define pulse pwn_dante
    "pwn_dante=p34"
#endif

/***********************/
/*    Define lists     */
/***********************/
#ifdef cal_NB1
```

```
  define list<loopcounter> ncyc_cal=<$VCLIST>
#else
  define list<frequency> N_offset=<$FQ3LIST>
#endif

/*****************************************************/
/* Assign cnsts to check validity of parameter range */
/*****************************************************/
#ifdef fsat
  "cnst10=plw10"          /* tsatpwr - set max at 0.00005W */
#endif

#ifdef cp_flg
#ifdef cal_NB1
  "plw16=plw12*pow((p12*4.0*cnst6)/1s,2)"
  "plw35=plw3*pow((pwn*4.0*cnst6)/1s,2)"
#else
  "plw16=plw12*pow(p12/pw_dip,2)"
  "plw35=plw3*pow(pwn/pw_dip,2)"

  define delay tau_cp
    "tau_cp=(2590.0/90.0)*pw_dip*l3"
#endif

  "cnst16=plw16"
  "cnst35=plw35"
#else
#ifdef N_sel
  "cnst32=spw32"
#endif
#endif

#ifdef c_flg
  "cnst22=spw22"          /* d_ad - set max at 80W" */
#endif

#ifdef cawurst_flg
  "spw23=plw23"
  "cnst23=plw23"          /* dpwrcadec - set max at 6.0W */
#endif

"cnst31=plw31"            /* dpwr2 - set max at 6W */
"cnst34=plw34"            /* 15N power level for DANTE pulses */

#ifdef Ddec
  "cnst4=plw4"            /* dpwr3 pl4 - set max at 10.5W */
  "cnst41=plw41"          /* dpwr3D pl41 - set max at 1.5W */
#endif

/***********************************/
/* Calculate parameters for D-CEST */
/***********************************/
#ifdef cos_flg
  define pulse p_dante
    "p_dante = 1s/cnst8 - 0.1u"

  "spw5=pow((4.0*pwn*l7*cnst9)/1s,2)*plw3"
#else
  define pulse p_dante
    "p_dante = (4.0*pwn_dante*cnst9)/cnst8"

  define delay d_dante
    "d_dante = 1s/cnst8 - p_dante"
#endif

#ifndef cal_NB1
  "l4 = (trunc((time_T1/1s)*cnst8+0.1))"
  "time_T1_adj = 1s*(l4/cnst8)"
  "time_T1_max_adj = larger(time_T1_adj, time_T1_max)"
```

```
#endif

/*************************************************/
/* Make adjustment for 1H decoupling power level */
/*************************************************/
#ifdef Hdec_adj
  "l5 = trunc((1s*90.0)/(2.0*p12*cnst5*cnst8) + 0.5)"
  "p15 = (1s*90.0)/(2.0*l5*cnst5*cnst8)"
  "plw15 = pow(p12/p15,2)*plw12"
#else
  "p15 = p12"
  "plw15 = plw12"
#endif

"cnst12=plw15"           /* tpwrml - set max at 5W" */

/************************/
/* Initialize variables */
/************************/
"l2=0"
"l3=0"
"plw2=0"
"spoal5=0.5"
"spoff5=0"
"spoal22=0.5"
"spoff22=0"

;"acqt0 = 0"
;baseopt_echo

/*******************************/
/* BEGIN ACTUAL PULSE SEQUENCE */
/*******************************/
1 ze
/*****************************************/
/*   Check Validity of Parameter Range   */
/*****************************************/
#ifdef fsat
  if "cnst10 > 0.00005" {
    2u
    print "error: tsatpwr pl10 is too high; < 0.00005W !!!"
    goto HaltAcqu
  }
#endif

  if "cnst12 > 5" {
    2u
    print "error: tpwrml pl12 is too high; < 5W !!!"
    goto HaltAcqu
  }

#ifdef cp_flg
  if "cnst16 > 4" {
    2u
    print "error: pl16 is too high; < 4W !!!"
    goto HaltAcqu
  }

  if "cnst35 > 60" {
    2u
    print "error: pl35 is too high; < 60W !!!"
    goto HaltAcqu
  }

