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Technical Note

Streamlined Data Processing for Determination of Equilibrium Dissociation Constants with Accurate Constant via Transient Incomplete Separation (ACTIS)

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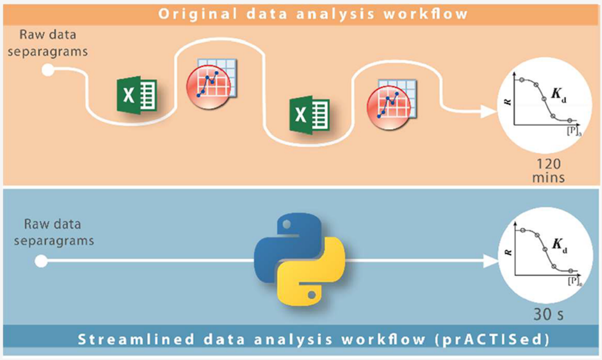

ABSTRACT: The determination of accurate equilibrium dissociation constants, K_d, of protein−small molecule complexes is important but challenging as all established methods have inherent sources of inaccuracy. Accurate Constant via Transient Incomplete Separation (ACTIS) is a new method for K_d determination using transient incomplete separation of the complex from the unbound small molecule in a pressure-driven flow inside a capillary. ACTIS is accurate, and its accuracy is invariant to variations in geometries of both the fluidic system and the flow. Furthermore, ACTIS is implemented using a simple fluidic system supporting its accuracy and providing a simple-to-follow/copy template for instrumentation. Despite the simple and robust instrumentation/acquisition, the current data processing workflow is cumbersome, time consuming, and prone to hard-to-trace human errors therefore hindering ACTIS' ability to become a practical reference method for K_d determination. This technical note describes a streamlined workflow for processing ACTIS data; the workflow is implemented as a set of open-source software tools called prACTISed (https://github.com/prACTISedProgram/prACTISed). These tools allow all steps of data processing to be performed in a fast and straightforward fashion. These practical software tools complement the simple instrumentation serving both developers and users of ACTIS.

Noncovalent complexes of proteins (P) with small-molecule ligands (L) play important roles in biology and medicine.[1,2] Such complexes (PL) are formed in a binding process schematically described by the following reaction equation:[3,4]

$$\mathrm{P} + \mathrm{L} \underset{K_d}{\rightleftharpoons} \mathrm{PL} \qquad (1)$$

where K_d is the equilibrium dissociation constant characterizing complex stability and defined through equilibrium concentrations of L, P, and PL:

$$K_d = [\mathrm{L}][\mathrm{P}]/[\mathrm{PL}] \qquad (2)$$

Knowing accurate K_d values is important but challenging.[5,6] There is no reference sample with a known K_d value or reference K_d-determination method producing accurate K_d. All current methods for finding the K_d of PL have inherent sources of inaccuracy.[7,8] As a result, K_d values determined by different methods for the same PL complex may differ by orders of magnitude,[9] and there is no reliable way to corroborate the results. Large inaccuracies in K_d values inevitably lead to misinterpretation of experimental results, mistaken conclusions, and misconceptions.[10]

We recently proposed a potential reference method for determination of K_d of protein−small molecule complexes. The method is termed Accurate Constant via Transient Incomplete Separation (ACTIS) and deemed to be free of inherent sources of inaccuracy.[8] In ACTIS, L and PL are separated from each other in a pressure-driven flow inside a capillary due to an interplay of a nonuniform flow-velocity profile and different rates of transverse diffusion of L and PL.

For ACTIS to become an established reference method, one must (i) prove the accuracy of the method, (ii) provide a simple and practical robust instrumentation for the method, (iii) show the applicability of the method to a wide range of binding pairs of interest, and (iv) provide a practical data processing workflow. We have systematically addressed these requirements. First, we showed that ACTIS is inherently accurate, and this accuracy is invariant to large variations in geometries of the fluidic system and the flow.[8,11] Second, this insensitivity to variations, in turn, allowed us to create a simple and robust fluidic system, which supports ACTIS accuracy.[12]

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Third, we showed ACTIS to be applicable to protein−small molecule and protein−aptamer binding pairs.[8,13] Although ACTIS can potentially be used for all molecular complexes, including protein−protein binding pairs, future experimental work needs to be done to show such applicability. Until now, ACTIS has lacked a practical data processing workflow.

