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Corresponding Author:	Harald Schwalbe GERMANY
Corresponding Author's Institution:	
Corresponding Author E-Mail:	schwalbe@nmr.uni-frankfurt.de
Order of Authors:	H. Berg
	M. A. Wirtz Martin
	A. Niesteruk
	C. Richter
	S. Sreeramulu
	H. Schwalbe
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TITLE:

NMR-based fragment screening in a minimum sample but maximum automation mode

AUTHORS:

Harald Berg[1][†], M. A. Wirtz Martin[1][†], A. Niesteruk[1,2], C. Richter[1], S. Sreeramulu[1], H. Schwalbe[1,2,*]

[1]Institute for Organic Chemistry and Chemical Biology, Center for Biomolecular Magnetic Resonance (BMRZ), Johann Wolfgang Goethe-University Frankfurt, Frankfurt, Germany

[2]German Cancer Consortium (DKTK) and DKFZ, Heidelberg, Germany

E-mail address:
berg@nmr.uni-frankfurt.de
wirtzmartin@nmr.uni-frankfurt.de
niesteruk@nmr.uni-frankfurt.de
ric@nmr.uni-frankfurt.de
sridhar@nmr.uni-frankfurt.de
schwalbe@nmr.uni-frankfurt.de

[†] joint first authors

*to whom correspondence should be addressed:
schwalbe@nmr.uni-frankfurt.de

KEYWORDS:

Fragment-based screening; Automation; Solution NMR-spectroscopy; Data collection and analysis; Screening platform; Fragment library

SUMMARY:

Fragment-based screening by NMR is a robust method to rapidly identify small molecule binders to biomacromolecules (DNA, RNA, or proteins). Protocols describing automation-based sample preparation, NMR experiments & acquisition conditions, and analysis workflows are presented. The technique allows for optimal exploitation of both ^{1}H and ^{19}F NMR-active nuclei for detection.

ABSTRACT:

Fragment-based screening (FBS) is a well-validated and accepted concept within the drug discovery process both in academia and industry. The greatest advantage of NMR-based fragment screening is its ability not only to detect binders over 7-8 orders of magnitude of affinity but also to monitor purity and chemical quality of the fragments and thus to produce high quality hits and minimal false positives or false negatives. A prerequisite within the FBS is to perform initial and periodic quality control of the fragment library, determining solubility and chemical integrity of the fragments in relevant buffers, and establishing multiple libraries to cover diverse scaffolds to accommodate various macromolecule target classes (proteins/RNA/DNA). Further, an extensive NMR-based screening protocol optimization with respect to sample quantities, speed of acquisition and analysis at the level of biological construct/fragment-space, in condition-space (buffer, additives, ions, pH, and temperature) and in ligand-space (ligand

analogues, ligand concentration) is required. At least in academia, these screening efforts have so far been undertaken manually in a very limited fashion, leading to limited availability of screening infrastructure not only in the drug development process but also in the context of chemical probe development. In order to meet the requirements economically, advanced workflows are presented. They take advantage of the latest state-of-the-art advanced hardware, with which the liquid sample collection can be filled in a temperature-controlled fashion into the NMR-tubes in an automated manner. ^{1}H/^{19}F NMR ligand-based spectra are then collected at a given temperature. High-throughput sample changer (HT sample changer) can handle more than 500 samples in temperature-controlled blocks. This together with advanced software tools speeds up data acquisition and analysis. Further, application of screening routines on protein and RNA samples are described to make aware of the established protocols for a broad user base in biomacromolecular research.

INTRODUCTION:

Fragment-based screening is now a commonly used method for identifying rather simple and low molecular weight molecules (MW <250 Da) that show weak binding to macromolecular targets including proteins, DNA and RNA. Initial hits from primary screens serve as the basis to conduct a secondary screen of commercially available larger analogues of the hits and then to utilize chemistry-based fragment growth or linking strategies. For a successful fragment-based drug discovery (FBDD) platform, in general, a robust biophysical method is required for detecting and characterizing weak hits, a fragment library, a biomolecular target and a strategy for follow-up chemistry. Four commonly applied biophysical methods within the drug discovery campaigns are thermal shift assays, surface plasmon resonance (SPR), crystallography and nuclear magnetic resonance spectroscopy (NMR).

NMR spectroscopy has displayed varied roles within the different stages of the FBDD. Apart from ensuring the chemical purity and solubility of the fragments in a fragment library dissolved in an optimized buffer system, ligand observed NMR experiments can detect fragment binding to a target with low affinity and the target observed NMR experiments can delineate the binding epitope of the fragment, thus enabling detailed structure-activity relationship studies. Within epitope mapping, NMR-based chemical shift changes cannot only identify the orthosteric binding sites but also allosteric sites that might be cryptic and only accessible in so called excited conformational states of the biomolecular target. If the biomolecular target already binds an endogenous ligand, the identified fragment hits can be easily classified as allosteric or orthosteric by performing NMR-based competition experiments. Determining the dissociation constant (K_D) of the ligand-target interaction is an important aspect in the FBDD process. NMR-based chemical shift titrations, either ligand or target observed can be readily performed to determine the K_D. A major advantage of NMR is that the interaction studies are performed in solution and near to physiological conditions. Thus, all conformational states for the analysis of ligand/fragment interaction with its target can be probed. Further, NMR-based approaches are not only restricted towards screening of well-folded soluble proteins, but also are being applied to accommodate larger target space including DNA, RNA, membrane bound and intrinsically disordered proteins[1].

Fragment libraries are an indispensable part of the FBDD process. In general, fragments act as the initial precursors which eventually become part (substructure) of the

100 new inhibitor developed for a biological target. Several drugs (Venetoclax[2], Vemuraf-
101 enib[3], Erdafitinib[4], Pexidartnib[5]) have been reported to have started as fragments and
102 are now successfully used in the clinics. Typically, fragments are low molecular weight
103 (<250 Da) organic molecules with a high aqueous solubility and stability. A carefully
104 crafted fragment library containing typically a few hundred fragments, can already
105 promise efficient exploration of chemical space. The general composition of fragment
106 libraries has evolved overtime and most often were derived by dissecting known drugs
107 into smaller fragments or designed computationally. These diverse fragment libraries
108 mainly contain flat aromatic or heteroatoms and adhere to the *Lipinski Rule of 5* [6], or
109 to the current commercial trend *Rule of 3* [7], but avoid reactive groups. Some fragment
110 libraries were also derived or composed of highly soluble metabolites, natural products
111 and or their derivatives[8]. A general challenge posed by most of the fragment libraries
112 is ease of downstream chemistry.

114 The Center for Biomolecular Magnetic Resonance (BMRZ) at the Goethe-University
115 Frankfurt, is a partner of the iNEXT-Discovery (Infrastructure for NMR, EM and X-rays
116 for Translational research-Discovery), a consortium for structural research infrastruc-
117 tures for all European researchers from all fields of biochemical and biomedical re-
118 search. Within the previous initiative of iNEXT which ended in 2019, a fragment library
119 comprising of 768 fragments was crafted with the aim of "minimum fragments and
120 maximum diversity" covering a large chemical space. Further, unlike any other frag-
121 ment library, the iNEXT fragment library was also designed based on the concept of
122 "poised fragments" with the aim to ease downstream synthesis of complex, high affinity
123 ligands and henceforth known as in-house library (Diamond, Structural Genomic Con-
124 sortium and iNEXT).

126 Establishing FBDD by NMR requires manpower, knowledge, and instrumentation. At
127 the BMRZ, optimized workflows to support technical assistance to fragment screening
128 by NMR have been developed. These include quality control and solubility assessment
129 of the fragment library [9], buffer optimization for the chosen targets, ^{1}H or ^{19}F- observed
130 1D-ligand based screening, competition experiments to differentiate between or-
131 thosteric and allosteric binding, 2D-based target observed NMR experiments for
132 epitope mapping, and for characterizing the interaction with secondary set of deriva-
133 tives of the initial fragment hits. BMRZ has established automated routines for the
134 analysis, as also previously discussed in the literature [10,11], of small molecule-protein
135 interactions and has in place all the necessary automated infrastructure for NMR-
136 based fragment screening. It has implemented saturation transfer difference NMR
137 (STD-NMR), water-ligand observed via gradient spectroscopy (waterLOGSY), and
138 Carr-Purcell-Meiboom-Gill-based (CPMG-based) relaxation experiments to identify
139 fragments within a wide range of affinity regimes as well as state-of-the-art automated
140 NMR instrumentation and software for drug discovery. While NMR-based fragment
141 screening is well established for proteins, this approach is less commonly used for
142 finding new ligands interacting with RNA and DNA. BMRZ has established proof of
143 concept for new protocols enabling the identification of small molecule-RNA/DNA in-
144 teractions. In the following sections of this contribution, application of screening rou-
145 tines on protein and RNA samples is reported to make aware of the established pro-
146 tocols for a broad user base in biomacromolecular research.

