Practical Considerations and Guidelines for Spectral Referencing for Fluorine NMR Ligand Screening

Yann Ayotte, Simon Woo, and Steven R. LaPlante*

ABSTRACT: Fluorine (19F) NMR strategies are increasingly being employed for evaluating ligand binding to macromolecules, among many other uses. 19F NMR offers many advantages as a result of its sensitive spin 1/2 nucleus, 100% natural abundance, and wide chemical shift range. Moreover, because of its absence from biological samples, one can directly monitor ligand binding without background interference from the macromolecule. Therefore, all these aforementioned features make it an attractive approach for screening compounds. However, the detection of ligand binding, especially those with weak affinities, can require interpretations of minor changes in chemical shifts. Thus, chemical shift referencing is critical for accurate measurements and interpretations. Unfortunately, one cannot rely on spectrometer indirect referencing alone, and internal chemical references have sample-dependent issues. Here, we evaluated 10 potential candidate compounds that could serve as 19F NMR chemical references. Multiple factors were systematically evaluated for each candidate to monitor the suitability for 19F NMR screening purposes. These factors include aqueous solubility, buffer compatibility, salt compatibility, aqueous stability, tolerability to pH changes, temperature changes, and compound pooling. It was concluded that there was no ideal candidate, but five compounds had properties that met the screening requirements.

INTRODUCTION

Fragment-based lead discovery (FBLD) involves the screening of low-molecular-weight compounds to identify binders to essential disease target proteins or nucleic acids. Nuclear magnetic resonance (NMR) is a very useful tool for FBLD due to its ability to detect weak binding events in a label- and immobilization-free environment. Traditional ligand-detected experiments have mostly been performed using proton (1H) NMR, but fluorine (19F) experiments have increasingly gained in popularity in recent years. The large, background-free chemical shift dispersion of the fluorine moiety, combined with its 100% natural abundance and high sensitivity to molecular interactions, has made it an attractive tool in the field of drug discovery.1−17

Binding events are usually detected by monitoring changes in chemical shifts, signal width, and/or peak intensities. Variation in the chemical shift is expected to occur if a difference between the bound and free states is experienced by the 19F nucleus.18 However, these observations can be skewed, as minor but significant changes in chemical shifts are common due to the wide spectral dispersion of the 19F nucleus coupled with spectrometer instabilities and sample-dependent shift changes. Thus, one cannot always rely on spectrometer indirect referencing alone.

In general, the IUPAC recommendations favor internal referencing or substitution methods,19 with internal referencing being generally more practical for a drug screening context. The IUPAC also recommends the use of CCl3F as a reference compound for 19F NMR, but this molecule presents several practical limitations: it has limited aqueous solubility, is highly volatile at ambient temperature, and possesses ozone-depleting properties, which restricts its commercial availability.20 Analogously, currently recommended 19F quantitative NMR references are intended to be used in organic solvents and are less optimal for screening, as their aqueous solubility is also limited.21 Therefore, there are no definitive guidelines for 19F NMR screening, and the choice of reference depends on lab-specific preferences or arbitrary reasons.1

Some essential characteristics should be considered in the choice of a 19F NMR shift reference, keeping in mind that requirements can be project-dependent. These characteristics include solubility and stability in aqueous media, compatibility with common buffer components, absence of promiscuous binding to protein systems, chemical shift compatibility with standard NMR experimental screening parameters, and minimal chemical shift changes from variations in sample...
RESULTS AND DISCUSSION

Example: Inconclusive Results in the Absence of an Internal Shift Reference. As with routine 1H NMR applications, spectrometer indirect referencing is often preferred for 19F NMR shift calibration. However, inconsistencies can arise from one sample to the next due to the reasons already described above.

One ramification is that interpretations can become ambiguous. An example is illustrated in Figure 1, which shows a 19F NMR screen aimed at determining whether two compounds bind to a target protein. In panel A, the differential line broadening/shifting (DLBS) method would suggest that compound 1 could bind to the target protein given that the 19F spectrum of free compound 1 (blue spectrum) experiences a distinct change in chemical shift upon the addition of target protein (red spectrum). However, a confirmational T2-CPMG (Carr–Purcell–Meiboom–Gill) experiment shows that compound 1 does not appear to bind the target protein.22 On the other hand, no significant changes in relaxation rates (i.e., slopes as a function of delay periods) are observed between the samples containing free 1 (blue) and 1 with added protein (red). On the other hand, in panel B the changes in DLBS and T2-CPMG data (red vs blue spectra) support that compound 2 indeed binds to the target protein. Thus, perhaps the chemical shift changes in panel A were due to either spectrometer drift or local chemical environment changes rather than a binding event. The lack of a significant difference in the T2-CPMG spectra in the presence of protein supports this hypothesis; therefore, an analysis based on shift alone could result in the misinterpretation that compound 1 bound to the target protein. Hence, it is crucial that a 19F chemical shift reference be employed in such a context.

