Using the VICTOR Nivo for cell-based HTRF assays.

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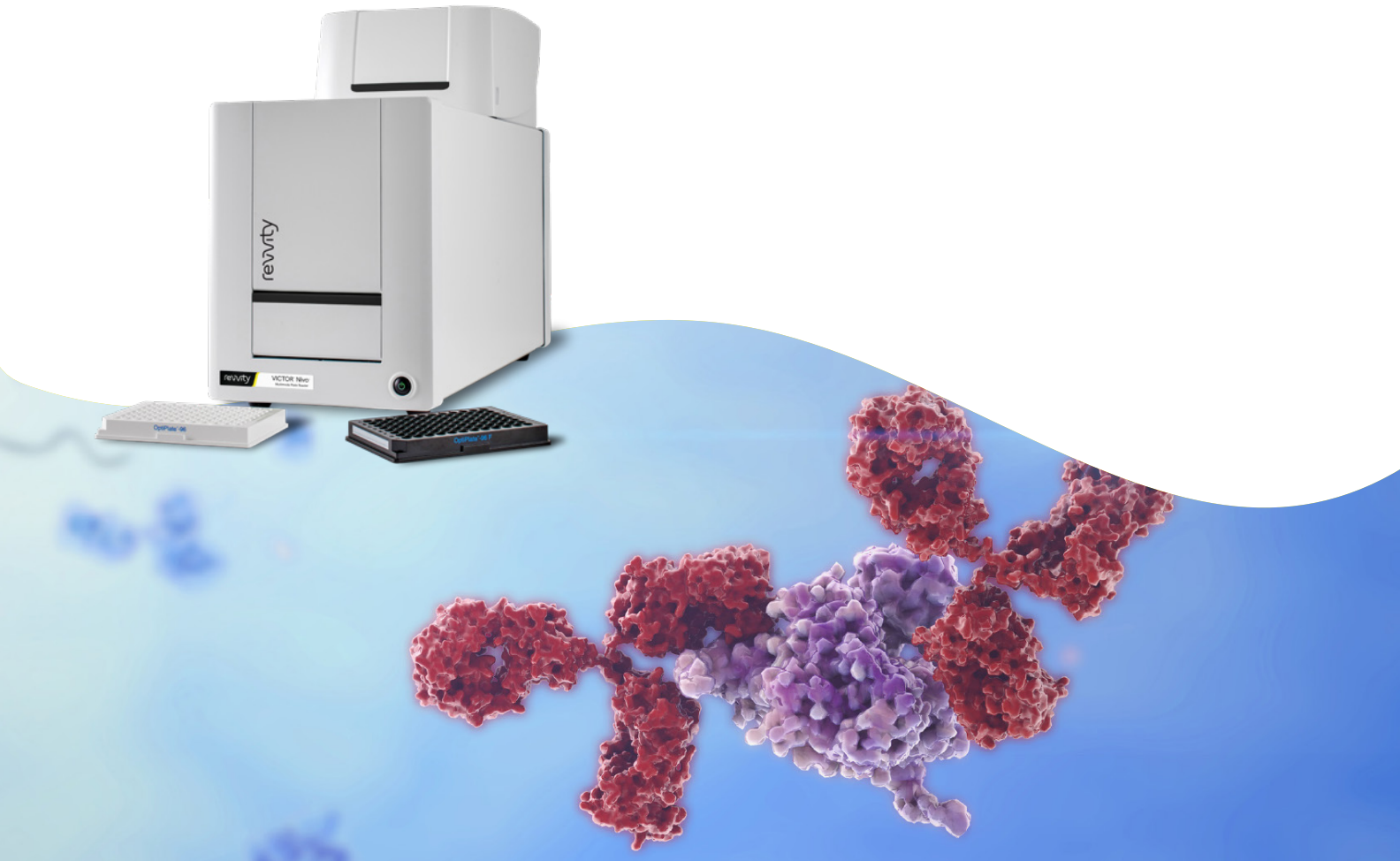
Introduction