148 **PROTOCOL:**

1.   Fragment library

1.1.   In-house fragment library

NOTE: Within the framework of one of the joint research activities of the iNEXT, a robust and downstream chemistry friendly first generation fragment library was developed[12] and subsequently a second generation of the library was put together in collaboration with Enamine and is known as the DSI (Diamond-SGC-iNEXT)-poised fragment library (from now on termed as "In-house library"). This library can be made available at the BMRZ for screening purposes.

1.1.1. Assess the fragment library for its integrity and solubility using a previously reported NMR-based protocol[9].

NOTE: The in-house library consists of 768 fragments with a very high chemical diversity (>200 Singletons). Performing the screening in fragment mixtures can significantly speed up the screening campaign; however, the number of fragments in a mix is limited due to signal overlap in ^{1}H-NMR spectrum. The higher chemical diversity offered by the in-house library allows for the preparation of mixtures containing 12 different fragments without any significant chemical shift overlap in the ^{1}H observed NMR spectra.

1.1.2. 103 fragments within the 768 fragments possess a fluorine atom. For ^{19}F screening purposes, divide all 103 fragments that possess a fluorine group into 5 mixes based on minimum ^{19}F chemical shift overlap. To minimize signal overlap in the ^{19}F screening, use the chemical shift information from single compound measurements to design mixtures with maximum number of fragments and minimal signal overlap. Each mix has 20-21 fragments with distinct ^{19}F chemical shifts allowing for unambiguous assignment of fragments.

1.2.   User defined/provided fragment library

1.2.1. Perform screening campaigns with the user defined or provided fragment library; however, the following steps need to precede the screening campaign.

1.2.2. If not specified by the user beforehand, perform NMR-based quality control of fragments with advanced software tools available at the BMRZ ([9], Chapter 6.1.1).

1.2.3. Check the solubility of the fragments in buffer-of-choice for the biomolecular target, structure integrity, and concentration of fragments prior to use.

1.2.4. Design the mixture to decrease both signal overlap in NMR spectra and measurement time.

1.2.5. Design mixtures according to step 4.2.

1.2.6. Screen single fragments or a subset of mixtures instead of the entire library.

2.   Sample preparation

NOTE: High-throughput screening by NMR utilizes a pipetting robot for sample preparation. NMR spectra, but also stabilities over several days of signal acquisition of proteins, RNAs and DNA are extremely sensitive to temperature fluctuations and therefore temperature-controlled automated systems will greatly facilitate the stability of the samples being pipetted. For this purpose, an additional add-on device, which works between 4 to 40 °C, is coupled to the pipetting robot for liquid handling of the NMR samples in a temperature-controlled environment.

2.1.   Ligand mixture preparation

2.1.1. Prepare screening samples for NMR measurements using a sample preparation robot. The flexible configuration of the robot allows for a wide range of applications (e.g., recovery of the samples from NMR tubes back into storage containers or general liquid handling tasks). Use NMR-tubes with different diameters (1.7, 2.0, 2.5, 3.0 and 5.0 mm). The sample robot system along with the advanced control software reads the barcode assigned for each container type and executes the liquid filling protocol optimally.

2.1.2. For the preparation of the in-house library ligand mixtures, use barcoded vials. The barcoded vials guarantee the highest level of reliability and optimal traceability of the samples.

2.1.3. Distribute 768 compounds into 8 plates of 96-well format. The stock concentration of each individual fragment is 50 mM in d_6-DMSO/D_2O (9:1). In total, prepare 64 mixes each containing 12 fragments. The final concentration of each fragment in a mix is 4.2 mM.

NOTE: The pipetting robot can accommodate a variety of container types with varied geometries (cryo- or auto sampler vials, 96-well plates round or square deep, barcoded standard vials, microcentrifuge tubes) and assists efficient execution of the liquid transfer to a variety of NMR tubes and racks.

[Place **Figure 1** here]

2.2.   Screening sample preparation blank (reference ligand spectrum) and with target (ligand in the presence of target)

2.2.1. For the preparation of the NMR screening samples, in the presence of the target biomolecule (protein/RNA/DNA) and the ligand mixture, use 3 mm NMR HT sample changer tubes selected from the Bruker NMR portfolio of standard NMR tubes.

2.2.2. Transfer the biomolecular target (e.g., [1]H Screening: 10 µM RNA or Protein) in a defined screening buffer into the 3 mm NMR tube (final volume of 200 µL) manually or using the pipetting robot.

2.2.3. Transfer 10 µL (e.g., [1]H Screening) of the ligand mixture in the next step using the robotic system into the barcoded 3 mm NMR tubes containing the target biomolecule and mix using the inbuilt protocol of the control software.

249　NOTE: The barcode number of the NMR tube is conveniently and automatically incor-
250　porated into the acquired NMR-dataset, thus ensuring ID oriented workflow without
251　any mix-up. The pipetting robot temperature control accessory allows to keep the pre-
252　pared samples in the NMR tubes under constant temperature.
253
254　2.3.　In-house defined conditions and parameters
255
256　2.3.1. Establish optimal buffer conditions for performing screening of RNA and protein
257　against the in-house fragment library. For RNA, use the following sample condition:
258　25 mM KPi, 50 mM KCl, pH 6.2. Mg^{2+} is optional.
259
260　2.3.2. Proteins are extremely sensitive for solution conditions; use buffers optimal for
261　the target of choice. For each of those buffers, acquire additional reference spectra of
262　the ligands to serve as blank for the analysis.
263
264　2.4.　User specified conditions
265
266　NOTE: In cases in which the in-house established conditions are not suitable for the
267　targets to be screened from a potential user, the following steps should be imple-
268　mented.
269
270　2.4.1. Perform ^{1}H-NMR of the buffer alone to ensure minimal interference from the
271　components of the buffer in performing and analysing the ligand observed screening
272　experiments. Interfering components could be suitably replaced with deuterated equiv-
273　alents.
274
275　2.5.　Limitations in sample production (target quantities)/conditions and availability
276
277　NOTE: Isolation or recombinant production of certain biomacromolecules can in cer-
278　tain cases prove challenging and result in limited availability of the target to pursue a
279　successful drug screening campaign. In cases of limited or unlimited availability of the
280　targets, the following alternatives could be utilized for conducting a successful NMR-
281　based fragment screening.
282
283　2.5.1. If limited, use ^{19}F-NMR based screening. Typical fluorinated ligands have a
284　single ^{19}F signal; therefore, use cocktails with 25-30 fragments without any signal
285　overlap. There are fewer signals to analyse, no signal interference from buffer com-
286　ponents, and fewer signals to rely on for hit identification.
287
288　2.5.1.1.　　If unlimited, use larger screens like ^{1}H-NMR. The larger fragment li-
289　brary can be screened. Typically, fragments are composed of more than one proton,
290　which means more signals to rely upon for the analysis.
291
292　**3.　　NMR acquisition conditions**
293
294　3.1.　In-house generally defined conditions
295
296　3.1.1. Spectrometer equipped with HT sample changer (Automation)
297

3.1.1.1.   For high-throughput screening, use 96-well plates that can only be measured using a HT sample changer. The HT sample changer also offers the possibility to temper each rack individually.

3.1.1.2.   For optimal signal-to-noise, use a spectrometer with a cryogenic probe that is either helium or nitrogen cooled. An automated tunning and matching module (ATM) are necessary for automation.

3.1.2. Parameter sets & pulse sequences

NOTE: Many NMR experiments can characterize binding events. The hit identification varies depending on the experimental setup. The following experiments are routinely used in BMRZ screening campaigns. Nevertheless, changes can be made for user defined screening campaigns and according to user specifications.

3.1.2.1.   If usingTopSpin software, include the parameter set for ligand-based experiments: SCREEN_STD, SCREEN_T1R, SCREEN_T2, SCREEN_WLOGSY. The parameter set includes all necessary parameters and the pulse sequences: STD: stddiffesgp.3; $T_{1\rho}$: t1rho_esgp2d; T_2: cpmg_esgp2d; and waterLOGSY: ephogsygpno.2.

3.1.2.2.   For all the listed experiments, use excitation sculpting[13] as water suppression. For a reference, use 1D excitation sculpting (zgesgp). The number of scans depends on the sensitivity of the system (magnetic field strength and probe head), the sample concentration, and choice of the experiment. A recommendation is: 1D with NS=64, $T_{1\rho}$ & T_2 with NS=128, STD with NS=256 and waterLOGSY with NS= 384 or 512.