#ifndef cal_NB1
  if "tau_cp > 14m" {
    2u
    print "error: dipsi-2 het-cp duration is too long; < 14ms !!!"
    goto HaltAcqu
```

```
  }

  if "tau_cp < 6m" {
    2u
    print "error: dipsi-2 het-cp duration is too short; > 6ms !!!"
    goto HaltAcqu
  }
#endif
#else
#ifdef N_sel
  if "cnst32 > 100" {
    2u
    print "error: dpwr2_sl spw32 is too high; < 100W !!!"
    goto HaltAcqu
  }
#endif
#endif

#ifdef c_flg
  if "cnst22 > 80" {
    2u
    print "error: d_ad pl22 is too high; < 80W !!!"
    goto HaltAcqu
  }
#endif

#ifdef cawurst_flg
  if "cnst23 > 8" {
    2u
    print "error: dpwrcadec pl23 is too high; < 8W !!!"
    goto HaltAcqu
  }
#endif

  if "cnst9/cnst8 > 0.25" {
    2u
    print "error: N15_B1/N15_SW ratio is too large; < 0.25 !!!"
    goto HaltAcqu
  }

  if "cnst31 > 9" {
    2u
    print "error: dpwr2 pl31 is too high; < 9W !!!"
    goto HaltAcqu
  }

#ifndef cal_NB1
  if "time_T1 > 1000m" {
    2u
    print "error: time_T1 is too long; < 1000ms !!!"
    goto HaltAcqu
  }

  if "time_T1_max > 1100m" {
    2u
    print "error: time_T1_max is too long; < 1100ms !!!"
    goto HaltAcqu
  }
#endif

  if "aq > 100m" {
    2u
    print "error: aq is too long; < 100ms !!!"
    goto HaltAcqu
  }

  if "d1 < 1.5s" {
    2u
    print "error: d1 is too short; > 1.5s !!!"
```

```
    goto HaltAcqu
  }

#ifdef Ddec
  if "cnst4 > 15" {
    2u
    print "error: dpwr3 pl4 is too high; < 15W !!!"
    goto HaltAcqu
  }

  if "cnst41 > 4" {
    2u
    print "error: dpwr3D pl41 is too high; < 4W !!!"
    goto HaltAcqu
  }

  if "pwd > 160u" {
    2u
    print "error: pwd is too long; < 160u !!!"
    goto HaltAcqu
  }

  if "pwd < 100u" {
    2u
    print "error: pwd is too short; > 100u !!!"
    goto HaltAcqu
  }

; d11 LOCKDEC_ON /* Not required for AvanceIII-HD */
  50u LOCKH_ON
  d11 H2_PULSE
  4u pl41:f4
#endif

2 d11 do:f3              /* loop back to here - NS times (per fid) */
/************************/
/* Update list pointers */
/************************/
  2u
#ifdef cal_NB1
  "ncyc_cal.idx=l2"
#else
  "N_offset.idx=l2"
#endif
  2u rpp11 rpp12 rpp25

/*****************************************/
/* Continue with checks that are run time */
/*****************************************/
#ifdef cal_NB1
  if "ncyc_cal/cnst8 > 2.0" {
    2u
    print "error: evolution time during B1 cal is too long !!!"
    goto HaltAcqu
  }
#endif

/***********************************************/
/* H heat compensation period - always do this  */
/***********************************************/
  10u fq=cnst1:f1        /* 1H SFO1+cnst1(Hz) @ tofNH */
  4u pl15:f1             /* power for 1H decoupling */
  (2u cpds1 ph26):f1     /* 1H dec ON */

#ifndef cal_NB1
  if "abs(N_offset) > 10000Hz" {
    time_T1_max_adj
  }
  else {
```

```
    if "time_T1_adj < time_T1_max_adj" {
      "DELTA = time_T1_max_adj - time_T1_adj"
      DELTA
    }
  }
#endif

  2u do:f1                /* 1H dec off */

/*************************************************/
/* Destroy residual 1H magnetization prior to d1 */
/*************************************************/
  10u fq=0:f1             /* 1H SFO1 @ tof(water) */
  4u pl1:f1               /* power pl1 for 1H pulses */

