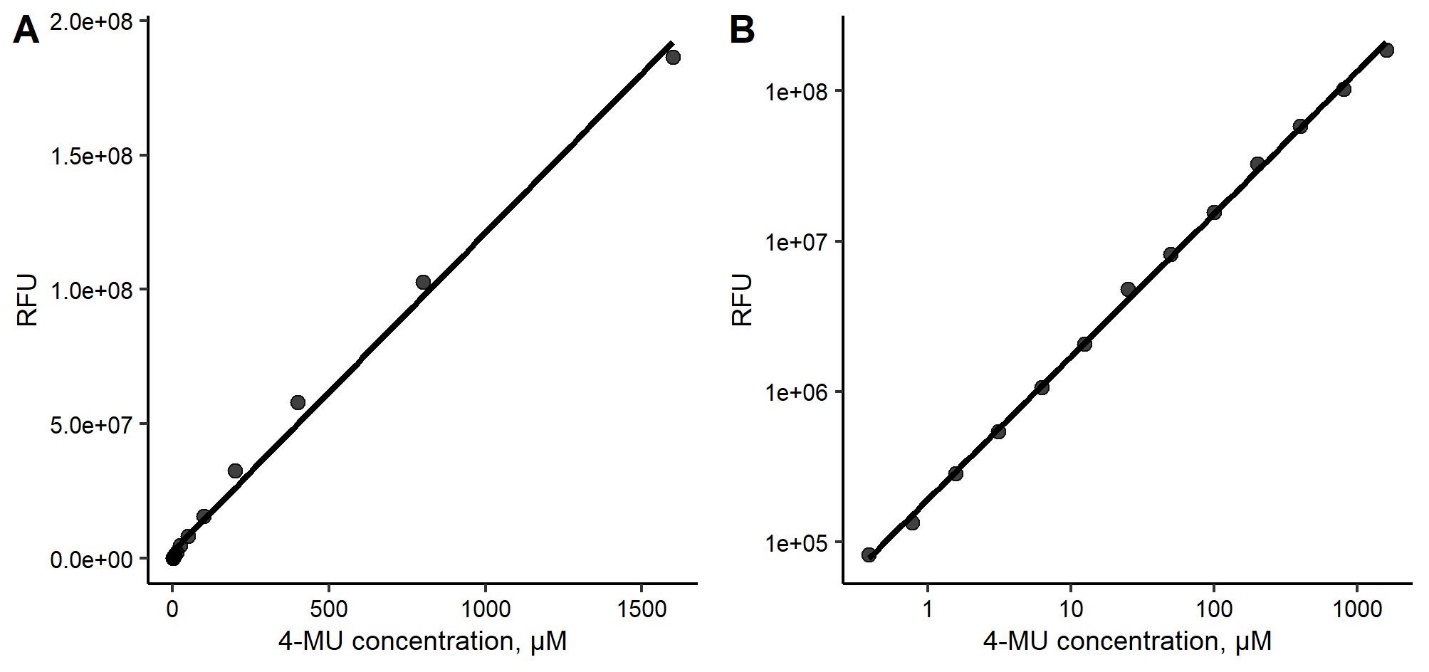
MUNANA App

A User Manual

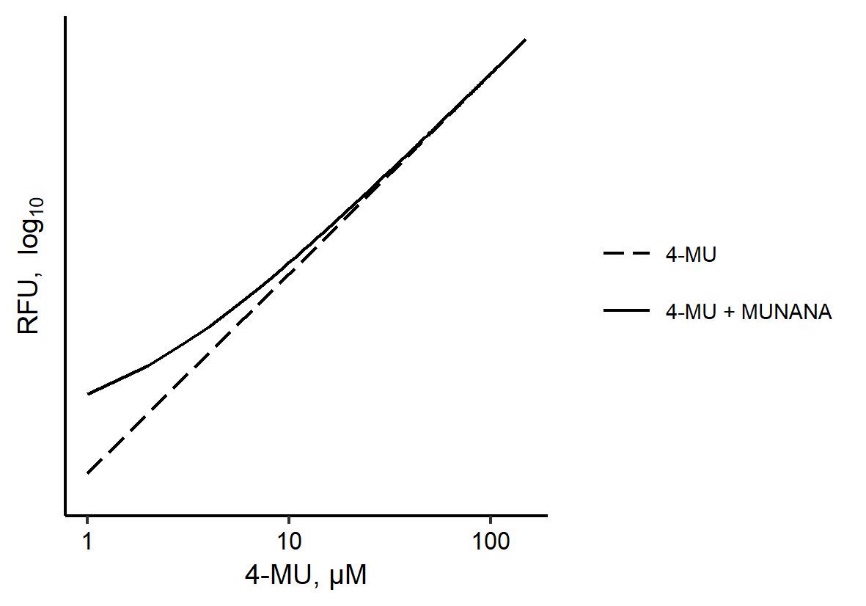
# Introduction

The MUNANA App is designed to study enzymatic activity of influenza A neuraminidase (NA) and the effects of specific monoclonal antibodies on its function in MUNANA assay. During the assay a weakly fluorescent 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUNANA) is converted to a brightly fluorescent 4-methylumbelliferone (4-MU). Measuring fluorescence intensities in real time allows one to estimate reaction velocities at different substrate concentrations. Knowing these parameters, one can calculate parameters of Michaelis-Menten equation (Km and Vmax) of the enzyme and how they change in the presence of antibodies.



*Fig. 1. Calibrating titration of 4-MU in linear scale (A) and log scale (B).*

General scheme of data processing in MUNANA assay is described by Marthe et al., 2013. Here we introduced two improvements to that algorithm. First, we log-transform optical signal and 4-MU concentrations from calibrating titration to build more precise models converting optical data into absolute 4-MU concentrations (Fig. 1). Nevertheless, a user can calculate linear models based on non-transformed data, by setting calibration method to ‘Linear’. Second, we use a more precise correction to eliminate the effect of weak substrate fluorescence on estimate of 4-MU concentration. In the original article, authors assume that during entire assay substrate concentration remains constant. Obviously, this is not true. In the beginning of the reaction, substrate concentration is maximal; meaning the contribution of its fluorescence to entire signal is also maximal. During the assay, NA converts the substrate; therefore, its contribution to fluorescence proportionally decreases over time (Fig. 2). Knowing the fold difference in brightness of the substrate and product, one can make a correction of original data to eliminate the effect of substrate fluorescence. This application allows a user to estimate the difference between substrate and product fluorescence and use this number for data correction.