Selection of 19F Reference Compounds. A set of commonly used 19F references was chosen for this study. Their names and abbreviations are as follows: BTF: benzotrifluoride; DBF: 1,2-difluorobenzene; HFB: hexafluorobenzene; KF: potassium fluoride; NaBF₄: sodium tetrafluoroborate; PhF: fluorobenzene; TFA: trifluoroacetic acid; TFE: trifluoroethanol; TF MBA: 2-(trifluoromethyl)benzoic acid; Triflate: sodium trifluoromethanesulfonate.

Solubility Tests. A chemical shift reference should exhibit sufficient solubility under aqueous conditions to be practical for NMR screening. The solubility of the 10 reference candidates was therefore assessed at 200 μM nominal concentration in 100% D₂O. Data were also acquired on samples at 200 μM nominal concentration in 100% DMSO-d₆ for comparisons of signal intensities. Figure 2 illustrates the respective signal intensities, as well as the overlay spectra (false-right column), in D₂O and DMSO-d₆ for each molecule. PhF was completely insoluble in D₂O, while BTF, DFB, and HFB exhibited poor signal-to-noise in aqueous solution. These four candidates were therefore eliminated from further testing due
to their insufficient solubility and, thus, incompatibility as a 19F NMR screening reference. Even though it appeared to be insoluble in DMSO, KF showed sufficient signal-to-noise under aqueous conditions and was therefore advanced to further evaluations. Similarly, TFA showed a lower signal intensity in DMSO as well as a broader, nonsymmetrical peak line shape, which could perhaps be due to an exchange phenomenon. Given an acceptable intensity profile in D2O, TFA was also considered for additional studies. Finally, NaBF4, TFE, TFMBA, and Triflate all showed adequate solubility profiles and were also selected for the next rounds of testing.

**Buffer Compatibility Tests.** Another important attribute for a fluorine reference compound should be compatibility with various buffer conditions. The remaining six reference candidates were then evaluated for their compatibility with four common buffer components: sodium phosphate, potassium phosphate, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), and tris(hydroxymethyl)-aminomethane (tris). An analysis of the 19F NMR spectra of the six candidates in these buffers is shown in Figure 3. Given the reasonable peak intensities in all conditions, it is apparent that all six are compatible with these buffers.

**Salt Compatibility Tests.** For screening purposes, a fluorine reference should also be compatible with various salts. The compatibility with four commonly used salts (sodium chloride, potassium chloride, magnesium chloride, and calcium chloride) was then assessed as shown in Figure 4. With the exception of KF, all compounds were compatible with the four salts investigated. However, KF exhibited insolubility in the presence of magnesium chloride. This effect was further confirmed by titrating KF against a lower concentration (1 mM) of MgCl2 (Figure S1), and significant loss of signal intensity and broad line widths could be observed, suggesting the formation of larger unknown entities in the sample. In a similar fashion, reduced signal intensity with a wider line shape was observed for KF in the presence of calcium chloride (Figure 4), which suggested limited compatibility with this salt as well. This is in line with the relatively limited solubility expected for MgF2 and CaF2.23a,b KF was therefore eliminated as a potential reference candidate. The remaining five molecules were then tested against a wide range of buffer components and additives (Figures S2−S5), and no significant incompatibility could be observed in any of the tested conditions.

**Aqueous Stability Tests.** Given that some NMR screening studies can last for several days, the aqueous stability of each remaining candidate was then assessed at three different time points: 0 h, 24 h, and one week. Figure 5A shows that signal intensities remain relatively stable across all time points for the five compounds. Similarly, minor variations in chemical shift of less than 1 Hz were noted for periods up to a week (Figure 5B).

