Briefings in Bioinformatics, 2025, **26(2)**, bbaf104
https://doi.org/10.1093/bib/bbaf104
Problem Solving Protocol

Regularly updated benchmark sets for statistically correct evaluations of AlphaFold applications

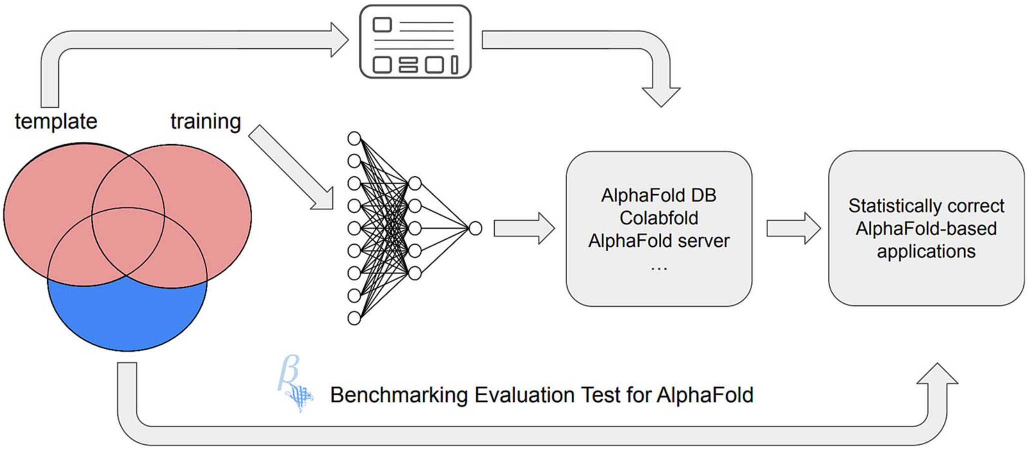
Laszlo Dobson[1,2,*], Gábor E. Tusnády[1,2], Peter Tompa[1,3,4]

[1]Institute of Molecular Life Sciences Research, Centre for Natural Sciences, Magyar Tudósok Körútja, Budapest, Hungary
[2]Department of Bioinformatics, Semmelweis University Tűzoltó u, Budapest, Hungary
[3]VIB-VUB Center for Structural Biology, Vlaams Instituut voor Biotechnologie (VIB), Brussels, Belgium
[4]HUN-REN Office for Supported Research Groups (TKI), Cell Cycle Laboratory, National Institute of Oncology, 1122 Budapest, Hungary
[*]Corresponding author. Institute of Molecular Life Sciences Research, Centre for Natural Sciences, Magyar Tudósok Körútja, Budapest, Hungary.
E-mail: dobson.laszlo@ttk.hu

Abstract

AlphaFold2 changed structural biology by providing high-quality structure predictions for all possible proteins. Since its inception, a plethora of applications were built on AlphaFold2, expediting discoveries in virtually all areas related to protein science. In many cases, however, optimism seems to have made scientists forget about data leakage, a serious issue that needs to be addressed when evaluating machine learning methods. Here we provide a rigorous benchmark set that can be used in a broad range of applications built around AlphaFold2/3.

Graphical Abstract



Keywords: AlphaFold; data leakage; protein structure; benchmark set; structure prediction

Introduction

The creation of AlphaFold2 (AF2) in 2020 was bound to revolutionize protein science, by significantly raising the standards of structure prediction at CASP XIV [1]. Within a year, the method was published [2], soon followed by a database of predicted all-atom structures for almost every known protein sequence [3]. Based on this spectacular feat, thousands of papers appeared that analyzed AF2 structures to predict and/or assess various structural and physicochemical features of proteins in specific areas, such as protein stability [4], conformational heterogeneity [5], protein function [6], structure, [7] or interfaces [8] of protein complexes, domain definitions [9], or disordered regions that do not fold into unique structures [10]. A similar or even more sweeping campaign of applications can be expected now with the appearance of AF3, with its advanced architecture and features, probably more reliably predicting protein-ligand (another protein), protein–nucleic acid and protein–small molecule interactions and complex structures [11].