*Fig. 2. Contribution of MUNANA fluorescence into optical signal from reaction.*

The user can estimate values of Km and Vmax, the parameters of Michaelis-Menten equation, fitting non-linear least-squares models to obtained velocity data.

All plots and data could be saved into a MS Word file or exported as an Excel table for further analysis.

# How to install

This application is developed using R programming language. However, it also relies on Java. Both should be installed on a computer to launch it.

The following instruction is valid for Windows machines:

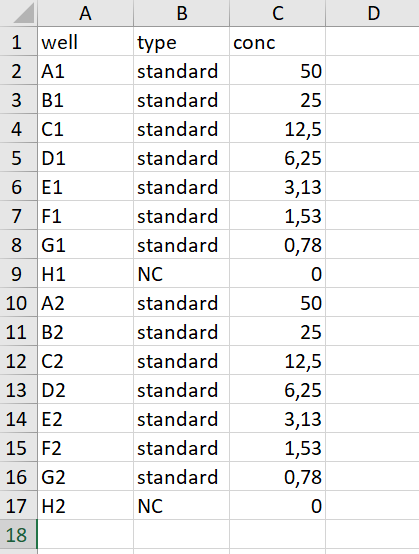
1. Install R (version 4.0.5 or higher): <https://cran.r-project.org/bin/windows/base/>
2. (*Optional*) Install RStudio: <https://www.rstudio.com/>
3. Install Java for Windows (64 bit version) if it is not already present on the machine: <https://www.java.com/en/download/manual.jsp>
4. Copy the folder containing R scripts into desired location.
5. Execute before\_first\_start.R script. Pay attention if the rtools package is properly installed (no warning messages popped up). If it is not installed, try to install it separately from here: <https://cran.r-project.org/bin/windows/Rtools/>. Then re-run before\_first\_start.R script.
6. Open the App.R script in RStudio or any other text editor. On the top of the file, change the path variable to the current location of this file. Pay attention to the direction of slash symbols (should be ‘/’, not ‘\’).
7. Repeat the same procedure with run.r file.
8. Open the MUNANA App.bat file in any text editor. Change the first address for the location of R.exe file in your machine, and the second address for current location of the run.r file.
9. Double click on MUNANA App.bat file to launch the application. It should appear in default web browser.