#ifdef Ddec
  20u UNBLKGRAMP
#else
  20u UNBLKGRAD
#endif

  (pwh ph26):f1

  2u
  p50:gp0*0.5
  d16

  (pwh ph27):f1

  2u
  p50:gp0
  d16

#ifdef Ddec
  4u BLKGRAMP
#else
  4u BLKGRAD
#endif

#ifdef mess_flg
  4u pl11:f1
  (dly_pg1 ph26):f1
  2u
  (dly_pg2 ph27):f1
#endif

#ifdef Ddec
  "DELTA = d1 - 300m - d11 - 6m"

#ifdef fsat
  4u pl10:f1
  300m cw:f1 ph26
  2u do:f1
  d11 H2_LOCK
  6m LOCKH_OFF
  DELTA cw:f1 ph26
  2u do:f1
  4u pl1:f1
#ifdef fscuba
  hscuba
  (pwh ph26):f1
  (pwh*2 ph27):f1
  (pwh ph26):f1
  hscuba
#endif /*fscuba*/
#else  /*fsat*/
  4u pl1:f1
  300m
  d11 H2_LOCK
```

```
  6m LOCKH_OFF
  DELTA
#endif /*fsat*/
  50u LOCKH_ON
  15u H2_PULSE
  20u UNBLKGRAMP

#else /*Ddec*/
#ifdef fsat
  4u pl10:f1
  d1 cw:f1 ph26
  2u do:f1
  4u pl1:f1
#ifdef fscuba
  hscuba
  (pwh ph26):f1
  (pwh*2 ph27):f1
  (pwh ph26):f1
  hscuba
#endif /*fscuba*/
#else  /*fsat*/
  4u pl1:f1
  d1
#endif /*fsat*/
  20u UNBLKGRAD
#endif /*Ddec*/

/*****************************************************/
/* Eliminate all magnetization originating on 15N */
/*****************************************************/
  4u pl3:f3
  (pwn ph26):f3

  2u
  p50:gp0
  d16

/***************************/
/* This is the real start */
/***************************/
#ifdef cp_flg
  10u fq=cnst1:f1

#ifdef cal_NB1
  10u fq=cnst3:f3
#endif

  (pwh ph26):f1
  2u pl16:f1 pl35:f3

#ifdef cal_NB1
  (center (pw_dip ph27):f1 (pw_dip ph27):f3)
#else
3 (center (pw_dip*3.556 ph11):f1 (pw_dip*3.556 ph11):f3)
  (center (pw_dip*4.556 ph12):f1 (pw_dip*4.556 ph12):f3)
  (center (pw_dip*3.222 ph11):f1 (pw_dip*3.222 ph11):f3)
  (center (pw_dip*3.167 ph12):f1 (pw_dip*3.167 ph12):f3)
  (center (pw_dip*0.333 ph11):f1 (pw_dip*0.333 ph11):f3)
  (center (pw_dip*2.722 ph12):f1 (pw_dip*2.722 ph12):f3)
  (center (pw_dip*4.167 ph11):f1 (pw_dip*4.167 ph11):f3)
  (center (pw_dip*2.944 ph12):f1 (pw_dip*2.944 ph12 ipp12):f3)
  (center (pw_dip*4.111 ph11):f1 (pw_dip*4.111 ph11 ipp11):f3)
  lo to 3 times l3
#endif

  2u pl3:f3
  (pwn ph1):f3

  10u fq=0:f1
```

```
#else /*cp_flg*/
  (pwh ph26):f1

  2u
  p51:gp1
  d16

#ifdef N_sel
  "DELTA = taua - 2u - p51 - d16 - pwn_sl*0.5"
  DELTA

  (center (pwh*2 ph26):f1 (pwn_sl:sp32 ph26):f3)
#else
  "DELTA = taua - 2u - p51 - d16"
  DELTA