3.1.2.3.   For the ^{19}F screening, use both 1D and T_2 experiments: -1D: F19CPD (pp=zgig) for $^{19}F\{^1H\}$-probe head and F19(pp=zg) for $^{19}F/^1H$-probe head - SCREEN_19F_T2 (pp = cpmgigsp)

3.1.2.4.   Use a spectral width of 220 ppm and an excitation frequency at -140 ppm. The experiment time is between 1 and 5 hours (ensure the long-term stability of the biomacromolecule) depending on the hardware and sample concentration. For T_2, the CPMG time should alternate between 0 ms and 200 ms.

3.1.3. Processing

3.1.3.1.   Record the STD, $T_{1\rho}$ and T_2 experiments as pseudo 2D. To process the two single 1D spectra, IconNMR uses the au-program proc_std either with or without the option relax. The first option provides the reference 1D and the difference of two spectra. The second option yields two separate spectra with short and long relaxation time. The waterLOGSY is a single 1D which should be phased with a negative for the solvent signal.

3.2.   User specific conditions

3.2.1.1.   Adapt any of the previously mentioned parameters to user-defined conditions. For example, if a facility user-provided protein is not stable at the generally

used temperature, optimization experiments can be conducted varying temperature, concentration, buffer conditions etc.

4. Data Analysis

4.1. Fragment library QC (d6-DMSO/specific buffer) and quantification

4.1.1. CMC-q

NOTE: Quality control of fragment libraries is essential prior to initiation of screening campaigns. Furthermore, long-term stability of fragment library needs to be ensured for the application of several screening campaigns, which is why periodic evaluation of the quality of the library must be conducted. For this purpose, the integrated software CMC-q and CMC-a from TopSpin is used for quality and quantity assessment. The CMC-q and CMC-a are software modules within Topspin which enable smooth acquisition, analysis including structure verification using ^{1}H-NMR spectrum obtained from small organic molecules [9].

4.1.1.1.   For integrity, prepare assessment samples with a fragment concentration of 1 mM in d6-DMSO. Prepare samples in an automated manner with a pipetting robot by filling liquid sample collection into a 3 mm NMR-tube.

4.1.1.2.   For solubility assessment, use sample consisting of 1 mM compound in 50 mM sodium phosphate buffer at pH 7.4, 150 mM sodium chloride, 90% H_2O/ 10% D_2O and 1 mM of 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TMSP-Na).

4.1.1.3.   Collect NMR spectra at 298 K or 293 K using a 600 MHz NMR spectrometer equipped with triple resonance 5 mm TCI cryogenic probe and a HT sample changer, which can handle 579 samples at once.

4.1.1.4.   For setting up CMC-q software follow the instructions of the user manual, which implements the creation of an IconNMR user, the activation of FastLaneNMR, and changing the HT sample changer.

4.1.1.5.   Calibrate the 90° pulse and save it in the TopSpin prosol table.

4.1.1.6.   Place the 96 sample well plate in one of the 5 rack positions in the HT sample changer.

4.1.1.7.   To load an SDF file (structure data file) that should contain its proposed chemical structure, a unique identifier, and the position in the HT sample changer of each sample in a batch, go to **Browse** in the CMC-q Setup window and click **Open** after selecting a file that ends in .sdf.

4.1.1.8.   In the CMC.q Batch Automation settings, set the verification type that defines the experiment that will be measured, the IconNMR user and define the Solvent.

4.1.1.9.   Define SDF files for the Path for SDF file, the molecule ID and the sample position.

398
399 4.1.1.10.   Start the acquisition by clicking on **Start**. Click on **Start Acquisition**
400 again. The CMC-q Setup can also be saved by clicking on **Save**.
401
402 4.1.1.11.   For in-detail description of CMC-q setup steps, follow the user manual
403 instructions from Bruker.
404
405 4.1.2. CMC-a
406
407 4.1.2.1.   For CMC-a, use thesoftware module within Topspin that enables analy-
408 sis including structure verification using 1H-NMR spectrum obtained from small or-
409 ganic molecules[9].
410
411 4.2.   Mixture design
412
413 NOTE: A proper mixture design plays an important role for screening using NMR as a
414 platform. A high number of fragments per mixtures allows for faster screening but in-
415 creases the risk of false positive and negatives. A lower number decreases that risk
416 but increases the time it takes to conduct the screening. In general, a signal overlap
417 has to be avoided when creating mixtures. Using the In-house library, this can be ne-
418 glected for the ^{1}H screening as the library was specifically designed to be diverse and
419 show little signal overlap while maintaining a high chemical diversity. This in turn
420 means that no special design procedure has to be undergone for creating the 64
421 mixes.
422
423 4.2.1. As the ^{19}F screening relies on the fragments of the in-house library that contain
424 fluorine and the library was not created to reduce the signal overlap for these specific
425 fragments, design a proper mixture.
426
427 4.2.2. Measure single compound spectra for all fragments containing ^{19}F.
428
429 4.2.3. Note the chemical shift information of each signal.
430
431 4.2.4. According to this information, choose 20-21 fragments per mixture. This in
432 turn gives 5 mixtures each containing 20-21 fragments with no signal overlap and al-
433 lows a semi-automated analysis of the data.
434
435 4.3.   Perform hit identification within a ligand observed biomacromolecule-ligand in-
436 teraction
437
438 NOTE: There are different definitions of a hit between the ^{19}F and ^{1}H screening pro-
439 cedure. The following hit identifications were set up by us and follow specific rules.
440 The subject of hit determination is a very subjective manner and can differ from user
441 to user. Nevertheless, it is of utmost importance that the rules for hit identification do
442 not change once agreed upon to maintain validation and credibility.
443
444 **4.3.1. ^{1}H Screen**
445

446   4.3.1.1.   To confidently determine hits, acquire 1D ^{1}H spectra, waterLOGSY and
447   T_2 relaxation experiments both in the presence and absence of target to identify bind-
448   ers. All three experiments have the potential to show a binding event. If a CSP of
449   greater than 6 Hz is visible in the sample spectra compared to the blank spectra, this
450   is considered as an indication for a hit. The same goes if a strong positive signal in the
451   waterLOGSY as well as more than 30% T_2 reduction in the sample spectra is visible.
452   Binding events can be showcased in all three experiments, when comparing the sam-
453   ple containing spectra with their respective blank spectra. However binding events
454   may not be visible in all three experiments. Because of this it was agreed upon that at
455   least two of the before described events must occur in order to classify a fragment as
456   a binding hit.

457

458   4.3.1.2.   Use the FBS tool to define the state of fragments into binding, ambigu-
459   ous, unknown, aggregates and not-binding.

460

461   4.3.1.3.   When finished with a mix, approve it within the FBS tool.

462

463   4.3.1.4.   In the summary tab within the FBS project, click on **Create a screening**
464   **report**. This will open a window that creates a .xlsx file. The user may then opt to
465   choose between all ligands, binding ligand only, not binding ligand only and ambigu-
466   ous ligands to be reported in the spreadsheet.

467

468   4.3.2. ^{19}F Screen

469

470   4.3.2.1.   To differentiate between non-binder, week-binder, and strong-binder, di-
471   vide the quotient between the 200 ms target measurement and the 200 ms blank
472   measurement by the quotient of the 0 ms target measurement and the 0 ms blank
473   measurement is used:

474

$$Hit - Score = \frac{\frac{Target^{200ms}}{Reference^{200ms}}}{\frac{Target^{0ms}}{Reference^{0ms}}}$$

475

476

477   NOTE: This gives values ranging from 0 to ~1 (the hit-score), making it possible to
478   assign thresholds for each binding state.

479

480   4.3.2.2.   Use the average of the reference 200 ms measurement as a baseline
481   threshold, to mark cases where the hit-score exceeds 1. This can occur, if the imported
482   integrals contain negative values or the reference measurement is higher than the
483   target measurement. A hit-score of ≤ 0.67 is considered a weak-hit, < 0.33 a strong
484   hit, and anything > 0.67 as no-hit. An example is shown in **Figure 2**.