# How to use

All necessary data is provided in three Excel files: standard table, sample table, and RFU data table.

**Standard table**

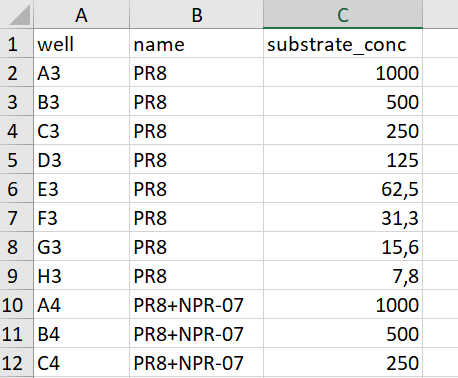
This file describes the location of well containing serial dilutions of the reaction product with known concentrations as well as wells containing working buffer only (Fig. 3). The table must contain three columns: ‘well’, ‘type’, and ‘conc’. The first column contains well names (start with capital letters). Second column has one of two values: either ‘standard’ (for wells containing reaction product) or ‘NC’ (for wells containing working buffer). The ‘conc’ column contains concentrations of the reaction product in μM.



*Fig. 3. An example of a standard table.*

**Sample table**

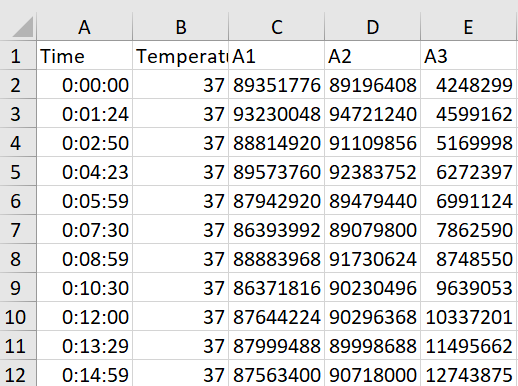
This file contains a table with three columns: ‘well’, ‘name’, and ‘substrate\_conc’ (Fig. 4). The first column is similar to one from first file (but there should no common wells in two files belonging to one experiment). The ‘name’ column contains names of samples studied in the experiment. All wells containing the same sample must have identical names; otherwise, they recognized as different samples. The last column contains concentrations of the substrate present in wells. Samples could be analyzed in duplicates, triplicates, etc. The application calculates reaction velocities for each well independently.



*Fig. 4. An example of a sample table.*

**RFU data table**

RFU table is an Excel file containing optical data from a spectrophotometer (Fig. 5). It should contain columns of two kind: ‘Time’, containing time points at which the measurements were taken, and well names with optical data. Any other columns, if present, are negated. This table could be directly obtained from the software of the SpectraMax i3x reader. The user must specify time format used in this file before uploading it into the application. By default, it has an Excel format, meaning it is presented as a fraction of a day passed since the moment the assay started and normally represented in Excel in H:MM:SS format. However, time information could be provided in seconds, minutes, or hours. The user should specify time format before uploading the file.



*Fig. 5. An example of RFU data table.*

Templates for all three tables could be generated by the application. The user must specify the ranges of columns used in each of them and hit ‘Save Template’ button.

**Reaction velocities calculation**

When the user uploads three necessary files to the app, first 10 rows from each table appear in the right section of the first page. If the files meet application’s requirements, no error messages appear instead of them. Next, the user should navigate to the ‘Standard’ tab. On the left side, a selection dialog will appear which allows to use either log-linear or liner method to calculate calibration models. By ticking ‘Brightness Correction’ box, the user can introduce correction for substrate fluorescence, which makes velocities estimates more precise. To do this, the user should specify the difference in brightness of the substrate and product (see below for the instructions how to estimate this value).

Standard plot could be displayed in two modes: to show averaged or individual data (if calibrating titrations have been made in duplicates, triplicates, etc).

Below the standard plot, three plots representing main parameters of linear regressions appear. They show how intercept, slope, and R2 change over the time of the assay. By default, the Y scales is adjusted automatically to show all data. However, a user can change it by clicking on corresponding tick boxes and entering the upper and lower limits of the Y axis.

Navigating to the ‘Progress curves’ tab, the user can see progress curves generated during the assay. They show accumulation of the reaction product (in μM) in sample wells. Three modes of this plot are available. By default, the application shows all progress curves for each sample in a separate panel. By selecting ‘All in one’, the user may put all the curves in one plot, or by selecting ‘By sample & concentration’, the user can generate a series of plots for each level of substrate concentration for each sample.