Cell-based assays are routinely employed in R&D to reveal compound induced effects *in vitro*. In this technical note, we describe the usage of the VICTOR® Nivo™ multimode plate reader for alpha-tubulin acetyl-K40 detection in a cellular assay based on HTRF® technology (Figure 1). Tubulin acetylation is widely studied due to its dysregulation in human neurological disorders.[1] αβ-tubulin heterodimers form microtubules that are essential polymers in the cytoskeleton. Via posttranslational modifications (PTMs), cells regulate microtubule structure and function. Acetylation of α-tubulin on K40 plays a key role among other PTMs.[2]

To demonstrate the HTRF assay principle on the VICTOR Nivo, we used mouse fibroblasts (NIH-3T3 cells) for which increased tubulin acetylation after inhibition of HDAC6 was shown already.[3] We performed two experiments on the VICTOR Nivo: first, we evaluated the optimal cell number for the assay and secondly, we investigated the effect of the test compounds Trichostatin A and Tubacin in the alpha-tubulin acetyl-K40 HTRF assay.[4]



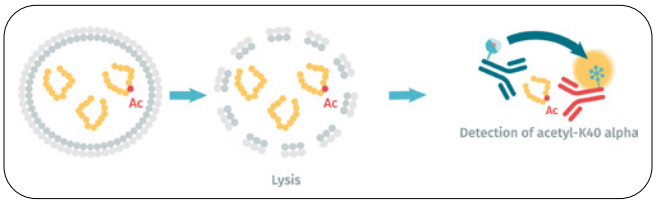


Figure 1: alpha-tubulin acetyl-K40 HTRF Assay Principle. For cell-based HTRF lysates are prepared after incubation of NIH-3T3 cells with test compound and transferred to a 384-well assay plate. In a next step, anti-alpha-Tubulin acetyl-K40-Tb^{3+} Cryptate and anti-alpha-Tubulin acetyl-K40-d2 antibodies are added to the lysates. After formation of the antibody-analyte-sandwich and upon excitation of the donor, energy will be transferred to the acceptor resulting in a specific FRET signal.

Material and methods

Cell culture

For the assay NIH-3T3 (#CRL-1657, ATCC), cells were cultured in DMEM (# MEM-XA, Capricorn), 10% FCS, 2 mM L-Glutamine, 100 µg/mL Streptomycin, 100 Units/mL Penicillin. For cell splitting, DPBS was used to rinse the cells and Trypsin-EDTA (0.05%) in DPBS was used for detachment. The cells were cultivated in an incubator at 37 °C, 5% CO_2 and 90% humidity.

Cell number optimization

To evaluate the optimal cell number for the alpha-tubulin acetyl-K40 HTRF assay (#63ADK072PEG, Revvity), NIH-3T3 cells were harvested and counted. The cell suspension was diluted further in standard culture media to prepare samples in a range from 50 000 down to 3 125 cells/well. Of these samples, 100 µL/well were transferred into a 96-well CulturPlate (#6005680, Revvity). The plate was placed into a cell incubator for two days.

After incubation, the HTRF assay was conducted according to the manufacturers' protocol.[5] In short, cells were lysed within 30 minutes using the lysis buffer contained in the assay kit and 16 µL/well of the lysates were transferred into a white 384-well OptiPlate (#6007290, Revvity).

The Anti-alpha-Tubulin acetyl-K40-Tb^{3+} Cryptate antibody and Anti-alpha-Tubulin acetyl-K40-d2 antibody were diluted in Detection buffer. Of each antibody solution, 2 µL/well were added to the cell lysates.