485

486   [Place **Figure 2** here]

487

488   4.4.   Data analysis

489

490   4.4.1. Prepare data for analysis

491

492   NOTE: It is important that the acquired data has no visible flaws. This means that data
493   where the shimming was problematic, or water suppression was insufficient should
494   not be considered for analysis. Rather it is recommended to record data again and
495   making sure that everything is fine with the sample (e.g., no air bubbles), with the
496   temperature, the shimming, and water suppression. Data correctness can always be
497   assessed when comparing DMSO signals.
498
499   4.4.2. ^{1}H screening
500
501   4.4.2.1.   To analyse ^{1}H screening data, use the FBS tool (needs additional li-
502   cense) in TopSpin 4.0.9.
503
504   4.4.2.2.   Follow the instructions in the FBS tool manual to start with the data anal-
505   ysis. The following steps summarize the procedure reported in the manual.
506
507   4.4.2.3.   Store the BMRZ NMR data from screening campaigns such that each
508   different screening mixture has its own directory in which a subdirectory holds the dif-
509   ferent experiments measured on the sample.
510
511   4.4.2.4.   For using the FBS tool, store the reference spectra that has all data
512   saved from samples without the biomolecular target but with the mixtures as well as
513   the single compound measured in different /nmr directories. This is important as FBS
514   tool will ask for the directory path of each individually.
515
516   NOTE: The FBS tool will recognize a directory as a screening project if the following
517   datasets were stored in the same directory where the mixtures of a screening sample
518   are stored (csv, FragmentScreen XML documents and BAK file).
519
520   4.4.2.5.   When using TopSpin 4.0.9, create a direct path to the directory contain-
521   ing the acquired data, a so-called DIR. Choose the /nmr directory in which all mixtures
522   should have a distinct directory.
523
524   4.4.2.6.   To start the FBS tool of a screened sample drag the symbol FBS project
525   into the middle of the TopSpin window. In the chosen directory the FBS project symbol
526   should appear if previously said datasets were copied into it.
527
528   4.4.2.7.   Copy csv, FragmentScreen XML documents and BAK file into the nmr
529   directory where all the data is.
530
531   4.4.2.8.   The window Fragment Based Screening Options should automatically
532   open when first loading a new FBS project. In this window choose a cocktail file. The
533   cocktail file is a csv file containing the assignment of the name of the mixes, the name
534   of each fragment and their division into the mixes. Also define a reference ligand spec-
535   tra folder which has all measured spectra of the single fragments. Lastly, define a
536   reference blank experiment folder, which is usually the folder containing the datasets
537   of the mixes without the investigated target.
538
539   4.4.2.9.   The Fragment Based Screening Options has a tab called Spectra types
540   that lets one define the investigated spectra as well as the colour for displaying the

spectra. Set the **Spectype** according to the beforehand processed data. In the **Display** layout tab, define the spectra that will be compared with each other according to their spectypes.

4.4.2.10.   Press **Ok** to start the FBS project.

4.4.2.11.   While looking at the data, a separate window will open, summarizing all cocktail mixes and all ligands of each mix in a table. By double clicking on a cell, the respective datasets will open, comparing for example ^{1}H 1D Blank spectra with the dataset containing the target.

4.4.2.12.   Before assigning binders make sure that reference peaks (DMSO of all measurements as well as the single compounds) match with each other and have the same chemical shift. If differences are observed, correct them by using the serial processing option from TopSpin.

4.4.2.13.   The serial processing option is under the **Process** tab under **Advanced**. It applies changes to all selected spectra from a dataset. This way, Spectypes can easily be assigned to experiment numbers and all spectra can be shifted at once to align with the reference.

4.4.3. ^{19}F Screening

4.4.3.1.   For the first analysis of the ^{19}F mixtures, create an integration file for each mix. To define the integration region, click on the **Integrate** function in the **Analyse** tab. Make sure that for every fragment in the mixture a clear integration region for the corresponding ^{19}F-singal is defined.

4.4.3.2.   Use the **Save/Export integration regions** button to export the integration-file for future use. Save any used integration-files in C:\Bruker\TopSpin4.0.9\exp\stan\nmr\lists\intrng, or the corresponding path of the TopSpin installation directory.

4.4.3.3.   For ^{19}F data, open a dataset either with or without the investigated target.

4.4.3.4.   To load the integration-file into the current spectrum, open the **Analyse** tab again, go to **Integrate** and using the **Read/Import integration regions** button, load the corresponding integration-file. This will load any defined regions of that file into the current spectrum.

4.4.3.5.   Save and return to find a list of all integrated regions in the **Integrals** tab. Copy this into a spreadsheet or any other tool used for the further analysis of the data.

4.4.3.6.   Repeat this procedure for every mix, with and without target.

4.4.4. Data Management

4.4.4.1.   For ease of use and productivities sake, have a uniform work-flow set up for the further analysis and storage of the acquired data. For both the ^{1}H and the ^{19}F screening, use a specifically designed spreadsheet for each.

591
592 NOTE: For the ^{1}H screening this was purely used for data management and to sum-
593 marize each target while for the ^{19}F screening it used the in chapter 0 explained quo-
594 tient to automatically label each fragment as hit/no hit after the integral data was cop-
595 ied into it. This reduces the risk of human error during the analysis, assuming the file
596 was set up properly, and makes sharing of information easier, as all the important
597 information is gathered in one place in a file that can be opened by virtually anyone
598 without the need of further programs for taking an initial look at the data.
599
600 **REPRESENTATIVE RESULTS:**
601 **Quality control of fragment library**
602 The fragments from the in-house library were delivered as 50 mM stock solutions in
603 90% d6-DMSO and 10% D_2O (10% of D_2O ensures minimization of compound degra-
604 dation due to repeated freeze-thaw cycles[14]). Single compound samples consisted of
605 1 mM ligand in 50 mM phosphate buffer (25 mM KPi pH 6.2 + 50 mM KCl + 5 mM
606 $MgCl_2$), pH 6.0 in 90% H_2O/9% D_2O/1% d6-DMSO. ^{1}H-NMR experiments of fragments
607 from the iNEXT library were measured on a 500/600 NMR spectrometer. This data
608 was further used for identifying the single compounds in ^{1}H screening campaigns us-
609 ing the CMC-q software which allows the user to fully acquire spectra in an automated
610 manner and the analysis addon CMC-a the quality (solubility and integrity) of frag-
611 ments was assessed. The results from the automated analysis from CMC-a are shown
612 as a graphical output similar to what is represented in **Figure 3**. The graphical output
613 shows a representation of a 96-well plate. A red colored circle means that this frag-
614 ment shows inconsistency in structure or concentration. Green colored wells indicate
615 that the fragment is consistent.
616
617 [Place **Figure 3** here]
618
619 Approximately, 65% and 35% of the fragments were classified as consistent and in-
620 consistent, respectively, in both DMSO and buffer. Further, 30% of the inconsistent
621 classified ligands turned consistent after a careful manual inspection of the spectra[9].
622
623 **^{19}F Mixture design**
624 103 fragments containing one or several fluorine groups from the in-house library were
625 divided into 5 mixes (A, B, C, D, E). Each mix has 20 to 21 fragments. In this case the
626 mixtures had to be carefully designed to avoid signal overlap. ^{19}F transverse relaxation
627 experiments were measured for each mixture that apply CPMG pulse trains. These
628 experiments can be modified by varying the relaxation delays. The ^{19}F chemical shift
629 of mixes A-E can be seen in **Figure 4**.
630
631 [Place **Figure 4** here]
632
633 **Sample preparation**
634 The sample preparation in the ^{19}F screening procedure was either done manually or
635 with automated pipetting using a pipetting robot. The fragments in each mixture had a
636 concentration of 2.5 mM in 90% d6-DMSO and 10% D_2O. The final volume of a
637 screening sample was 170 µL with 5% D_2O as a locking agent. Each mixture was
638 pipetted two times, one in a buffer containing solution (without target) and one into a
639 target containing buffer solution. The ratio of target and fragment was set to 1:1, re-
640 sulting in a final target/ligand concentration of 50 µM. Additionally control samples are

641 the target biomolecule in screening buffer without a mixture to ensure target integrity
642 as well as a control sample with only buffer and D_2O to ensure buffer quality.
643
644 NMR screening data of ^{19}F-1D and ^{19}F-CPMG-T_2 were measurements as described in
645 section 3.1. For example, in the case of RNA a jump-return echo sequence (pp =
646 zggpjrse, [15]) was acquired for the single target sample in buffer.
647