Data processing in ACTIS involves the handling of several types of information: (i) dependency of signal on time termed separagram (approximately 50 per experiment), (ii) signal averaged over a small time window on a separagram, (iii) signal-correction factor, (iv) corrected signal, (v) fraction of unbound ligand, (vi) binding isotherms (dependency of this fraction on the initial protein concentration), and, finally, (vii) K_d value. Accordingly, data processing includes multiple steps and manipulation of digitized information of different natures. In the proof of principle works, different software tools and data formats were used to process different steps, and data transfer between them was performed manually.[8] This rudimentary workflow is lengthy (hours per experiment) and convoluted (include several switches between software tools and data formats) (see Note S1 for details). Lengthy and convoluted workflows are prone to human errors and are difficult to learn, thus hindering the adaptation of such workflows in general. A practical method such as ACTIS requires a far more practical workflow to become a truly accessible tool.

Practical workflows need to be comprehensible, replicable, and adaptable. These requirements for a practical workflow necessitate using open workflows based on open concepts and on open-source software tools.[14−21] The Python open source environment has been a package of choice in designing such software tools due to its rich and versatile library ("Batteries included") and its ease of integration into other software pipelines.[22,23] Python facilitates comprehensibility by creating user-friendly tools, replicability by taking advantage of the high automation tools in its library, and adaptability by providing extensive user support for the customization of those tools.[24,25] Therefore, since ACTIS is based on open concepts, which have been previously published, an open and practical workflow can be created using the Python open source environment.

Here, we report on developing such an open and practical workflow for streamlined data processing in ACTIS. We implemented this streamlined workflow as a set of open-source software tools called prACTISed (processing ACTIS experimental data) using Python. The proposed workflow requires straightforward user input of the experimental parameters from the experimentalist and rapidly (less than 30 s) produces a comprehensive output including publication-ready graphs and professional analysis reports. prACTISed is also able to trap and minimize user input errors. The streamlined prACTISed workflow was applied to previously evaluated sets of data, and the same K_d values were obtained as with the previous rudimentary workflow. The prACTISed workflow takes less than 30 s as opposed to approximately 2 h for the original rudimentary workflow, demonstrating the superior performance of prACTISed. The workflow and software tools are publicly accessible, allowing us and other researchers to further develop them as needed.

■ METHODS

General Workflow for Data Processing. The general workflow of ACTIS data processing is depicted in Figure 1. The workflow is split into three parts: (i) working file preparation, (ii) compensation procedure, and (iii) evaluation. The details of each part of the workflow can be found in the caption of Figure 1. The underlying algorithms and math of ACTIS data processing were developed and described in our previous publications.[8]

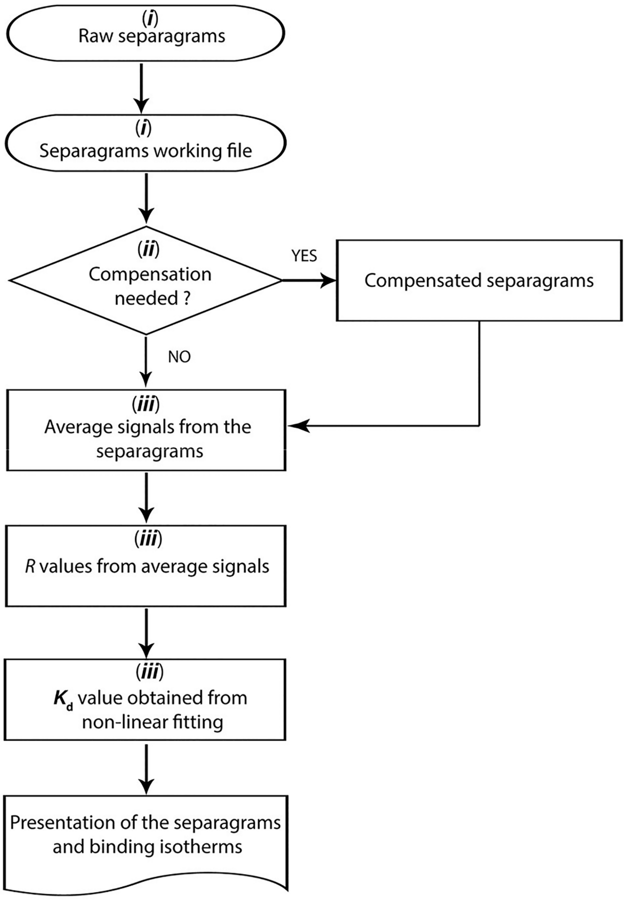

(i)
Raw separagrams
(i)
Separagrams working file
(ii)
Compensation needed ?
YES
Compensated separagrams
NO
(iii)
Average signals from the separagrams
(iii)
R values from average signals
(iii)
Kd value obtained from non-linear fitting
Presentation of the separagrams and binding isotherms