The apparent insight provided by these noted, and many other original applications gave cause for optimism, having

Received: December 11, 2024. **Revised:** January 27, 2025. **Accepted:** February 26, 2025

reinvigorated a broad range of structure-related studies, with artificial intelligence promising to expand the proteome coverage enabled by laborious and time-consuming experiments. We noted, however, that many of these studies did not take a stringent approach to selecting proteins that had not been used to train AF2, leading to data leakage, which is inevitably leads to statistical inaccuracies when applying machine learning methods. Most of these papers are still highly valuable and point to relevant applications and evaluations. It is of relevance that our perusal of literature could not be done right after AF2 publication, only after an intense period of diverse analyses based on its applications. We figure the same will happen after AF3 code and/or access will be made broadly available, thus our conclusion and suggested solution also apply across a wide range of future applications.

AF2 has not been trained on protein complexes, its performance in predicting their structures is remarkable, probably because protein–protein interactions have a similar biophysical background to protein folding, which AF2 has been trained to model – these capabilities are promised to further improve with AF3. Intriguingly, the capabilities of AF2 to model complexes is not limited to those built on domain-domain interactions [7], it can also well handle more transient interactions driven by short linear motifs [9]. It should be noted, however, that although the evaluation of multimers appears to be not affected by the choice of benchmarks, the bound state of the respective single chains used for the training of AF2 does cause data leakage and compromises the resulting statistical inference.

To overcome this limitation we suggest a deep homology search for proteins in the training/template sets of AF. Although the solution may seem trivial, considering how distant homologous structures can be still captured by AF and the many different versions of AF each with a different training cutoff, we prepared benchmark sets to facilitate this process. Here we provide Benchmarking Evaluation Test for AlphaFold (BETA), containing predefined datasets for AF applications, and demonstrate through a range of case studies that it resolves the issue of data leakage-derived statistical uncertainties in AF-based analyses.

Materials and methods

Defining benchmark sets

BETA was created as follows: PDB [6], SwissProt, [12] and BIOGRID [13] were downloaded on the 21 May 2024. Considering the first day of the following month of each cutoff date (30 April 2018, 31 May 2020, 15 February 2021, 30 September 2021, 15 July 2022, 01 November 2022, 01 January 2023, and 01 January 2024) we performed homology searches based on both sequence and structure. AF can be also used without incorporating template structures, and for training AF NMR structures were not used as models, therefore for cutoff dates related to training (30 April 2018 and 30 September 2021) NMR structures were not included in the background database. These dates were selected based on how different AF versions training/template libraries were selected (see Fig. 1). Homology searches were carried out as follows: (i) Sequence search (PSI-BLAST [14]) of all structures after cutoff date against structures before cutoff date; (ii) Structure search (Foldseek [15]) of all structures after the cutoff date against structures before cutoff date; (iii) Sequence search (PSI-BLAST) of SwissProt proteins against structures before cutoff date. In the case of PSI-BLAST (e-value 0.0001, three iterations, maximum target sequences 50 000), all queries having any hit longer than 10 amino acids with at least 20% sequence identity were filtered In Foldseek searches (maximum target structures 50 000), all queries having any hit longer than 10 amino acids with at least 0.25 TM-Score were filtered. We used Voronota [16] to detect all interchain interaction in PDB structures, considering the first oligomerization state stored on PDBe. We used BIOGRID (only ‘direct interactions’) to search for interactions in SwissProt sequences. The pipeline and relevant resulting collections of different BETA benchmark sets are shown on Fig. 1.

