Replication in bioanalytical studies with HDX MS: aim as high as possible

“While more complicated and time consuming, the higher order the replication, the more biologically relevant the information.”

Keywords: biopharmaceutical • comparability • error • hydrogen exchange • MS • reproducibility

How many measurements provide confidence?

To have some confidence in the significance of a measurement, replicates are required. If you have had your cholesterol and HDL levels checked more than once, you are at least casually familiar with analytical significance and replication. For example, if you are two points outside the ‘good/normal’ HDL range, is that as significant as being 50 points out of the range? The answer may depend on the repeatability of the measurement: perhaps in one replicate measurement the number is two points out and on another measurement it is not. Which is correct? It is not always easy to know how many times the levels must be measured before there is some confidence in the number. At the same time, many thousands of samples must be measured to create the reference ranges of ‘good/normal’ versus ‘bad/abnormal.’

Replication may involve many variables

In replication, not only must the number of repeat measurements be considered, but also the type of replication. As examples, the following four cases of HDL measurement replication are quite different: six replicates of the same blood sample, three blood samples taken within 15 min of each other and samples taken on the first Wednesday of each month for a year or one sample taken every 2 years. Different still is the average value of replicates from one race compared with replicates from another race. In short, there can be many parameters in replication that

must be considered, not the least of which is biological variation.

Replication in hydrogen deuterium exchange MS

We bring up these concepts to introduce the discussion of replication in measurements of protein dynamics with hydrogen deuterium exchange (HDX) MS. In the most common type of HDX MS experiment, a protein is incubated in deuterium and the resulting mass increase measured with a mass spectrometer [1]. Protein conformation and dynamics affect how much deuterium is incorporated into the protein during the exchange process; therefore, the results report on the nature of the protein structure. HDX MS is finding use in many areas [2] including in the biopharmaceutical industry [3] where protein comparability can be a central question. Just as measurement of HDL levels in a single patient can vary depending on the time of day, fasting status, age, genetics, etc., so too can the levels of deuterium in a sample according to parameters such as temperature, pH, time, analysis instrumentation and analyst [4]. An important question, therefore, is how reproducible are HDX MS measurements? To define reproducibility, however, the type of replicate must be considered.

Questions of reproducibility arise for an HDX MS experiment as for any analytical measurement. How many replicates are required before there is reasonable confidence in the measured value? What difference(s) between measurements would be considered ‘significant’ or, more importantly,

Jamie A Moroco

Department of Chemistry & Chemical Biology, Northeastern University, Boston, MA 02115, USA

John R Engen

Author for correspondence:
Department of Chemistry & Chemical Biology, Northeastern University, Boston, MA 02115, USA
Tel.: +1617 373 2855
j.engen@neu.edu

**FUTURE
SCIENCE** part of

fsg

meaningful? If one analyst in Laboratory A makes a measurement, how well can that measurement be reproduced with the identical sample by another analyst in Laboratory B with similar equipment and expertise? The answers to these questions depend on a number of factors and clearer understanding of what types of measurements are possible and which ones have what type(s) of error.

Hierarchy of HDX MS replication levels

We have defined [5] five categories or levels of replication in HDX MS experiments: biological, manipulation, labeling, analysis and processing. There is a hierarchy to these replicate levels with biological replication being the highest order and processing replication being lowest order. Types of replication lower in order may be found in those of higher order (e.g., there could be six analysis replicates in each of three biological replicates and so forth). Considering each type of replication from the lowest to highest order, processing replicates are repeats of software-based data analysis, but on the same set of data. This replication tests the computational parameters and data processing reliability. Analysis replicates are replicate LC/MS injections of the identical labeled sample and test all variables from the point of injection into the LC/MS system onward. The next highest level, labeling replicates, are independent deuterium additions to the same protein stock material, and test sample conditions during labeling (such as timing, pH and temperature) as well as all LC/MS parameters. Manipulation replicates are not always done but make allowance for steps just prior to the addition of deuterium, such as incubation of a protein with a ligand or a membrane. Finally, the highest order is the biological replicate. This type of replicate encompasses independent protein expression (or isolation from source tissue), protein purification, labeling, analysis and data processing. It is one thing to obtain similar values in analysis replicates, but quite another to obtain similar values in biological replicates. We argue that the most relevant answers come from biological replication (high order), not from analysis replication (low order). One could imagine even higher order replication (above biological replicates) such as comparison of three or more biological replicates from one cell type (or species) with three or more biological replicates from another cell type (or species), etc.

The higher the order, the better

Historically, time and energy have inhibited venture into higher order replication in HDX MS. Fifteen years ago in HDX MS, there was only primitive processing software and it took so much time to analyze a single sample, there was hardly time or energy to do

many replicates. With modern instrumentation and technology, it is not uncommon to do at least five labeling replicates with ease. It is also possible to routinely do multiple biological replicates along with multiple labeling and analysis replicates. What is clear from our experience with higher-order replicates is that biological variability can sometimes be high, and this may provide interesting, new and useful information. Two cases in point are protein binding to small molecules and protein interactions with lipids.