Figure 1. ACTIS data processing workflow. The workflow for ACTIS data processing consists of three parts: (i) Working file preparation (ovals). A working file for separagrams is prepared by converting the raw separagrams into an accessible format for data analysis. The user also enters total protein concentrations $[P]_0$ and the total ligand concentration $[L]_0$ to the working file. Other pertinent experimental parameters are added to the working file for future reference. (ii) Signal compensation (diamonds). A signal compensation procedure is applied to the separagrams if needed. For instance, this procedure is applied for mass spectrometry detection separagrams due to the masking effect of the protein on small-molecule ionization.[8] (iii) Evaluation (boxes). Signal values are obtained from the separagrams at different values of $[P]_0$. Fractions of unbound ligand R for different $[P]_0$ are calculated from the signal values. The K_d value is determined by performing a nonlinear fitting on the binding isotherm of R vs $[P]_0$. Lastly, the binding isotherm and separagrams are plotted for presentation.

Software and Sample Data Availability. prACTISed is a publicly available software released under the GNU General Public License v3.0. It can be used, copied, and distributed freely. It offers a graphical user interface. Python scripts (runnable under Windows, Linux, and macOS), source code, user guide, and example working files are available on the prACTISed program GitHub repository (https://github.com/prACTISedProgram/prACTISed). Modifications can be made to the ACTIS algorithm by adapting the open-source code, to provide flexibility to ACTIS developers and users.

■ RESULTS AND DISCUSSION

Concept of ACTIS. This section, which is adapted from our previous work, provides a brief introduction into the open concept of ACTIS. ACTIS is based on very fast (typically achieved in less than 1 min) transient incomplete separation (TIS) of the complex from the small molecule. TIS of two species always occurs in a pressure-driven laminar flow inside a capillary if their diffusion coefficients differ.[26−30] (Refer to refs 8 and12 for more details and discussion on the concept, separagrams, binding isotherms, K_d determination, and applications of ACTIS.)

Briefly, a short plug of an equilibrium mixture of the protein and ligand is injected into a capillary, and the plug is propagated inside the capillary by a pressure-driven flow. Different rates of transverse diffusion of the large complex PL and small free ligand L in a flow with a nonuniform (e.g., parabolic) flow-velocity profile cause their transient incomplete separation (TIS) in the longitudinal direction (Figure 2A).

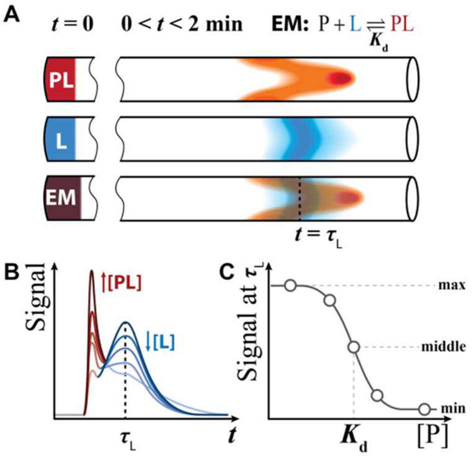


Figure 2. Simplified schematic of determining K_d by ACTIS. (A) A short plug of the equilibrium mixture (EM) of P and L is propagated through a capillary. Differences in transverse diffusion of PL and L cause their longitudinal separation. (B) Longitudinal separation results in two peaks, and a cumulative signal from L and PL is measured at time τ_L, which is the characteristic time of transverse diffusion of L. The signal is measured at a constant concentration of L and varying concentrations of P. (C) A binding isotherm "signal-at-τ_L vs concentration of P" is built, and K_d is found as the concentration of P, which corresponds to the signal in the middle between the maximum and minimum signals. Adapted from ref 11.