A case study: prediction of protein disorder

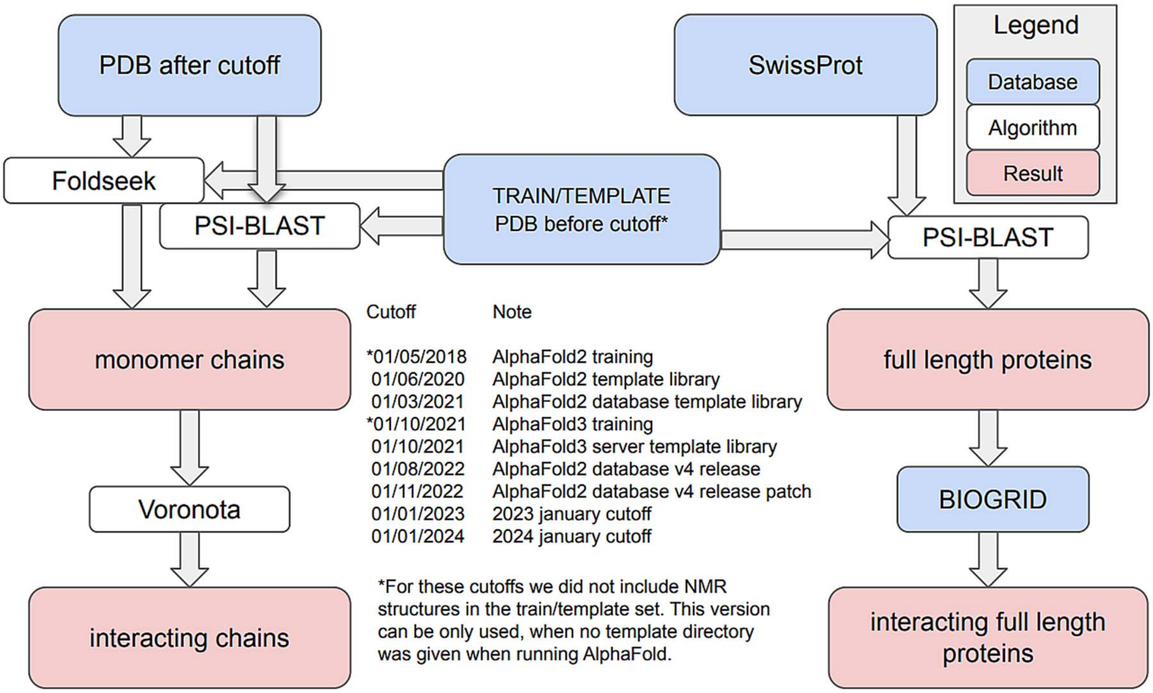
To demonstrate the importance of our methodology, we assessed how prediction methods of intrinsically disordered proteins/regions (IDPs/IDRs) would be affected by utilizing BETA (Fig. 2A). Previously, the confidence measure local-distance difference test (pLDDT) was shown to be a good predictor of protein disorder [10]. We downloaded all PDB structures, where the most probable oligomeric state was a monomer according to PDBe [6]. Sequences were filtered to 40% identity using CD-HIT [17], and disorder of residues was inferred, if coordinates of their side-chains were missing from the PDB structures. By adhering to the straightforward definition of missing coordinates, we: (i) accept the consensus in the IDP community, as reflected in state-of-the-art protein disorder annotation databases DisProt [18] and MobiDB [19], (ii) define a straightforward and clear methodology, so readers/users do not get lost in details of the pipeline, and (iii) use an approach where no cutoffs required (that can be always argued). As the resolution of structures may have no effect on the choice of disordered residues [20], at the moment it is not considered in standard approaches of selecting for disordered residues, for example, in the latest release of the DisProt database [18] or in formulating respective community guidelines [21]. Also, in accord with guidelines, segments shorter than 10 amino acids were omitted, and the remaining residues (with side-chain coordinates) were labeled as ordered. We used SIFTS [22] to cross-reference UniProt identifiers and positions with PDB chains and residues, then downloaded AF2 structures from the AlphaFold protein structure database. Structures were categorized into ‘homologous structures’ and ‘BETA structures’, considering the AF database training/template cutoff, which in this case is 01 March 2021. Supplementary Table S1 contains all residues incorporated in the analysis (with cross-referenced PDB and AF positions), with ordered and disordered residue classifications (inferred from PDB structures), AF2 pLDDT values, as well as BETA and homologous structure classifications.