Example one: kRas

In recent HDX MS studies [6] of small molecule binding to kRas, biological variability became apparent. kRas binds tightly to GDP and in order to compare the conformational consequences of binding to GTP, the GDP must be switched out for GTP. As the binding constants for both GDP and GTP are subnanomolar, removing one small molecule and replacing it with the other can be challenging. In various preparations of kRas in which the GDP had been switched for GTP to different degrees, the regions of kRas experiencing differences in HDX in the GDP- or GTP-bound form remained constant, but the magnitude of deuterium incorporation could vary. A mixture of GDP- and GTP-bound forms coexisting in the solution skewed the mass from what was observed when the population of bound kRas molecules was 100% GDP- or 100% GTP-bound. By monitoring the deuterium levels in regions of kRas sensitive to the small molecule ligands, information about the population of bound proteins could be obtained. This helped provide quality control for the sample handling steps, including the efficiency of switching ligands prior to labeling, and monitoring the ability of other competitive small molecules to displace GDP/GTP. Without biological replication, that is, only MS measurements of several analysis replicates originating from one sample preparation, we would not have realized there could be such a distribution of bound forms existing in solution.

Example two: membrane proteins

A second example of the value of biological replication comes from our HDX MS work with membrane proteins [7–14]. Depending on the batch of lipid mimetics (liposomes or nanodiscs), there can be variability in deuterium incorporation in membrane proteins. Differences in the measurements could be the result of multiple situations, including different liposome composition between each preparation, changes to the protein:lipid ratio (given the error of protein concentration determination) or because the interaction between protein and lipid is actually different from one preparation to the next. By running multiple

combinations of each type of replication, the source of variability can be determined. For example, multiple labeling replicates and biological replicates can be performed with one batch of liposomes and then repeated with a second batch of liposomes. Such replication can be quite time consuming, but is necessary to clarify what is responsible for the differences measurements and pinpoint where variability is being introduced.

Closing thoughts

While more complicated and time consuming, the higher order the replication, the more biologically relevant the information. What we would like to advocate is that biological replication needs to become the new norm in HDX MS. Biological replication is certainly what provides the most meaningful measures of comparability in different preparations of biopharmaceuticals or biosimilars. As HDX MS is

used more and more for analysis of complex biology involving protein machines and complexes, biological replication will also become critical. Just as a course of medical therapy is not based on a few HDL measurements, conclusions about protein action and function should not be based on only a few HDX MS measurements.

Financial & competing interests disclosure

We acknowledge funding from the NIH grant R01-GM101135 and a research collaboration with the Waters Corporation. JR Engen is a consultant of the Waters Corp. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

- Engen JR, Wales TE, Shi X. Hydrogen exchange mass spectrometry for conformational analysis of proteins. *Encyclopedia of Analytical Chemistry* doi: 10.1002/9780470027318.a9780470029201 (2011).
- Pirrone GF, Iacob RE, Engen JR. Applications of hydrogen/deuterium exchange MS from 2012 to 2014. *Anal. Chem.* 87(1), 99–118 (2015).
- Berkowitz SA, Engen JR, Mazzeo JR, Jones GB. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. *Nat. Rev. Drug Discov.* 11(7), 527–540 (2012).
- Iacob RE, Engen JR. Hydrogen exchange mass spectrometry: are we out of the quicksand? *J. Am. Soc. Mass Spectrom.* 23(6), 1003–1010 (2012).
- Engen JR, Wales TE. Analytical aspects of hydrogen exchange mass spectrometry. *Annu. Rev. Anal. Chem. (Palo Alto Calif)* doi: 10.1146/annurev-anchem-062011-143113 (2015) (In press).
- Lim SM, Westover KD, Ficarro SB *et al.* Therapeutic targeting of oncogenic K-Ras by a covalent catalytic site inhibitor. *Angew. Chem. Int. Ed. Engl.* 53(1), 199–204 (2014).
- Hebling CM, Morgan CR, Stafford DW, Jorgenson JW, Rand KD, Engen JR. Conformational analysis of membrane proteins in phospholipid bilayer nanodiscs by hydrogen exchange mass spectrometry. *Anal. Chem.* 82(13), 5415–5419 (2010).
- Morgan CR, Hebling CM, Rand KD, Stafford DW, Jorgenson JW, Engen JR. Conformational transitions in the membrane scaffold protein of phospholipid bilayer nanodiscs. *Mol. Cell. Proteomics* 10(9) M111.010876 (2011).
- Morgan CR, Miglionico BV, Engen JR. Effects of HIV-1 Nef on human N-myristoyltransferase 1. *Biochemistry* 50(16), 3394–3403 (2011).
- Kim M, Sun ZY, Rand KD *et al.* Antibody mechanics on a membrane-bound HIV segment essential for GP41-targeted viral neutralization. *Nat. Struct. Mol. Biol.* 18(11), 1235–1243 (2011).
- Nasr ML, Shi X, Bowman AL *et al.* Membrane phospholipid bilayer as a determinant of monoacylglycerol lipase kinetic profile and conformational repertoire. *Protein Sci.* 22(6), 774–787 (2013).
- Karageorgos I, Wales TE, Janero DR *et al.* Active-site inhibitors modulate the dynamic properties of human monoacylglycerol lipase: a hydrogen exchange mass spectrometry study. *Biochemistry* 52(29), 5016–5026 (2013).
- Parker CH, Morgan CR, Rand KD, Engen JR, Jorgenson JW, Stafford DW. A conformational investigation of propeptide binding to the integral membrane protein gamma-glutamyl carboxylase using nanodisc hydrogen exchange mass spectrometry. *Biochemistry* 53(9), 1511–1520 (2014).
- Barclay LA, Wales TE, Garner TP *et al.* Inhibition of pro-apoptotic BAX by a noncanonical interaction mechanism. *Mol. Cell* 57(5), 873–886 (2015).