The cumulative signal from the ligand (both free and protein bound) is detected at the capillary exit resulting in a separagram containing two unresolved peaks: a nondiffusive peak for PL and a diffusive peak for L.[11] ACTIS is a quasi-titration method; i.e., to determine K_d, TIS is performed for a series of equilibrium mixtures (EMs) with a constant total concentration of the ligand ($[L]_0 = [L] + [PL]$) and varying total concentration of the protein ($[P]_0 = [P] + [PL]$). The result is a set of separagrams (Figure 2B). The cumulative signal from the ligand (L and PL) is determined at a time corresponding to the maximum of the diffusive peak for a run with pure ligand. Such a signal is determined and used to calculate the experimental fraction of free ligand R for each separagram, which is defined as

$$R = [L]/[L]_0 \tag{3}$$

As follows from the definition, $R = 1$ in the absence of the protein and asymptotically decreases to zero, with the growth of protein concentration. The dependence of R on $[P]_0$ constitutes a classic binding isotherm (Figure 2C). Finally, the value of K_d is determined by fitting the binding isotherm with a theoretical dependence of R on $[P]_0$ while using K_d as a fitting (varied) parameter. For the simple binding stoichiometry shown in eq 1, the theoretical dependence is in the following equation:

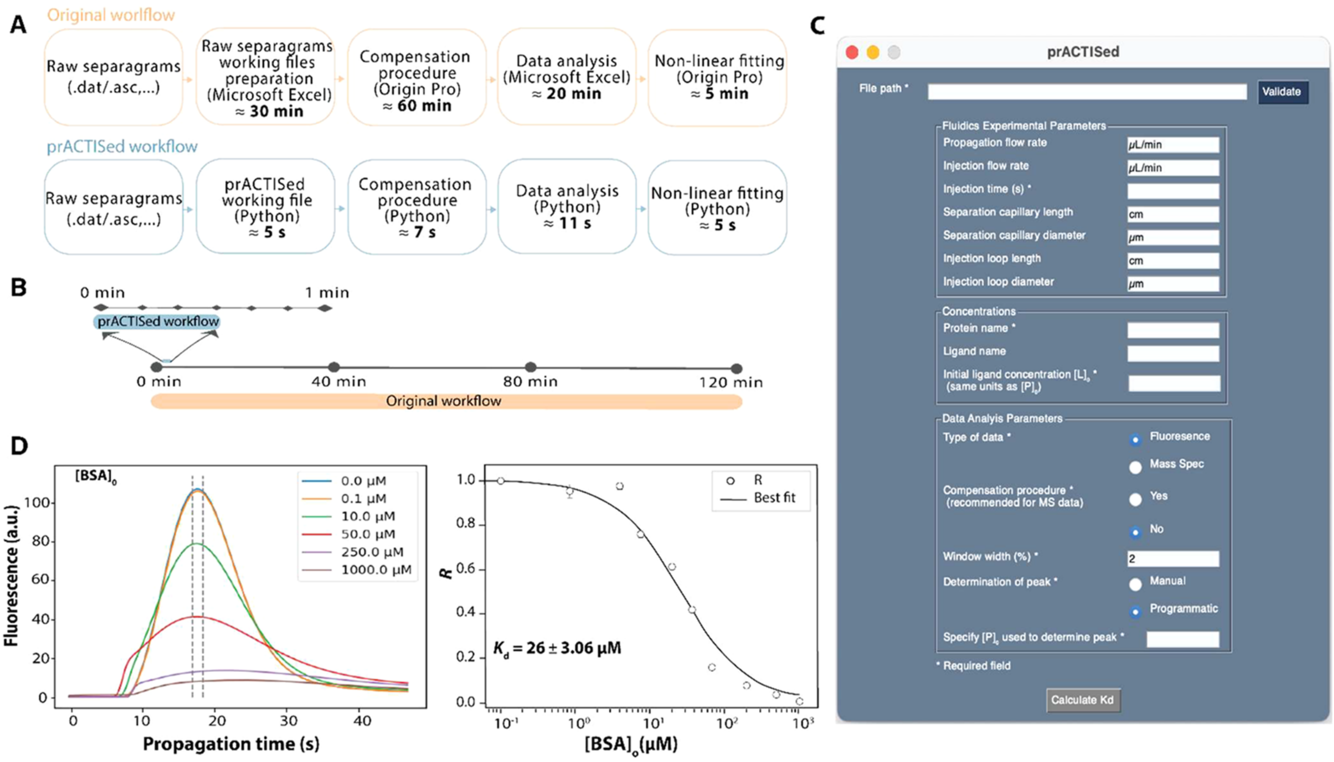
$$R = \frac{[L]_0 - K_d - [P]_0}{2[L]_0} + \sqrt{\left(\frac{[L]_0 - K_d - [P]_0}{2[L]_0}\right)^2 + \frac{K_d}{[L]_0}} \tag{4}$$

Detection can potentially skew signals in a systematic way; e.g., protein may affect the efficiency of small-molecule ionization in MS detection. In such a case, a signal-compensation procedure is needed to ensure the resulting K_d is not biased.[8]