  (center (pwh*2 ph26):f1 (pwn*2 ph26):f3)
#endif

  DELTA pl3:f3

  2u
  p51:gp1
  d16

  (pwh ph27):f1

  2u
  p52:gp2
  d16

  (pwn ph1):f3

  2u
  p53:gp3
  d16

  "DELTA = taub - 2u - p53 - d16"
  DELTA

  (center (pwh*2 ph26):f1 (pwn*2 ph26):f3)

  DELTA

  2u
  p53:gp3
  d16

  (pwn ph27):f3
  2u
  (pwh ph26):f1
#endif /*cp_flg*/

  2u
  p54:gp4
  d16

/*******************************************/
/* Start time_T1 relaxation/exchange period */
/*******************************************/
  10u fq=cnst1:f1
  4u pl15:f1
#ifndef cos_flg
  4u pl34:f3
#endif

#if defined(Ddec) && !defined(cal_NB1)
  4u pl4:f4
  (pwd ph27):f4
```

```
  2u pl41:f4
  (2u cpds4 ph26):f4
#endif

#ifdef cawurst_flg
  4u pl23:f2
  (2u cpds2 ph26):f2
#endif

  (2u cpds1 ph26):f1

#ifdef cal_NB1
  10u fq=cnst3:f3

#ifdef F2P
  (pwn_dante ph26):f3
#endif

  if "ncyc_cal > 0" {
#ifdef cos_flg
4   0.1u ipp25
    if "l7%2 == 0" {
      (p_dante:sp5 ph25):f1
    }
    else {
      (p_dante:sp5 ph26):f1
    }
#else
4   (p_dante ph26):f3
    d_dante
#endif
    lo to 4 times ncyc_cal
  }

#ifdef F2P
  if "l3 % 2 == 0" {
    (pwn_dante ph28):f3
  }
#endif

#else /*cal_NB1*/
  10u fq=N_offset:f3

  if "abs(N_offset) <= 10000Hz" {
    if "l4 > 0" {
#ifdef cos_flg
4     0.1u ipp25
      if "l7%2 == 0" {
        (p_dante:sp5 ph25):f1
      }
      else {
        (p_dante:sp5 ph26):f1
      }
#else
4     (p_dante ph26):f3
      d_dante
#endif
      lo to 4 times l4
    }
  }
#endif /*cal_NB1*/

  2u do:f1

#ifdef cawurst_flg
  2u do:f2
#endif

#if defined(Ddec) && !defined(cal_NB1)
```

```
  2u do:f4
  2u pl4:f4
  (pwd ph29):f4
#endif

  10u fq=0:f1 fq=0:f3
  4u pl11:f1 pl3:f3

  (dly_pg1 ph26):f1
  2u
  (dly_pg2 ph27):f1

  2u
  p55:gp5
  d16

/*********************************/
/* 15N Frequency labeling period */
/*********************************/
#ifdef ip_flg
  "t1_max = (ni-1)*in0*2.0 + in0 + 2u"
  "DELTA = larger(t1_max-d0*2.0, TAU2)"
  "DELTA1 = larger(t1_max-d0*2.0, TAU2)*0.5"
  "DELTA2 = larger(t1_max-d0*2.0, TAU2)*0.5 + pwn*4.0/PI"

  10u fq=cnst1:f1
  4u pl12:f1
  (2u cpds5 ph26):f1

  (pwn ph2):f3

#ifdef c_flg
  if "d0 - pwc_ad*0.5 > 2u" {
    "DELTA = d0 - pwc_ad*0.5"
    DELTA
    (pwc_ad:sp22 ph26):f2
    DELTA
  }
  else {
    d0
    d0
  }
#else
  d0
  d0
#endif /*c_flg*/

  2u do:f1
  10u fq=0:f1
  4u pl1:f1

  2u
  p56:gp6*-1.0
  d16

  "DELTA = taub - 2u - 10u - 4u - 2u - p56 - d16"
  DELTA

  (center (pwh*2 ph26):f1 (pwn*2 ph3):f3)