Data Analysis
649 The ^{19}F screening procedure was applied to the TPP riboswitch thiM from *E. coli* and
650 protein tyrosine kinase (PtkA) from *M. tuberculosis* among several other targets[16]. The
651 ^{19}F screening library has 103 fragments that are divided into 5 Mixes labelled from Mix
652 A to E. Preparation of screening samples can be performed manually without the use
653 of a sample pipetting robot. 40 µM thiM RNA containing solution (buffer conditions)
654 was mixed with 3.2 µL from the mixtures. Further control samples were prepared con-
655 sisting of buffer only, buffer with 5% of DMSO (previously ensure the stability of the
656 biomacromolecule in the presence of the desired DMSO concentration) and buffer with
657 RNA. These 13 screening samples were prepared and transferred to 3 mm NMR-
658 tubes. Barcodes of NMR tubes are scanned and each mixture in the presence and
659 absence of RNA, as well as control samples were measured according to the afore-
660 mentioned ^{19}F NMR experiments performed at 298 K. Screening of thiM RNA against
661 the in-house library was performed by conducting T_2 measurements with CPMGs of 0
662 ms and 200 ms for each different sample. Proper shimming and water suppression
663 were monitored after finishing the measurements by comparing all DMSO peaks in
664 terms of line broadening and intensity loss of additionally measured ^1H 1D experi-
665 ments for all samples. Processing of obtained CPMG T_2 ^{19}F relaxation spectra was
666 performed using a previously prepared and automated macro in TopSpin, respectively.
667 Data analysis was performed following the instructions in the protocol section. The
668 integral data obtained from TopSpin (following the instructions in the protocol) can be
669 evaluated quickly and easily using a pre-made spreadsheet or any similar program,
670 by setting the correct conditions and thresholds. As described previously, thresholds
671 are useful in defining binder, weak binder, or non-binder. **Figure 5** shows typical re-
672 sults of CPMG spectra of thiM RNA and PtkA, respectively. In some cases, further
673 expert revision was needed.
674
675 [Place **Figure 5** here]
676

^1H Screening
Mixture design
679 The used in-house library is so diverse that for ^1H screening purposes no mixture de-
680 sign was performed. This means that 64 mixes were prepared by randomly choosing
681 12 to be mixing in one mixture.
682

Sample Preparation
684 For the ^1H screening of an exemplary SARS-CoV-2 RNA, automated pipetting using
685 a pipetting robot was performed to prepare the samples. The fragments in each mix-
686 ture had a concentration of 4.2 mM in 90% d6-DMSO and 10% D_2O. The final volume
687 of a screening sample was 200 µL with 5% D_2O as a locking agent. 64 samples each
688 containing a different mixture in 25 mM KPi, 50 mM KCl at pH 6.2 were pipetted with-

689 out target RNA. Respectively, 64 samples were pipetted with target RNA, each con-
690 taining a different mixture. The RNA:Ligand ratio was set to 1:20, resulting in an RNA
691 concentration of 10 µM and a ligand concentration of 200 µM.
692
693 **Data Analysis**
694 For the ^{1}H analysis, the FBS tool in TopSpin was used. To determine if a fragment is
695 a hit, 1D chemical shift, waterLOGSY, and T_2 relaxation experiments were conducted.
696 For T_2 relaxation, a decrease in intensity greater than 30% was counted as a hit, while
697 for the chemical shift a shift of greater than 6 Hz was the cut-off. The waterLOGSY
698 had to show a significant signal change (from negative to positive in this case). If any
699 two of these three criteria were positive, a fragment was counted as a hit. Two exam-
700 ples for this can be seen in **Figure 6**.
701
702 [Place **Figure 6** here]
703
704 Hit-1 shows a T_2 decrease of ~50% and a CSP ≥ 6 Hz. The waterLOGSY does not
705 show a significant enough change in signal to also be counted as positive. As two out
706 of three experiments are positive, this fragment is counted as a hit. For Hit-2, the T_2
707 shows a decrease of ~80% signal intensity and a clear signal change can be seen for
708 the waterLOGSY. The CSP is not enough in this case, but as the two previous criteria
709 are positive it is still counted as a hit.
710
711 **FIGURE LEGENDS:**
712 **Figure 1**: (**A**) High-throughput NMR sample preparation and NMR-tube filling robot
713 installed at BMRZ. (**B**) High-throughput sample changer with individual temperature-
714 controlled racks installed on a 600 MHz spectrometer at the BMRZ facility.
715
716 **Figure 2**: **Hit identification for the ^{19}F screening.** Section of ^{19}F CPMG NMR spectra
717 of an exemplary compound. This pictorial representation explains the properties of a
718 binder. ^{19}F-CPMG spectra of a compound acquired of mixture samples in the presence
719 and absence of RNA. The values represent the normed integral values of the corre-
720 sponding peak.
721
722 **Figure 3**: **Quality control of fragment library.** Schematic representation of CMC-a
723 based automated output. Fragment properties such as concentration and structural
724 integrity are assessed. Green stands for consistent, orange in this case stands for
725 inconsistent. Inconsistent fragments are revised manually following the shown work-
726 flow.
727
728 **Figure 4: ^{19}F 1D-NMR spectra of mixture samples from the in-house library.**
729
730 **Figure 5**: **Cut out of ^{19}F CPMG NMR spectra showing the intensity changes ob-**
731 **tained from different delay times of CPMG based experiments.** (A) Representation
732 of a binder (hit) and a non-binder in ^{19}F fragment-based screening performed on TPP
733 riboswitch thiM RNA from *E. coli*. (B) Representation of a binder and a non-binder in
734 ^{19}F fragment-based screening performed on PtkA from *M. tuberculosis*.
735
736 **Figure 6**: **^{1}H screening performed on an exemplary SARS-CoV-2 RNA showing**
737 **hit determination criteria.** Acquisition of three different experiments (^{1}H T_2 CPMG
738 (5/100 ms), waterLOGSY, and 1D ^{1}H).

DISCUSSION:

Versatility of the NMR-based fragment/drug screening. BMRZ has successfully implemented state-of-the-art automated NMR instrumentation as well as STD-NMR, waterLOGSY and relaxation experiments to identify fragments within a wide range of affinity regime for drug discovery. The installed hardware includes a high-throughput sample preparation robot and high-throughput sample storage, changer and data acquisition unit associated to a 600 MHz spectrometer. A recently purchased cryogenic probe for ^{1}H, ^{19}F, ^{13}C and ^{15}N ensures the required sensitivity for the proposed measurements and allows ^{1}H decoupling during ^{19}F detection. This probe is connected to the latest generation of NMR console that offers the possibility to use the advanced software tools from Bruker, including CMC-q, CMC-assist, CMC-se and FBS (included in TopSpin). The fragment-based screening (FBS) tool is included in the latest version of TopSpin and helps to analyse the high-throughput data comprising of STD, water-LOGSY, T_2/$T_{1\rho}$-relaxation experiments. The liquid sample collection can be filled into the NMR-tubes in an automated manner by using the sample filling robot. Typically, a block of 96 tubes (3 mm) are filled in approximately two hours. The 96-well-plate-racks are directly positioned in the HT sample changer, which reads the barcode of the block and assigns the NMR tubes to the experiments controlled by the automation software (*IconNMR*). Five 96-well-plate-racks can be stored and programmed in the HT sample changer at the same time. The temperature of each of the individual racks can be controlled and regulated separately. Additionally, each individual sample can be pre-conditioned (preheating and tube drying for removal of condensed humidity) to the desired temperature before the measurement.

Suitability for wide range of applications. One of the broad applications of this automated NMR-based screening is to identify and develop novel ligands binding to a biomacromolecular target (DNA/RNA/Proteins). These ligands can include orthosteric and allosteric inhibitors that typically bind non-covalently. Further, FBDD by NMR is typically used as a first step to select promising compounds, the requirements to be met are availability of the biomolecular target in sufficient quantities. This objective is divided into two major tasks.

Task one is to develop and characterize an in-house fragment library for the following reasons: initial and periodic quality control, characterization, and quantification of more than 1000 fragments; determination of solubility of the fragments in buffers optimized for each target, in particular for protein targets; and the establishment of several libraries to accommodate diverse scaffolds and extending towards other macromolecule classes. Task two is to integrate workflows for fragment-based drug design (FBDD) by NMR using: automated 1D-ligand observed screening (^{1}H and ^{19}F observed); automated replacement assays (competition experiments with (natural) ligand) to differentiate orthosteric and allosteric binding; automated secondary screenings with multiple fragments; automated 2D-protein screening, and secondary screening of a set of derivatives around an initial hit making use of the EU-OPENSCREEN library or any other library; and re-profiling screening of FDA-library against the chosen targets.