Streamlining the Data Processing. Data processing in ACTIS involves handling different types of information through multiple steps. The previous ACTIS data processing workflow involved an operator transferring different types of information manually from one software to another. Accordingly, this original workflow (Figure 3A) was lengthy (≈2 h) and convoluted; it involved four manual switches between three different proprietary software tools each working with its own data format. This lengthy and cumbersome workflow is prone to human errors and difficult to learn, thus hindering the adaptation of ACTIS for practical uses (Note S1). The purpose of our current work is to streamline data processing by putting all the steps involved in the data processing into a single environment, thus making data processing faster, more user friendly, more flexible, and less reliant on the operator. The streamlining was implemented with the Python programming language resulting in a set of open-source software tools called prACTISed.

prACTISed takes advantage of already established libraries in the Python environment to perform all the steps involved in the general workflow of ACTIS data processing, such as the csv and pandas library used to extract and compile raw data files into an Excel workbook. The pandas library was also used to perform the compensation procedure and data analysis. The scipy library was used for nonlinear curve fitting, and matplotlib was used for plotting. The data processing with prACTISed is also done on a significantly faster time scale (less than 30 s), hence speeding up the experimental process (prACTISed workflow in Figure 3A). A time requirement comparison of the original workflow and the prACTISed workflow is shown in Figure 3B.

prACTISed provides a graphical user interface where all the experimental and data analysis parameters are inputted (Figure 3C); from these inputs, prACTISed then creates a specially formatted single input Excel file that contains all parameters (concentrations, experimental fluidic parameters, time window for signal averaging, etc.) and the respective signal lines (Notes S2 and S3). This single input file approach allows every required value to be present in one location and to be easily manipulated by users to determine the effects of altering



prACTISed

File path *

Fluidics Experimental Parameters
Propagation flow rate μL/min
Injection flow rate μL/min
Injection time (s) *
Separation capillary length cm
Separation capillary diameter μm
Injection loop length cm
Injection loop diameter μm

Concentrations
Protein name *
Ligand name
Initial ligand concentration [L]$_0$ * (same units as [P]$_0$)

Data Analyis Parameters
Type of data * ● Fluoresence ○ Mass Spec
Compensation procedure * (recommended for MS data) ○ Yes ● No
Window width (%) * 2
Determination of peak * ○ Manual ● Programmatic
Specify [P]$_0$ used to determine peak *

* Required field

Figure 3. prACTISed workflow time requirements and graphical user interface. (A) Time requirements for the data processing steps in the original workflow and in the prACTISed workflow. (B) Comparison of time requirements of the data processing of between the original workflow and the prACTISed workflow. (C) Graphical user input of prACTISed. (D) Sample separagrams and binding isotherm generated by prACTISed for inputted experimental conditions along with the calculated K_d value. More screenshots of the graphical user interface can be found in the Supporting Information.

different experimental and data analysis parameters. Previously, the values of parameters, such as the signal averaging time window, fitting parameters, etc., would have to be adjusted by the users in various locations (Note S1). prACTISed also outputs graphs that are suitable for presentation and publication; examples are shown in Figure 3D.

Generating Input Files. Advantageously, ACTIS can be combined with any detection method which (i) can be coupled with a capillary, (ii) uniformly integrates the signal through the capillary cross-section, (iii) has sufficiently high signal readout speed, and (iv) has a concentration limit of quantitation below K_d values of the studied complexes.[31] There are many different types of detectors from a variety of manufacturers available that will be suitable to use with ACTIS. Practically, each of these detectors comes with its own software and data format. These data formats have to be converted into an input file by prACTISed. This conversion is done with a converter tool.

It is impossible to foresee and support all possible detectors and data formats. Therefore, we cannot provide a universal converter tool. However, we provide two converter tools, integrated into prACTISed, for a Beckman Coulter/SCIEX fluorescence detector (Karat32) and an API 5000 mass spectrometry/SCIEX detector (Analyst). Furthermore, we provide a working file preparation Python code that can serve as a template allowing users to quickly create their own converter tool for their respective detectors (see GitHub repository). A short guide on how to create a customized converter is provided in the Supporting Information (see Note S4).

Design Choices in prACTISed. A practical workflow must allow for (i) user friendliness, (ii) postanalysis data manipulation, (iii) ease of archiving and discussion of data, and (iv) adaptability of the workflow to the needs of different developers. These considerations informed many of our decisions when designing the software.