  "DELTA = taub - 2u - p56 - d16"
  DELTA

  2u
  p56:gp6*1.0
  d16
#else /*ip_flg*/
  (pwn ph2):f3
```

```
  2u
  p56:gp6*-1.0
  d16

  "DELTA = taub + pwh*2.0 - 2u - p56 - d16"
  DELTA pl1:f1

  (pwn*2 ph3):f3

  d0

  (pwh*2 ph26):f1

  2u
  p56:gp6
  d16

#ifdef c_flg
  "DELTA = taub - 2u - p56 - d16 - pwc_ad"
  DELTA
  (pwc_ad:sp22 ph26):f2
#else
  "DELTA = taub - 2u - p56 - d16"
  DELTA
#endif

  d0
#endif /*ip_flg*/

#if defined(cp_flg) && defined(cal_NB1)
  10u fq=cnst1:f1 fq=cnst3:f3
  (ralign (pwh ph26):f1 (pwn ph26):f3)

  2u pl16:f1 pl35:f3
  (center (pw_dip ph27):f1 (pw_dip ph27):f3)

  2u pl1:f1
  (pwh ph26):f1

  10u fq=0:f1 fq=0:f3

  "DELTA = BigT1 - 10u"
  DELTA
#else
  (center (pwh ph26):f1 (pwn ph4):f3)

  2u
  p57:gp7
  d16

  "DELTA = taua - 2u - p57 - d16"
  DELTA

  (center (pwh*2 ph26):f1 (pwn*2 ph26):f3)

  DELTA

  2u
  p57:gp7
  d16

  (center (pwh ph27):f1 (pwn ph29):f3)

  2u
  p58:gp8
  d16

  "DELTA = taua - 2u - p58 - d16"
  DELTA
```

```
  (center (pwh*2 ph26):f1 (pwn*2 ph26):f3)

  "DELTA = taua - 2u - p58 - d16 + (pwn - pwh)*0.5"
  DELTA

  2u
  p58:gp8
  d16

  (pwh ph28):f1

  BigT1
#endif

  (pwh*2 ph26):f1

  2u
  p59:gp9*-1.0*EA
  d16

  "DELTA = BigT1 - 2u - p59 - d16 - 4u - 4u - de + pwh*2.0/PI"
  DELTA

#ifdef Ddec
  4u BLKGRAMP
#else
  4u BLKGRAD
#endif
  4u pl31:f3

/*****************/
/* Acquire data */
/*****************/
  go=2 ph31 cpds3:f3
  d11 do:f3 mc #0 to 2

#if defined(cal_NB1) && defined(F2P)
  F2PH(calclc(l3, 1), calclc(l2, 1))
#else
  F2QF(calclc(l2, 1))
#endif
  F1EA(calgrad(EA) & calph(ph4, +180), caldel(d0, +in0) & calph(ph2, +180) & calph(ph3
1, +180))

#ifdef Ddec
  d11 H2_LOCK
  d11 LOCKH_OFF
; d11 LOCKDEC_OFF        /* use statement for earlier hardware */
#endif

HaltAcqu, 1m
exit

ph1=0 2
ph2=1
ph3=0 0 1 1 2 2 3 3
ph4=0
ph11=1 3 3 1
ph12=3 1 1 3
ph25=2 0
ph26=0
ph27=1
ph28=2
ph29=3
ph31=0 2 2 0