Reaction velocities are calculated by fitting quadric regression models to each curve (y = ax2 + bx + c). The b coefficients represents reaction velocities during the entire assay if curves are linear. If the curves bent down, it reflects only initial velocities. To see them on the plots, the user should click on ‘Show velocities’ check box.

**Michaelis-Menten models**

Michaelis-Menten models are calculated by fitting non-linear least-squares models to the velocity data. To do this, the user should estimate approximate values of Vmax and Km by looking at a plot showing relationship between substrate concentration and reaction velocity and enter their guesses into the corresponding fields. Next, the application improves them based on real data and provides the result in the right part of the screen. If default guesses are not valid, an odd shaped curve may appear on the plot. To remove it from the plot and see raw data only, the user may tick off ‘Show model’ check box underneath the plot. This cycle should be repeated for each sample, since initial guesses of Km and Vmax values could be not valid for each sample. Adjusted Vmax and Km values are stored in the ‘Result table’. They are updated each time the user changes initial guesses for Km and Vmax, which may result in change of adjusted values.

**Michaelis-Menten plots**

The ‘Plot’ section contains a figure comparing all Michaelis-Menten curves generated on the previous step. By ticking on and off the check boxes on the left, the user can compare curves of desired samples. If all the check boxes are ticked off, the application shows all curves again.

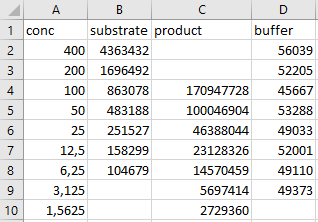
Underneath the plot with Michaelis-Menten curves, there is a scatter plot showing Km and Vmax values from selected samples. To the right of the plots, a fraction of the result table is shown. It contains the data only from selected samples.

**Saving report in the Word file**

Moving to the ‘Report’ tab, the user can save generated plots and calculated data in a Word file. He or she can enter the title of the experiment, select its data, and tick on items to include into the report. Generated file contains plots in the way they are presented in the application at the time when the user clicks the ‘Save Report’ button.

**Estimation of the brightness difference of reaction substrate and product**

To estimate the brightness difference of reaction substrate and product, the user should make serial dilutions of both and make several wells with working buffer only. Then, optical signal from all the wells should be measured using the working settings of the plate reader. Collected data should be provided to the application in the form of an Excel file containing a table of four columns: ‘conc’, ‘substrate’, ‘product’, and ‘buffer’ (Fig. 6). The first column should contain concentrations of the substrate or product (in μM). The ‘substrate’ and ‘product’ column should contain optical signals from wells with substrate and product, respectively. The last column should contain optical data obtained from wells with working buffer only.

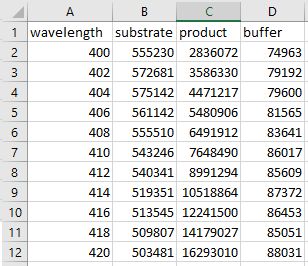


*Fig. 6. An example of a table with optical signals from reaction substrate and product.*

When the file with data is uploaded to the application, a plot showing titration of the substrate and product is generated. Two corresponding regression linear models are generated assuming that the slopes of two lines equal to -1, meaning dilution of the substances twice leads to a double reduction in their brightness and any deviation from this rule is random. The difference between the intercepts of two models represents the difference in the brightness of two substances.

**Spectrum analysis of substrate and product**

To carry out a fluorescence spectrum analysis of reaction substrate and product, the user should measure their optical signal at different wavelengths and upload this data to the application in a form of an Excel file. This file should contain four columns: ‘wavelength’, ‘substrate’, ‘product’, and ‘buffer’. All these columns should contain optical signal from corresponding substances recorded at indicated wavelengths. The application automatically determines maximal fluorescence intensity of the reaction product and marks it by vertical line. The user can specify the bandwidth a spectrophotometer will use to measure optical signal during assays. It is represented on a plot as a grey area.



*Fig. 7. An example of a table with optical signals from reaction substrate, product, and working buffer at different wavelengths.*

# References

1. Marathe BM, Lévêque V, Klumpp K, Webster RG, Govorkova EA. Determination of neuraminidase kinetic constants using whole influenza virus preparations and correction for spectroscopic interference by a fluorogenic substrate. PLoS One 2013 Aug 15;8(8):e71401.