Results

Benchmarking evaluation test for AlphaFold

To place AF2 – and by the same logic, AF3 – based structure–function studies on a solid ground, we present here the BETA, a dataset of structures and sequences that were not used in AF training (https://beta.pbrg.hu, https://github.com/brgenzim/Beta). In BETA, we list (i) monomeric PDB chains with no homologs before the selected date, (ii) interacting PDB chain pairs where participants have no homologs prior the selected date, (iii) complete protein sequences from SwissProt without any homologs in the PDB before the selected date, and (iv) complete interacting sequences from SwissProt where the interacting participants do not have homologs before the selected date (Fig. 1).

BETA can be utilized in many different ways to help construct a benchmark set for surveys or prediction methods, while also providing a convenient way for researchers without a computational background to browse data. We aimed to construct datasets for all users, including those accessing AF via the AlpaFold database [3], ColabFold [23] or the AlphaFold server [11], or running their



Cutoff	Note
*01/05/2018	AlphaFold2 training
01/06/2020	AlphaFold2 template library
01/03/2021	AlphaFold2 database template library
*01/10/2021	AlphaFold3 training
01/10/2021	AlphaFold3 server template library
01/08/2022	AlphaFold2 database v4 release
01/11/2022	AlphaFold2 database v4 release patch
01/01/2023	2023 january cutoff
01/01/2024	2024 january cutoff

*For these cutoffs we did not include NMR structures in the train/template set. This version can be only used, when no template directory was given when running AlphaFold.

Figure 1. Pipeline used to define the benchmarking evaluation test for AlphaFold (BETA). Blue and white panels represent databases and algorithms (respectively), red panel show results of the pipeline. Predefined cutoff dates and their rationale can be found in the middle.