**pH, %DMSO, Temperature, and Compound Pooling Tests.** We then evaluated the “sensitivity” of the compounds
chemical shifts given variations in pH, amount of DMSO, temperature, and in the presence of pools of other compounds. Figure 6A shows that the chemical shift of NaBF4 seems to be relatively sensitive to pH variations above pH values of 6 but that all the other molecules were rather stable across the pH range tested. Therefore, small variations in pH upon additions of various components, such as a protein or other compound, would only be expected to potentially cause more significant variations in the chemical shift of NaBF4.

Similarly, because compounds are usually added into a buffer from DMSO solvent stocks, there was concern that chemical shifts of the potential reference compounds could be sensitive to different concentrations of DMSO (e.g., for drug titration purposes, from different stock concentrations, or even variations in pipetting). Thus, we were interested in evaluating if the reference candidates experienced changes in chemical shifts as the percentage of DMSO was altered. Figure 6B shows the changes in chemical shifts upon addition of 1, 2, 3, or 5% (v/v) DMSO-d6. NaBF4 was once again more influenced by variations in the amounts of DMSO.

Although temperature is usually well-controlled during NMR experiments, some NMR pulse sequences can result in some sample heating. Moreover, insufficient equilibration of the sample temperature before acquisition can also result in variation during the experiment. The influence of temperature fluctuation on the reference’s chemical shifts was therefore probed by varying the temperature from 25 to 40 °C by 5 °C increments (Figure 6C). Most of the compounds show comparable changes in chemical shifts with varying temperature, but, interestingly, the chemical shift of NaBF4 was the least affected by changes in temperature.

Fragment-based NMR screens are often performed using mixtures of compounds (pools) in order to increase throughput, so an internal reference used in these screens should experience minimal effects on its chemical shift in the presence of other compounds. Each reference candidate was therefore tested in 20 different pools of small-molecule fragments containing 11–15 fragments per pool. Figure 6D shows the average change in chemical shift for each reference candidate when placed in the pools compared to the free compounds. It is noteworthy that NaBF4’s chemical shift is, on average, more affected, while triflate’s is the least affected. TFE, TFMA, and TFA exhibit comparable intermediate profiles. In light of the results observed in Figure 6A, the presence of multiple compounds in solution may induce slight changes in pH within the samples, and these changes could explain some of the observed effects. These results suggest that NaBF4

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**Figure 4.** Compatibility of the six candidates with four common salts. 1D 19F spectra are shown for compounds in each condition. Sodium and potassium chloride were tested at 200 mM, while magnesium and calcium chloride were tested at 50 mM. Compounds were tested at a nominal concentration of 200 μM, and solutions were prepared with 10% D2O.

**Figure 5.** Evaluation of compound stability under aqueous conditions. 1D 19F spectra were acquired at three time points: 0 h, 24 h, and one week. Signal intensities were measured at each time point (A) as well as variation in the 19F chemical shift between each time point (B). Signal intensities in (A) were normalized to t = 0 h for each molecule. Samples were measured at 200 μM in 50 mM sodium phosphate pH 7.4, 100 mM NaCl, 10% D2O.
would be a less-favorable option in the context of screening mixtures.

Ideally, an internal reference should not bind to the target macromolecule (usually a protein) that is being screened. Therefore, we screened the five remaining candidates against four different commercially available protein systems: bovine serum albumin (BSA), elastase, lysozyme, and trypsin. Binding was assessed at three different ligand-to-protein ratios (L/P) by monitoring changes in the $^{19}$F differential line broadening (DLB) of each reference’s signal upon addition of protein. Figure 7A shows that the addition of elastase, lysozyme, or trypsin results in little to no DLB effects under the various L/P tested. However, the addition of BSA results in relatively large DLB effects for TFA, triflate, and especially TFMBA (illustrated in Figure 7B) as the ligand-to-protein ratio approaches equimolar. Note that this is not unexpected considering that albumins are known to bind a wide variety of molecules. However, this observation suggests that these reference candidates may be more prone to binding target macromolecules. Interestingly, NaBF$_4$ exhibited minimal DLB across all the conditions tested, suggesting that it might be less susceptible to protein binding.

**Summary of the Main Pros and Cons for the Five $^{19}$F NMR References.** This study has shown that the evaluation of fluorine reference compounds for NMR screening must consider multiple parameters (Figure 8) and as a result is challenging. Overall, five reasonable candidate references emanated from this study. Table 1 summarizes the main pros and cons for each of these five references. Interestingly, all five contained a polyfluoro moiety (either a CF$_3$ group or BF$_4^-$) giving them sufficient signal-to-noise even if used at lower concentrations for referencing in NMR screens. Moreover, all five are commercially available and affordable.