Additionally, metabotyping of various cell lines (disease relevant) can be conducted in order to unravel the regulatory mechanisms that link cell cycle control and metabolism. Also, there is functional characterization of RNA/DNA/protein regulation elements in vivo and in vitro for optimization of construct/domain optimization (stability optimization

789 for structural investigations (Buffer, pH, temperature, and salt screening), and an ex-
790 tension of NMR-based fragment screening to membrane proteins and intrinsically dis-
791 ordered proteins, which are generally inaccessible to other techniques.
792
793 **Limitations.** Use of ^{19}F and ^{1}H fragments libraries have their pros and cons, few of
794 which will be mentioned in the following. The largest benefit of ^{19}F versus ^{1}H meas-
795 urements is the speed of both the actual measuring time and the subsequent analysis,
796 as the mixtures contain almost double the number of fragments and fewer experiments
797 must be conducted. The follow up analysis is also easier for ^{19}F screening, as there is
798 no interference from buffers and additionally offers a broader chemical shift range with
799 almost no signal overlap for an optimally designed fragment mixture. The spectra
800 themselves are greatly simplified, usually only having one or two signals per fragment,
801 depending on the number of fluorine atoms. The analysis of these spectra can there-
802 fore be automated, again cutting down on time. This comes at the cost of chemical
803 diversity, at least for the library used in this study. As only ~13% of the library contains
804 ^{19}F, but naturally all of them are useable in ^{1}H screening, the diversity of the ^{19}F screen-
805 ing fragments will be lower. This could be circumvented using specifically designed
806 ^{19}F libraries with more fragments and bigger chemical diversity. Another disadvantage
807 for ^{19}F screening is the low number of signals per fragment. Fragments generally are
808 composed of more than one hydrogen atom. Therefore, ^{1}H observed screening exper-
809 iments can rely on different signals for the same fragment for detecting binding. This
810 gives a higher degree of confidence when identifying hits for the ^{1}H screening,
811 whereas the ^{19}F screening must rely on the one or two signals given per fragment.
812
813 A detailed account on the modern automated NMR-based fragment screening instru-
814 mentation, software and analysis methods and protocols thereof has been presented.
815 The installed hardware includes a high-throughput sample preparation robot and a
816 high-throughput sample storage, changer and data acquisition unit associated to a
817 600 MHz spectrometer. A recently installed cryogenic probe head for ^{1}H, ^{19}F, ^{13}C and
818 ^{15}N ensures the required sensitivity for the proposed measurements and allows ^{1}H
819 decoupling during ^{19}F detection. Further, the latest generation of NMR console offers
820 the possibility to use advanced analytical software for aiding acquisition and on-the-fly
821 analysis. The above discussed technology, workflows, and the described protocols
822 should foster remarkable success to users pursuing FBS by NMR.
823

829

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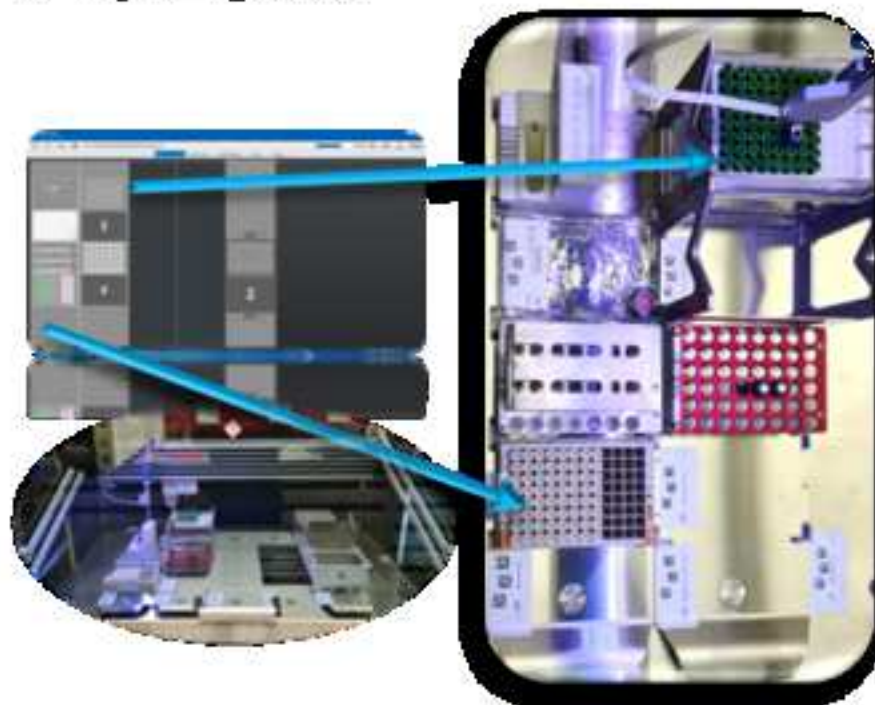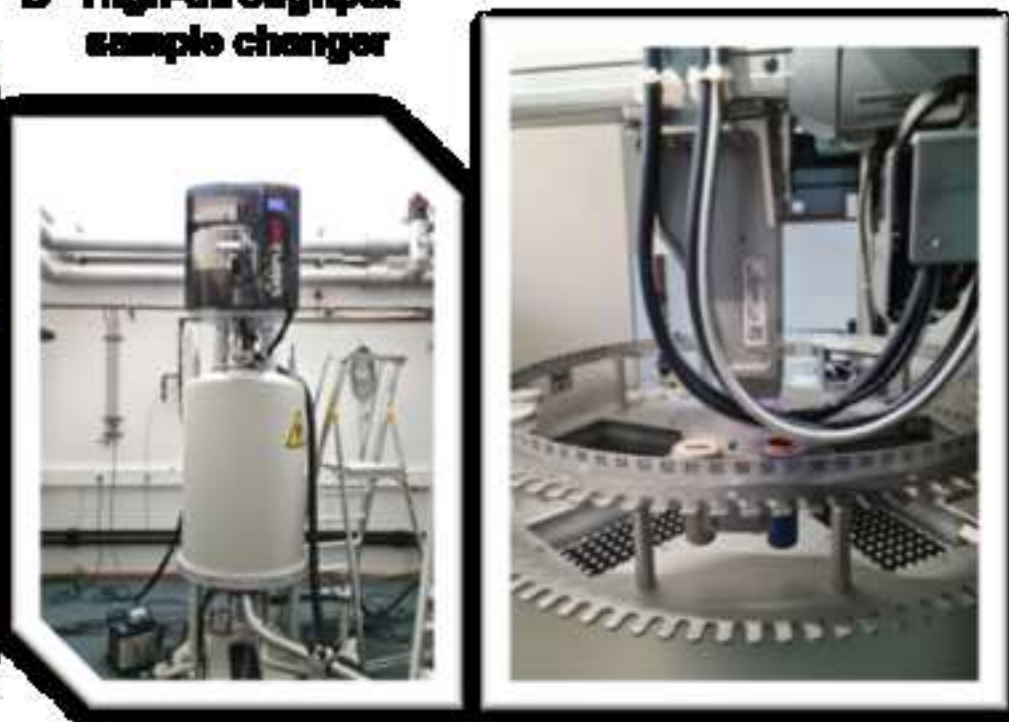873   cbic.202000476 (2020).
874