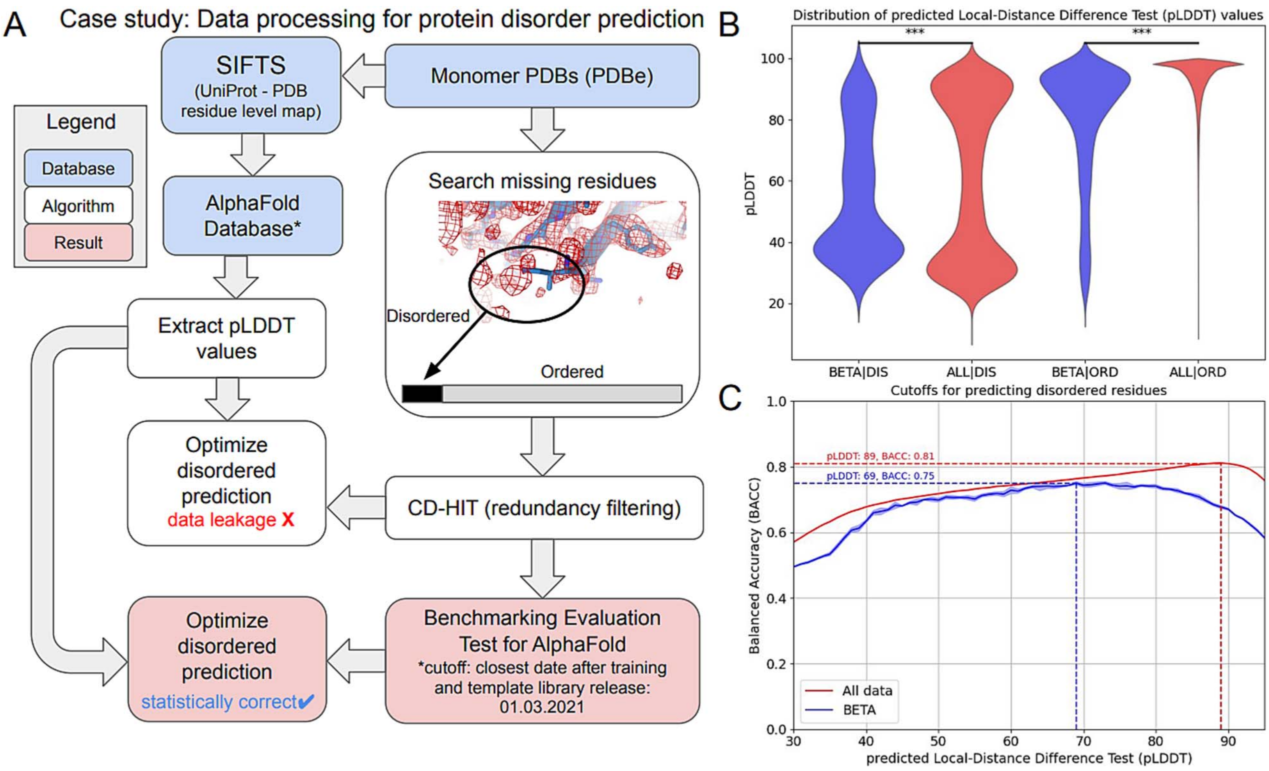


Figure 2. Pipeline and evaluation of the protein disorder prediction case study. A) Pipeline to define disordered residues from PDB and incorporating BETA into the pipeline. For more details, see Methods. B) Distribution of pLDDT values of disordered (DIS) and ordered (ORD) residues in all monomeric structures (red) and BETA structures (blue). There is highly significant difference between pLDDT values in the case of BETA against all structures, both for ordered and disordered residues. C) Correspondence between pLDDT and balanced accuracy considering all structures and BETA structures. The highest balanced accuracy (with suggested pLDDT cutoff is shown). The small area around curves show the standard error calculated from bootstrapping 50% of the data five times, while the curves show the mean value at a given point.

predictions on their own computer. For defining an appropriate benchmark set using BETA, one should first determine the appropriate cutoff date based on the input data (UniProt sequences or PDB codes) and the version of AF (AlphaFold database, ColabFold, AlphaFold server or running on one's own computer), and then select the first date after this cutoff to avoid using structures used during AF2/AF3 training or prediction. To demonstrate the general applicability of BETA, we outline a highly informative case study of predicting structural disorder, out of five relevant use cases (more details are available at Supplementary Material S1 and https://beta.pbrg.hu/): (i) building a protein disorder prediction method (further detailed as a case study); (ii) predicting antibody epitopes; (iii) predicting phase-separating regions in proteins; (iv) assessing the pathological effects of missense mutations; and (v) ranking interactions driven by short linear motifs. These examples explain how an appropriate benchmark dataset can be selected and used.

Case study: the effect of using BETA for protein disorder prediction

To set the precise pLDDT threshold to predict IDRs, we can define a set of proteins where we can assign labels to each residue, whether they belong to IDRs or not. Using a benchmark set of PDB structures (see Methods), we looked for the distribution of pLDDT values in ordered and disordered residues (Fig. 2B, red distributions). As the two distributions are different, one can assume that pLLDT value is an indeed good predictor for protein disorder. However, limiting the examined residues to those that are available in the BETA database, there is also a significance difference between ordered residues in BETA and all ordered residues (Kolmogorov–Smirnov (KS) Test, $P < 0.0001$), as well as between residues in IDRs (BETA against all structures, K-S Test, $P < 0.0001$) (Fig. 2B). To further highlight the gravity of differences caused, we also looked for the optimal pLDDT cutoff to achieve the highest balanced accuracy (Fig. 2C, Supplementary Table S2). Indeed, the cutoff for the highest accuracy is different for all structures (pLDDT = 0.89) from the cutoff defined using only BETA structures (pLDDT = 0.69). Notably, the number of residues in IDRs is relatively low in the BETA set defined by monomeric PDBs (total 1062 residues), which is likely caused by the fact that most novel structures deposited to PDB correspond to big complexes. To avoid statistical bias, we randomly sampled 50% of the residues five times to define the pLDDT cutoffs.

We have to add that we do not aim to build a perfect IDR predictor here, and these steps can be further optimized. Our goal was to show that defining a stringent benchmark set may heavily influence an application built on AF, both in the selected cutoff, but also on the measured accuracy which is lower when data leakage is ruled out.