;d1: relaxation delay
;d2: time_T1
```

```
;d3: time_T1_max
;d4: taua delay ~ 2.38 ms (< 1/4JNH)
;d5: taub delay = 2.68 ms (= 1/4JNH)
;d11: delay for disk i/o, 30ms
;d14: set to BigT1 500 us
;d16: gradient recovery delay, 200us
;tau_cp: dipsi2 het-cp duration (~1/JNH = 10.8 ms)
;pl1: tpwr - power level for pwh
;pl3: dhpwr2 - power level for 15N pulse pwn
;pl4: power level for 2H high power pulses
;pl10: tsatpwr - power level for water presat
;pl11: tpwrmess - power level for Messerle purge
;pl12: tpwrml - power level for 1H decoupling during time_T1
;plw15: tpwrml - adjusted power level for 1H decoupling during time_T1
;pl23: dpwrsed - power level for Ca/CO (wurst-2) decoupling
;pl31: dpwr2 - power level for 15N cpd3
;pl34: power level for 15N DANTE pulses
;pl41: power level for 2H waltz decoupling
;sp22: power level for pwc_ad
;sp32: power level for pwn_sl
;spnam5: shape for shaped D-CEST excitation
;spnam22: shape for pwc_ad
;spnam23: File name for Ca/CO decoupling during 15N CEST period
;spnam32: shape for pwn_sl
;p1: pwh - 1H 90 degree pulse
;p3: pwn - 15N 90 degree pulse
;p4: 2H high power pulse
;p12: pwmlev - 90 degree pulse for decoupling sequence
;p15: pwmlev - adjusted 90 degree pulse for decoupling sequence
;p16: pw_dip - 90 degree pulse for dipsi-2 het-cp
;p22: pwc_ad - 13C 180 pulse (adiabatic)
;p23: pwcadec - Ca/CO wurst-2 dec total length of supercycled pattern
;p32: pwn_sl for selective pulse on amide N15s
;p34: pwn_dante - 90 degree pulse at pl34
;p50: gradient pulse 50                                  [1000 usec]
;p51: gradient pulse 51                                  [500 usec]
;p52: gradient pulse 52                                  [1000 usec]
;p53: gradient pulse 53                                  [500 usec]
;p54: gradient pulse 54                                  [500 usec]
;p55: gradient pulse 55                                  [1000 usec]
;p56: gradient pulse 56                                  [625 usec]
;p57: gradient pulse 57                                  [500 usec]
;p58: gradient pulse 58                                  [500 usec]
;p59: gradient pulse 59                                  [256 usec]
;cpdprg1: 1H decoupling program during 15N CEST [dipsi3.p15]
;cpdprg2: 13C decoupling program during 15N CEST [p5m4sp180.p23]
;cpdprg3: 15N decoupling program during t2 [waltz16]
;pcpd3: f3 channel - 90 degree pulse for decoupling sequence (1/dmf2)
;cpdprg4: 2H decoupling program during 15N CEST [dipsi3]
;pcpd4: 2H pulse width for 2H decoupling
;cpdprg5: 1H decoupling program during t1 [waltz16.p12]
;cnst1: diff (Hz) between 1H decoupling position and water
;cnst3: diff (Hz) between 15N peak and carrier for B1 calibration
;cnst5: amount of rotation (degree) made by each element of cpdprg1
;cnst6: weak B1 field (Hz) for het-cp selective excitation
;cnst8: Frequency period (Hz) between adjacent excitation bands
;cnst9: Average N15 B1 (Hz) during D-CEST
;fq3list: frequency list (Hz) for 15N offsets in D-CEST
;vclist: variable counter list for 15N B1 calibration in D-CEST
;l3: loop counter for dipsi-2 cycles in het-cp
;l7: number of excitation bands for D-CEST
;inf1: 1/SW(X) = 2*DW(X)
;in0: 1/(2*SW(x))=DW(X)
;nd0: 2
;ns: 2*n
;FnMODE: Echo-Antiecho in F1
;FnMODE: QF in F2

;use gradient ratio:    gp 6 : gp 9
```

```
;                          80 : 39.6

;for z-only gradients:
;gpz0: 20%
;gpz1: 12%
;gpz2: 90%
;gpz3: 24%
;gpz4: 70%
;gpz5: -75%
;gpz6: 80%
;gpz7: 60%
;gpz8: 15%
;gpz9: 39.6%

;use gradient files:
;gpnam0: SMSQ10.32
;gpnam1: SMSQ10.32
;gpnam2: SMSQ10.32
;gpnam3: SMSQ10.32
;gpnam4: SMSQ10.32
;gpnam5: SMSQ10.32
;gpnam6: SMSQ10.32
;gpnam7: SMSQ10.32
;gpnam8: SMSQ10.32
;gpnam9: SMSQ10.32

;zgoptns: Df1180, Dfsat, Dfscuba, Dmess_flg, Dc_flg, Dcal_NB1, DN_sel, Dcawurst_flg, D
Hdec_adj, DDdec, Dip_flg, Dcp_flg, Dcos_flg, DF2P
```