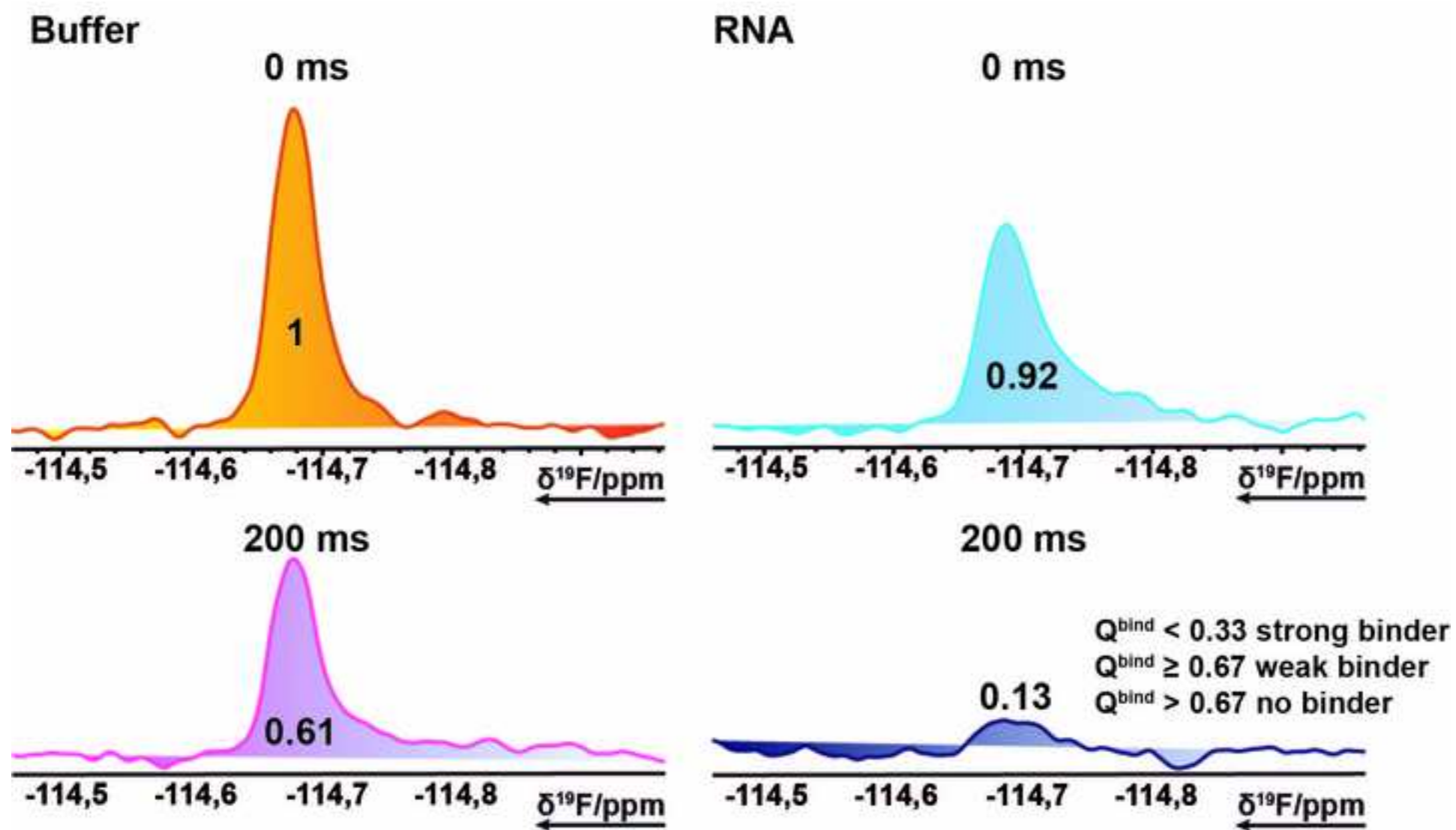Figure 1

A Pipetting robot

B High-throughput sample changer

Figure 2

Figure 3

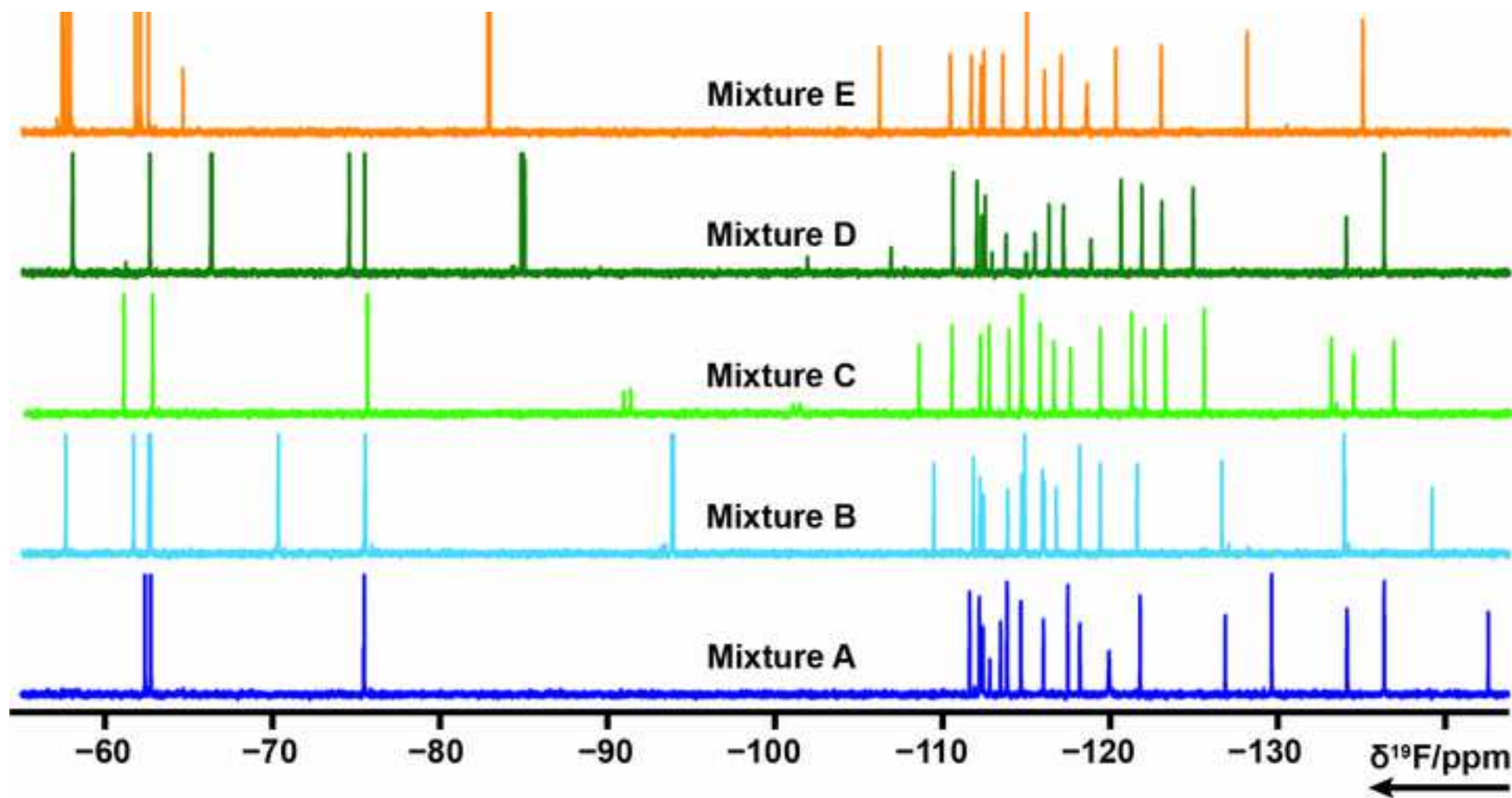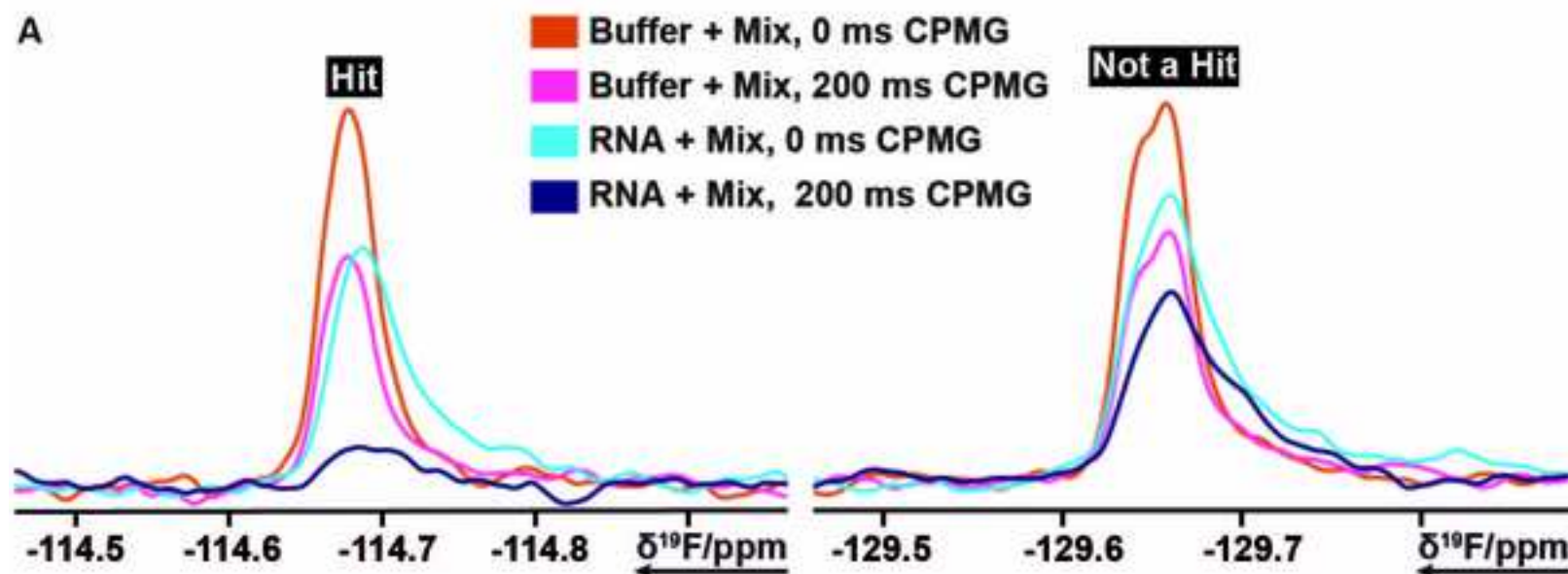Click here to access/download;Figure;Figure 3.jpg ⤓