Conclusion

While AF2 is already available through multiple resources, AF3 is currently available as a web server (or the standalone program), with a broad range of applications for predicting various features on the horizon. We noticed that many applications built on AF do not take into account the training set of AF. Our comparison on predicting protein disorder demonstrates that our conclusion and suggestion are useful for future applications of AF, thus we plan to update the underlying BETA dataset on a regular basis, taking into account how AF2/AF3 based resources will update their training and template libraries in the future. Of possibly even broader relevance of applicability, multiple other structure prediction methods have been published since AF was developed [24–26]. Although the website of BETA cannot offer an exact cutoff date for the training date of each method, researchers can still rely on the website (with the nearest training cutoff) or perform homology search with custom cutoff dates using the GitHub repository, when building applications on structure prediction tools.

Key Points

- When building applications on AlphaFold, scientists should consider the possibility of data leakage between AlphaFold training set and the independent test set of their method
- BETA provides multiple datasets with structures and sequences that were not used during the training of AlphaFold
- These datasets provide a diverse range of use cases
- The protocol was applied when building a simple disordered prediction method, showing different parameters required to optimize disordered prediction for proteins not used in AlphaFold training

Supplementary data

Supplementary data is available at *Briefings in Bioinformatics* online.

Acknowledgements

We thank Rita Pancsa for discussing the manuscript and Zsofia E. Kalman for the website logo.

Conflict of interest: None declared.

Funding

NKFIH [PD-146564 and K-146314 to L.D. and G.E.T., respectively]. The project was implemented with the support from the National Research, Development and Innovation Fund of the Ministry of Culture and Innovation under the RGH_24 (RGH 151464) Grant Agreement with the National Research, Development and Innovation Office (to P.T.)

Data availability

Supplementary Table 1 is available at https://zenodo.org/records/14711867.

Supplementary Table 2 is available Online.

References

1. Kryshtafovych A, Schwede T, Topf M. *et al.* Critical assessment of methods of protein structure prediction (CASP)-round XIV. *Proteins* 2021;**89**:1607–17. https://doi.org/10.1002/prot.26237
2. Jumper J, Evans R, Pritzel A. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* 2021;**596**:583–9. https://doi.org/10.1038/s41586-021-03819-2
3. Varadi M, Anyango S, Deshpande M. *et al.* AlphaFold protein structure database: Massively expanding the structural coverage of protein-sequence space with high-accuracy models.