**Example Demonstration of the Utility of TFMBA for $^{19}$F Chemical Shift Referencing in a Competition Study.** Given the ensemble of data described herein, our laboratory frequently uses TFMBA as an internal reference for $^{19}$F NMR screening studies. Figure 9 demonstrates an example of such a study for which the aim was to evaluate two compounds to determine whether they bound to a specific pocket (P-1) of the multipocketed target human rhinovirus polymerase (HRV). To do so, we had access to a known P-1 binder ($^{19}$F probe—see Figure 9B,C) that could serve as a displacement probe for other binders to pocket P-1. This probe also contained a CF$_3$ group, which increased the $^{19}$F NMR signal-to-noise while also exhibiting relatively sharp linewidths due to a fast rotation along the CF$_3$–phenyl bond. Given this, samples were prepared with the internal TFMBA $^{19}$F reference at a lower concentration of 5 μM. The low concentration of both the reference and the probe ($^{19}$F probe at 22 μM) would minimize any potential intermolecular association and interference. As a precaution, separate control experiments were run and showed that the TFMBA reference did not bind to the probe, HRV protein, or to the two test
compounds (data not shown). Samples were then prepared with the three compounds present simultaneously (TFMBA at 5 μM, 19F probe at 22 μM, test compound at 100 μM), both with and without HRV protein.

Key to the proper analyses of the data shown in Figure 9 was that all 19F NMR spectra were chemical shift referenced to the TFMBA peak as shown in Figure 9A, which then facilitated accurate interpretations. Given that the 19F probe and the test compounds were relatively weak binders, resonance averaging was expected due to fast exchange between the free and bound states (on the NMR time scale). Therefore, minor chemical shift changes would be expected upon binding. Upon addition of the HRV protein, the 19F NMR resonance of the 19F probe would shift downfield to that of the free 19F probe, indicating a binding event. In the presence of a competitor test compound, however, as shown in the upper spectrum of Figure 9C, the resonance of the 19F probe returned to its free-state chemical shift, indicating that it was competed out of pocket P-1 by the competitor test compound. In contrast, in the presence of a noncompetitor test compound, as shown in the upper spectrum of Figure 9B, the chemical shift of the 19F probe remained the same as that of the sample without the noncompetitor compound. This result indicated that the 19F probe remained bound, and the noncompetitor compound did not compete for pocket P-1. Without the chemical shift referencing to TFMBA, these results would have been much less convincing.

Additional Considerations. Several factors make identification of a universal 19F internal reference very challenging. The main one being the large chemical shift distribution of 19F compounds, which may require the use of a reference with a chemical shift close to the compound(s) of interest. For example, different references might be desired if mixtures are designed based on the presence of CF or CF3, since they may require different carrier frequencies and spectral width depending on the experimental setup used. Alternatively, the use of broadband 19F pulse sequences can help circumvent this difficulty.

If users also plan to include 1H NMR experiments for follow-up steps, then some 1H-containing references will be less appropriate due to the potential for signal overlap with the compounds of interest. For example, Figure S6 shows the one-dimensional (1D) 1H spectra of TFMBA and TFE. On the one hand, because of the absence of any aromatic protons on TFE, this mitigates potential problems of resonance overlap considering that aromatic protons are often favored during an NMR binding analysis due to the simplicity and the usual lack of any signal overlap with common buffer components. On the other hand, TFMBA possesses four aromatic protons,
which is likely to overlap various compounds of interest, as depicted in Figure S6B.

## CONCLUSIONS

In summary, we have evaluated various fluorine reference candidates under various conditions to assess their suitability for NMR drug discovery experiments. We also highlighted the complexity of choosing an appropriate reference molecule and provided some recommendations to guide these choices. Evidently, there are likely other interesting reference candidates out there, and therefore, a similar testing sequence could be extended to other compounds to identify additional candidates. After a screen, a molecule defined as nonbinder can also be used as project-specific internal reference for follow-up steps.\(^1\) However, care must be taken when changing experimental conditions to ensure compatibility.

Regardless of the references to be used, they should always be assessed against each screening target to rule out binding of the reference to the target or even destabilization of the latter. To streamline the process, a selected subset of references can be pooled together and tested against the protein/nucleic acid of interest before launching screening efforts.