First, in prACTISed, user friendliness is achieved by providing a graphical user interface with a clear and simple input form where all the necessary parameters are collected at once at the start of the program (Figure 3B). Moreover, the graphical user interface error trapping is implemented in this input form allowing the user to be notified when information is missing or when invalid information is entered before any data processing starts. The simple input form and error trapping allows novice ACTIS users to become familiar with prACTISed. A video is provided as a quick starter guide to prACTISed (see page S1 of the Supporting Information for the location of the video).

Second, postanalysis data auditing and processing is facilitated by an Excel sheet that compiles all the inputs and outputs (Note S3). The inputs can be modified and reanalyzed by prACTISed. This feature allows a greater control of the data; for example, removing outliers from the data, changing the signal averaging time window, changing signal fitting parameters, etc., can all be done from within this single Excel sheet.

Third, a professional analysis report feature is included in prACTISed. The resulting reports provide a comprehensive overview of the results, i.e., input parameters, plots, binding isotherm, and signal averages (Note S5). This feature facilitates the sharing and discussion of results. Furthermore, results can be easily archived or provided to third parties, such as collaborators or customers.

Fourthly, prACTISed is designed as a set of interoperable open-source software tools; these software tools carry out the different steps depicted in Figure 3A of the prACTISed workflow. This software architecture, where different software tools are assigned to the different steps in the workflow, allows the tools to be independently modified and, therefore, easily accommodate any future changes and required updates. For instance, the nonlinear fitting tool is adapted for 1:1 binding but could also be adapted to 1:n binding. Comprehensive documentation based on code modification templates, thorough coding comments, and written guides are provided to help developers adapt the software tools to their needs (see GitHub repository, https://github.com/prACTISedProgram/prACTISed).

Workflow Validation. Any new workflow must be validated to ensure analytical integrity. Therefore, we validated the results of prACTISed by applying it to our previously collected and analyzed set of data. Ideally, there is no difference between the results from prACTISed and the original workflow; i.e., K_d values and treatment of graphs and signals should be identical for both workflows. Comparing the prACTISed results to those previously obtained with the original validated workflow, there was no difference in the treatment of graphs and signals used, leading to no difference in K_d values obtained (Table S1). This comparison outcome confirms the prACTISed workflow to be valid. This confirmation allows novice users to be confident in prACTISed results, without needing the expert user skills and the time-consuming analysis requisite in the original workflow.

■ CONCLUDING REMARKS

We report a practical workflow for streamlined data processing in ACTIS. This streamlined workflow is implemented as a set of open-source software tools called prACTISed using the Python programming language. The proposed workflow only requires user input into a graphical user interface and rapidly (less than 30 s) produces a comprehensive output, including graphs of publication quality. Moreover, prACTISed is a validated data analysis workflow allowing novice users to perform ACTIS data analysis without requiring expert user skills. Several prACTISed design considerations enhance its practicality, namely (i) a simple form input to facilitate a user-friendly user interface, (ii) an Excel sheet containing inputs and outputs for ease of post analysis data manipulation, (iii) a professional analysis report to allow discussion and archiving of the data, and (iv) an easily modifiable software architecture design that allows developers to adapt prACTISed to their specific needs. As an example, we provide a working template for creating a prACTISed converter tool to accommodate any detector that could be used in ACTIS (see GitHub repository). Comprehensive resources and guides are provided to help a novice user to get quickly started with prACTISed and an experienced developer to adapt the prACTISed software tools to their specific needs (see GitHub repository, https://github.com/prACTISedProgram/prACTISed). prACTISed removes a major obstacle for ACTIS becoming of an accessible method and will be an indispensable tool for ACTIS developers and users with varying levels of experience.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c05607.

Original data processing workflow (Note S1), prACTISed graphical user interface (Note S2, Figure S1), prACTISed working file (Note S3, Figure S2), prACTISed converter tool template (Note S4, Figure S3), prACTISed analysis report (Note S5, Figure S4), and comparison of K_d values from the original workflow and from prACTISed (Table S1) (PDF)

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Author Contributions

Jean-Luc Rukundo provided guidance on software development and led manuscript writing. Shiv Jain and Jessica Latimer planned and developed the data analysis script. Shiv Jain developed foundational scripts and Python workflows. Jessica Latimer designed and developed the GUI, working file preparation, signal compensation. and report software tools. Sven Kochmann provided guidance on software architecture and design. Sergey N. Krylov conceptualized and supervised the project. All authors contributed to writing the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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