Nucleic Acids Res 2022;**50**:D439–44. https://doi.org/10.1093/nar/gkab1061

4. Pak MA, Markhieva KA, Novikova MS. *et al.* Using AlphaFold to predict the impact of single mutations on protein stability and function. *PloS One* 2023;**18**:e0282689. https://doi.org/10.1371/journal.pone.0282689
5. Stein RA, Mchaourab HS. SPEACH_AF: Sampling protein ensembles and conformational heterogeneity with Alphafold2. *PLoS Comput Biol* 2022;**18**:e1010483. https://doi.org/10.1371/journal.pcbi.1010483
6. PDBe-KB consortium. PDBe-KB: A community-driven resource for structural and functional annotations. *Nucleic Acids Res* 2020;**48**:D344–53. https://doi.org/10.1093/nar/gkz853
7. Bryant P, Pozzati G, Zhu W. *et al.* Predicting the structure of large protein complexes using AlphaFold and Monte Carlo tree search. *Nat Commun* 2022;**13**:6028. https://doi.org/10.1038/s41467-022-33729-4
8. Lee CY, Hubrich D, Varga JK. *et al.* Systematic discovery of protein interaction interfaces using AlphaFold and experimental validation. *Mol Syst Biol* 2024;**20**:75–97. https://doi.org/10.1038/s44320-023-00005-6
9. Bordin N, Sillitoe I, Nallapareddy V. *et al.* AlphaFold2 reveals commonalities and novelties in protein structure space for 21 model organisms. *Commun Biol* 2023;**6**:160. https://doi.org/10.1038/s42003-023-04488-9
10. Conte AD, Mehdiabadi M, Bouhraoua A. *et al.* Critical assessment of protein intrinsic disorder prediction (CAID) - results of round 2. *Proteins* 2023;**91**:1925–34. https://doi.org/10.1002/prot.26582
11. Abramson J, Adler J, Dunger J. *et al.* Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* 2024;**630**:493–500. https://doi.org/10.1038/s41586-024-07487-w
12. UniProt Consortium. UniProt: The universal protein knowledgebase in 2023. *Nucleic Acids Res* 2023;**51**:D523–31. https://doi.org/10.1093/nar/gkac1052
13. Oughtred R, Stark C, Breitkreutz B-J. *et al.* The BioGRID interaction database: 2019 update. *Nucleic Acids Res* 2019;**47**:D529–41. https://doi.org/10.1093/nar/gky1079
14. Altschul SF, Madden TL, Schäffer AA. *et al.* Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 1997;**25**:3389–402. https://doi.org/10.1093/nar/25.17.3389
15. van Kempen M, Kim SS, Tumescheit C. *et al.* Fast and accurate protein structure search with Foldseek. *Nat Biotechnol* 2024;**42**:243–6. https://doi.org/10.1038/s41587-023-01773-0
16. Olechnovič K, Venclovas C. Voronota: A fast and reliable tool for computing the vertices of the Voronoi diagram of atomic balls. *J Comput Chem* 2014;**35**:672–81. https://doi.org/10.1002/jcc.23538
17. Fu L, Niu B, Zhu Z. *et al.* CD-HIT: Accelerated for clustering the next-generation sequencing data. *Bioinformatics* 2012;**28**:3150–2. https://doi.org/10.1093/bioinformatics/bts565
18. Aspromonte MC, Nugnes MV, Quaglia F. *et al.* DisProt in 2024: Improving function annotation of intrinsically disordered proteins. *Nucleic Acids Res* 2024;**52**:D434–41. https://doi.org/10.1093/nar/gkad928
19. Piovesan D, Del Conte A, Clementel D. *et al.* MobiDB: 10 years of intrinsically disordered proteins. *Nucleic Acids Res* 2023;**51**:D438–44. https://doi.org/10.1093/nar/gkac1065
20. Monzon AM, Necci M, Quaglia F. *et al.* Experimentally determined long intrinsically disordered protein regions are now abundant in the protein data Bank. *Int J Mol Sci* 2020;**21**:4496. https://doi.org/10.3390/ijms21124496
21. Mészáros B, Hatos A, Palopoli N. *et al.* Minimum information guidelines for experiments structurally characterizing intrinsically disordered protein regions. *Nat Methods* 2023;**20**:1291–303. https://doi.org/10.1038/s41592-023-01915-x
22. Dana JM, Gutmanas A, Tyagi N. *et al.* SIFTS: Updated structure integration with function, taxonomy and sequences resource allows 40-fold increase in coverage of structure-based annotations for proteins. *Nucleic Acids Res* 2019;**47**:D482–9. https://doi.org/10.1093/nar/gky1114
23. Mirdita M, Schütze K, Moriwaki Y. *et al.* ColabFold: Making protein folding accessible to all. *Nat Methods* 2022;**19**:679–82. https://doi.org/10.1038/s41592-022-01488-1
24. Wohlwend J, Corso G, Passaro S. *et al.* Boltz-1 democratizing biomolecular interaction Modeling. *bioRxiv* 2024; 2024.11.19.624167. https://doi.org/10.1021/acs.nanolett.4c05426
25. Lin Z, Akin H, Rao R. *et al.* Evolutionary-scale prediction of atomic-level protein structure with a language model. *Science* 2023;**379**:1123–30. https://doi.org/10.1126/science.ade2574
26. Wu R, Ding F, Wang R. *et al.* High-resolution de novo structure prediction from primary sequence. *bioRxiv* 2022; 2022.07.21.500999. https://doi.org/10.1101/2022.07.21.500999

Briefings in Bioinformatics, 2025, **26(2)**, bbaf104
https://doi.org/10.1093/bib/bbaf104
Problem Solving Protocol