## MATERIALS AND METHODS

### Compounds and Libraries.

All compounds investigated in this work were ordered from external vendors. The suppliers

| reference compound | advantages | disadvantages |
|-------------------|------------|---------------|
| NaBF\(_4\)        | Minimal chance of resonance overlapping with common fragments | Chemical shift might fall outside the spectral width of some NMR sequences |
|                   | Absence of \(^1\)H | 2 species observed due to natural abundance of boron isotopes |
| TFA               | Absence of \(^1\)H | Appears to be more sensitive to variations in DMSO or pH |
| TFE               | Only aliphatic \(^1\)H | Can already be a residual from chemical synthesis |
| TFMBMA            | Decent all-around performance | Volatile and flammable |
| Triflate          | Absence of \(^1\)H | Aliphatic \(^1\)H |
|                   | | Known to promote protein changes at higher concentrations\(^2\) |
|                   | | Aromatic \(^1\)H |
|                   | | More fragment-like molecule, could be more prone to binding proteins |
|                   | | Appears to be slightly more sensitive to variations in temperature than other candidates |

Figure 9. Demonstration of the utility of \(^19\)F chemical shift referencing in a competition study. (A) \(^19\)F NMR spectra of TFMBMA (5 \(\mu\)M). (B) \(^19\)F NMR spectra of \(^19\)F probe at 22 \(\mu\)M, HRV at 22 \(\mu\)M, noncompetitor test compound at 100 \(\mu\)M. (C) \(^19\)F NMR spectra of \(^19\)F probe at 22 \(\mu\)M, HRV at 22 \(\mu\)M, competitor test compound at 100 \(\mu\)M.
and catalog numbers are provided in the Supporting Information. The fragment library used to assess pool effects was provided by NMX Research and Solutions Inc. (Fast-Screen 19F Fragment Library).

**NMR Sample Preparation.** Each compound was prepared as a 30 mM stock solution from the purchased powder or liquid in either dimethyl sulfoxide-d<sub>6</sub> or deuterium oxide (D<sub>2</sub>O). This solution was then diluted to give the desired final compound concentrations. NMR samples were stored at 4 °C in a SampleJet sample handler, and data were acquired at 25 °C unless otherwise specified.

**NMR Experiments.** All experiments were run on a 600 MHz Bruker Avance III spectrometer equipped with a helium HFCN cryoprobe. 1D 1H-decoupled 19F experiments were acquired using the standard Bruker sequence zgfhigqn. Spectra were acquired with 32 scans and a relaxation delay of 10 s. 1D 1H spectra were acquired using standard Bruker 1D 1H sequence with excitation sculpting (zgesgp) and a relaxation delay of 10 s. Spectra were acquired with 16 scans.

To avoid potential interference with another internal referencing, a deuterium lock on the magnet was used for 19F referencing. To ensure sufficient robustness of this method, repeated measurements were performed on three samples containing 200 μM trifluoroethanol over three time points (0, 12, and 24 h). Very good consistency was observed across time points, with average variations in chemical shifts below 0.1 Hz (Table S1).

**Data Interpretations.** Data visualization and analysis were done in Bruker’s TopSpin software (https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html).

**Proteins.** All proteins used in this report were purchased from external vendors. The suppliers and catalog numbers are provided in the Supporting Information.

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**ASSOCIATED CONTENT**

- Supporting Information
- Additional NMR figures; spectrometer stability of indirect referencing; information on compounds and proteins used in this report (PDF)

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**AUTHOR INFORMATION**

**Corresponding Author**

Steven R. LaPlante — Centre Armand-Frappier Santé Biotechnologie, Institut national de la recherche scientifique, Laval, Québec H7V 1B7, Canada; NMX Research and Solutions Inc., Laval, Québec H7V SB7, Canada; orcid.org/0000-0003-2835-5789; Email: stevenrlaplante@gmail.com

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**Authors**

Yann Ayotte — Centre Armand-Frappier Santé Biotechnologie, Institut national de la recherche scientifique, Laval, Québec H7V 1B7, Canada; NMX Research and Solutions Inc., Laval, Québec H7V SB7, Canada; orcid.org/0000-0002-9394-6553

Simon Woo — Centre Armand-Frappier Santé Biotechnologie, Institut national de la recherche scientifique, Laval, Québec H7V 1B7, Canada; NMX Research and Solutions Inc., Laval, Québec H7V SB7, Canada

Complete contact information is available at:

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**Notes**

The authors declare no competing financial interest.

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