Next to the cell lysates, the following controls were included in the experiment:

- **Positive control:** control lysate + Anti-alpha-Tubulin acetyl-K40-d2 + Anti alpha-Tubulin acetyl-K40-Tb^{3+} Cryptate
- **Negative control:** lysis buffer + Anti-alpha-Tubulin acetyl-K40-d2 + Anti alpha-Tubulin acetyl-K40-Tb^{3+} Cryptate
- **Blank control:** cell lysate + detection buffer + Anti alpha-Tubulin acetyl-K40-Tb^{3+} Cryptate

In this technical note, we will refer to the 'positive control' as 'control lysate' to avoid misinterpretation of results.

After two hours incubation at room temperature, the HTRF signal was detected on the VICTOR Nivo using the filter settings presented in Table 1.

Table 1: HTRF protocol settings for the VICTOR Nivo.

OPERATION	TRF-EndPoint
MEASUREMENT UNIT	COUNTS
MEASUREMENT TYPE	Dual emission
EXCITATION FILTER	320/75nm
EMISSION FILTER (1)	620/10nm
EMISSION FILTER (2)	665/8nm
DICHROIC MIRROR 1	D400
DICHROIC MIRROR 2	D400
DELAY TIME (µs)	70
EMISSION TIME (µs)	200
MEASUREMENT DIRECTION	Top measurement
MEASUREMENT TIME (ms)	500
Z-FOCUS (mm)	8.5
EXCITATION SPOT SIZE (mm)	2
EMISSION SPOT SIZE (mm)	2
FLASH ENERGY (mJ)	10
MEASUREMENT ORDER	Bi-directional by rows

Compound testing

The compounds Trichostatin A (#89730, Cayman Chemical) and Tubacin (#13691, Cayman Chemical) were selected to demonstrate the assay principle on NIH-3T3 cells. Both compounds are reported to inhibit HDAC6 thereby causing an increase in cellular microtubule acetylation.[3,4]

NIH-3T3 cells were seeded at 12 500 cells/well in 50 µL standard media on the first day of experiment. After overnight incubation, the test compounds were added at 50 µL/well. A separate 96-well plate was used for each test compound. Both compounds were dissolved in DMSO and 12-point dilution series were prepared in media (0.5% final DMSO concentration). Trichostatin A was tested in a range of 1.4 x 10^{-11} - 2.5 x 10^{-6} M and Tubacin in a range of 1.4 x 10^{-9} - 2.5 x 10^{-4} M.

DMSO was tested as vehicle control. In addition, some wells with cells were left untreated.

After further 18 hours of incubation, the assay was conducted as described already for the cell number optimization experiment. The plate layouts for the assay are shown in Figure 2.