CMCA-based auto analysis

Inconsistent (auto)

Manual intervention

compare DMSO and buffer spectra

-same compound in two wells
-too little stock compound
-degradation
-inconsistent with structure
-large % impurities

not similar (impurity)

Similar

insoluble

soluble

estimate impurity (integrals)

< 15 %

consistent

disregard compound

Figure 4

Mixture E

Mixture D

Mixture C

Mixture B

Mixture A

−60 −70 −80 −90 −100 −110 −120 −130 δ¹⁹F/ppm

Figure 5

Click here to access/download;Figure;Figure 5.jpg

RNA
TPP riboswitch aptamer domain (80 nt)

A

Hit

Not a Hit

Buffer + Mix, 0 ms CPMG
Buffer + Mix, 200 ms CPMG
RNA + Mix, 0 ms CPMG
RNA + Mix, 200 ms CPMG

-114.5 -114.6 -114.7 -114.8 δ^{19}F/ppm

-129.5 -129.6 -129.7 δ^{19}F/ppm

Protein
PtkA

B

Not a Hit

Hit

Hit

Buffer + Mix, 0 ms CPMG
Buffer + Mix, 200 ms CPMG
Protein + Mix, 0 ms CPMG
Protien + Mix, 200 ms CPMG

-114.4 -114.6 -114.8 δ^{19}F/ppm

-133.9 -134.0 -134.1 -134.2 δ^{19}F/ppm

Figure 6

Click here to access/download;Figure;Figure 6.jpg ⬇

Hit = at leat 2 experiments satisfied

① T2 ≥ 30% decrease ② waterLOGSY positive ③ CSP ≥ 6 Hz

Material	Catalog Number	Manufacturer	Comments
Bruker Avance III HD		Bruker	600 MHz NMR Spectrometer
Matrix Clear Polypropylene 2D Barcoded Open-Top Storage Tubes	3731-11 0.75ML V-BOTTOM TUBE/LATCH RACK	ThermoFisher Scientific	Barcoded Tubes
Matrix SepraSeal und DuraSeal	4463 Cap Mat, SeptraSeal 10/CS	ThermoFisher Scientific	
SampleJet		Bruker	HT Sample Changer
SamplePro Tube		Bruker	Pipetting Robot

Dear Editors, Dear Reviewers,

Thank you for the very careful review of our manuscript and for further constructive suggestions to improve its quality. We have addressed all your suggestions and concerns. Please see below our detailed point-by-point response to all reviewer comments for your reviewing process. We hope this revised version meets your expectations and we are looking forward to your response.

Sincerely yours,

Harald Schwalbe

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

-Done to the best of our knowledge

2. Please revise the following lines to avoid previously published work: 304-307, 498-501.

-We have now re-phrased

3. Please provide the complete address of all the affiliations.

-Corrected

4. Please provide an institutional email address for each author.

-Corrected

5. Please ensure that the summary clearly describes the protocol and its applications (10-50 words). Please consider removing the lines 27-30.

- Corrected

6. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

- Corrected

7. Please define all abbreviations before use (STD, CPMG)

- Corrected

8. Please ensure that the corresponding reference numbers appear as numbered superscripts after the appropriate statement(s) for in-text formatting.

- Corrected

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

-Corrected

10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials (SamplePro Tube, Matrix, ThermoFisher Scientific, Eppendorf, SampleJet, Prodigy, TopSpin, etc.)

- Corrected

11. Please include a one line space between each protocol step.

- Corrected

12. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

-We have highlighted the relevant section

13. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

-

14. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

- Corrected

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

-Corrected

16. Figure 1: Please remove trademark (™) and registered (®) symbols from the figure (SamplePro Tube, SampleJet). Please use generic terms to label the figure.

- Corrected

17. Figure 2: Please Label each figure in the panel. What do the numbers (1, 0.92, 0.61, 0.13) represent? Please specify them in the figure or the Figure Legend.
- Corrected

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors described the protocol of automated NMR fragment-based screening. It will guide more scientists to identify hits against various targets. The following minor concerns should be addressed.

Minor Concerns:
L97-99: the fragment library should adhere to RO3 but not Lipinski RO5.

-is appropriately mentioned in the text:

"These diverse fragment libraries mainly contain flat aromatic or heteroatoms and adhere to the Lipinski Rule of 5 [6], or to the current commercial trend Rule of 3 [7], but avoid reactive groups."

L162: Format error should be revised.

-Correct as best as possible

L191-194: Why 10% D2O is used in the stock solution? First, DMSO is chemically inactive; Second, 10% D2O may lead to a solubility issue.

-explanation included in the text "*10% of D_2O ensures minimization of compound degradation due to repeated freeze-thaw cycles [12]*"

L207-208: it seems that 5% DMSO is introduced in the final screening buffer, and it is worth noting that not all biomolecules remain stable in such a concentration. Thus higher stock concentration and thus lower DMSO concentration in the final screening sample may help.

-suggestion noted

L281: The experimental time is 1-5 hours? If it is 5 hours/sample, the total screening time would be really long. In such case, the long-term stability of the biomolecules should be taken into account.

-suggestion noted

L288: at 5% DMSO, do the authors some times observe non-specific binding of DMSO? (no?)

-no

Previous work on automated NMR FBS should be cited, e.g., C. Peng et al., J. Med. Chem. 2016; J. Gao et al., PLoS One 2014, 9:e88098

-has been appropriately cited

Figure 5: STD spectra should be included to better evaluate the performance of this NMR FBS protocol.

-Figure 5 focuses only on the ^{19}F-detected relaxation experiments for identifying binders

Reviewer #2:

Manuscript Summary:

The manuscript describes fragment screening for proteins and RNA. This will be of interest to other scientists.

I have no background in NMR screening, but it appears that the methods are described in a way that they can be reproduced by others.

Major Concerns:

The manuscript appears to be rather an unfinished draft than the final version. Text for one section and information about the instrumentation is lacking. Some section headers do not fit to the following text and the language needs improvement.

It is clear that the introduction will not be a comprehensive review of fragment screening, but some references to key papers and reviews would nevertheless be very useful for readers less experienced with the topic.

Minor Concerns:

Fragment screening by X-ray crystallography should be added to the list of alternative screening methods mentioned in the introduction.

-included:

"*Four commonly applied biophysical methods within the drug discovery campaigns are thermal shift assays, surface plasmon resonance (SPR), crystallography and nuclear magnetic resonance spectroscopy (NMR).*"

Reviewer #3:

Manuscript Summary:
This well-written manuscript by Berg and coworkers reported an automated protocol for NMR-based screening process. The reviewer found this work would fit into JoVE with significant value to the field of FBDD. The detailed comments are as following:

Major Concerns:
1. The references, especially in the introduction section, were light. The authors should include more citations regarding the important development in the fields of FBDD and NMR-based screening.

-
Minor Concerns:
2. Page 4, line 59 "<250 Da" should be "< 250 Da).

-Corrected
3. Page 4, line 122 "STD-NMR, waterLOGSY and CPMG-based" should be defined.

- Corrected
4. Page 7, line 162, the section "2020),Chapter Error!" should be corrected.

- Corrected
5. Line 176 "40oC" should be "40 oC)

- Corrected
6. Line 179, the authors left one bullet point highlighted, which should be corrected.

- Corrected
7. Line 196, "96 well" should be "96-well".

- Corrected
8. Line 202, the authors left one bullet point highlighted, which should be corrected.

- Corrected
9. Line 273 WLOGSY is used instead of waterLOGSY. The authors should keep their abbreviations consistent.

- Corrected
10. Line 306 "a 1H-NMR" should be "an 1H-NMR".

-
11. Line 334, the authors left one bullet point highlighted, which should be corrected.

- Corrected
12. Line 386, "200ms" should be "200 ms".

- Corrected
13. Line 408, the authors left one bullet point highlighted, which should be corrected.

- Corrected
14. Line 456, the authors left one bullet point highlighted, which should be corrected.

- Corrected
15. Line 485, "D2O" should be "D2O".

- Corrected
16. Line 486, "MgCl2" should be MgCl2.

- Corrected
17. Line 557 "25mM" should be 25 mM.

- Corrected

Overall, this is a good manuscript. I recommend publication of this work in JoVE after addressing the listed concerns.