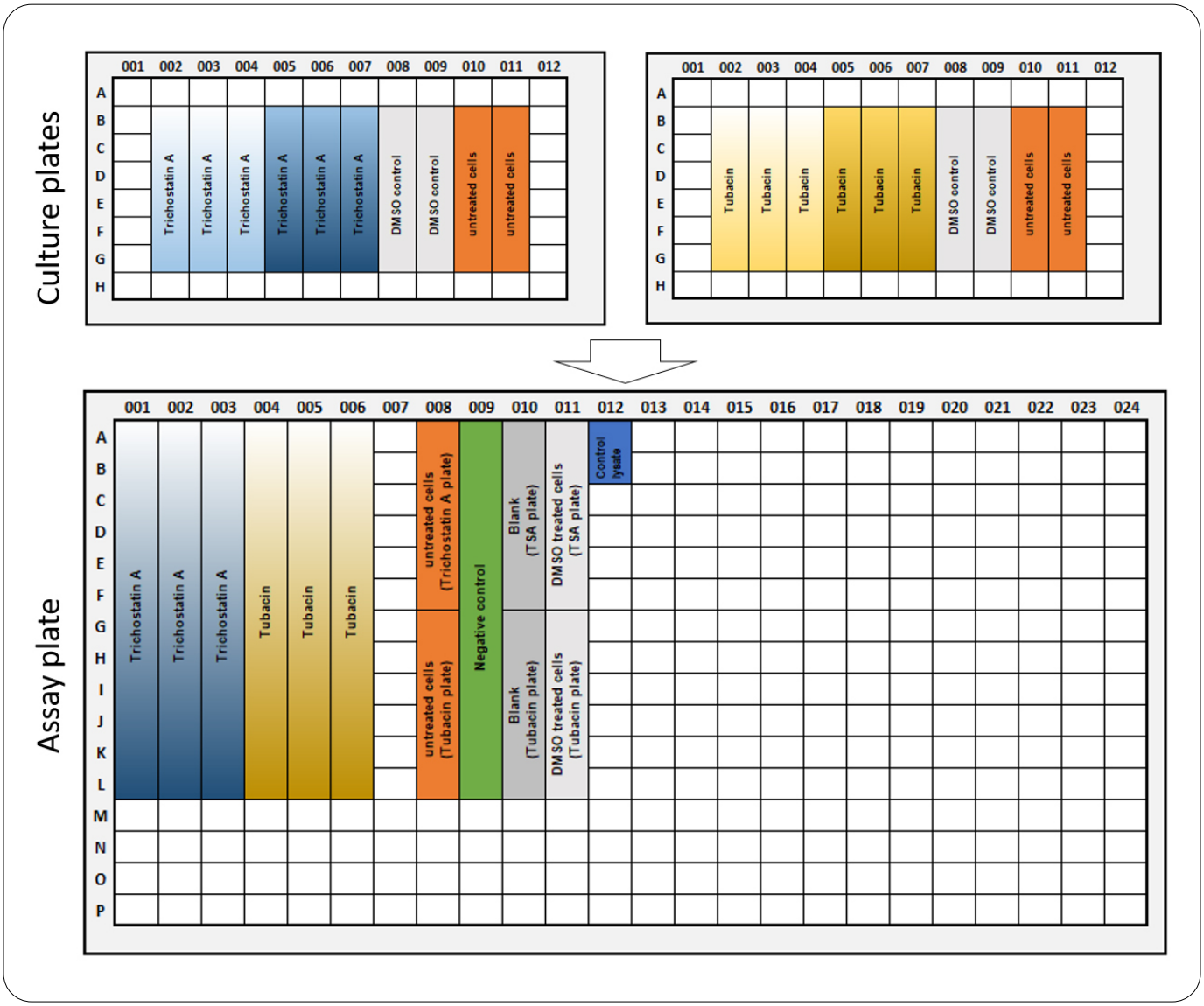


Figure 2: Plate layout for compound profiling in the alpha-Tubulin acetyl-K40 HTRF assay. The compounds Trichostatin A and Tubacin were tested in 12-point dose response on NIH-3T3 cells in 96-well CulturPlates (triplicates per sample, concentration decreasing from the top to bottom wells). Lysates were transferred to a 384-well assay plate for the HTRF readout.

Data analysis

Data analysis was conducted using MyAssays® Desktop (version 8.2.28.751). Results are easily imported from the VICTOR Nivo into MyAssays® Desktop and data is processed automatically according to a predefined protocol. This allows standardized data analysis also for multiple assay plates. A step by step description for the configuration of a data analysis in MyAssays® Desktop is provided in the appendix.

Results

During the cell density optimization experiment, a set of controls as well as lysates from different cell densities were tested to prove assay performance. As a guideline, the assay manual states that the window between control lysate and negative control is acceptable greater than two. As shown in figure 3, the ratio was greater than four in our experiment. The blank control, which contained cell lysates but only the anti-alpha-Tubulin acetyl-K40-Tb^{3+} Cryptate antibody produced results comparable to the negative control, due to the incomplete FRET pair.

We tested lysates of five different NIH-3T3 cell densities. The HTRF ratios obtained were as expected density-dependant (Figure 3). As recommended in the assay protocol, a cell seeding density of 12 500 cells/ well also proved to be suitable for NIH-3T3 cells and was therefore selected for further experiments.

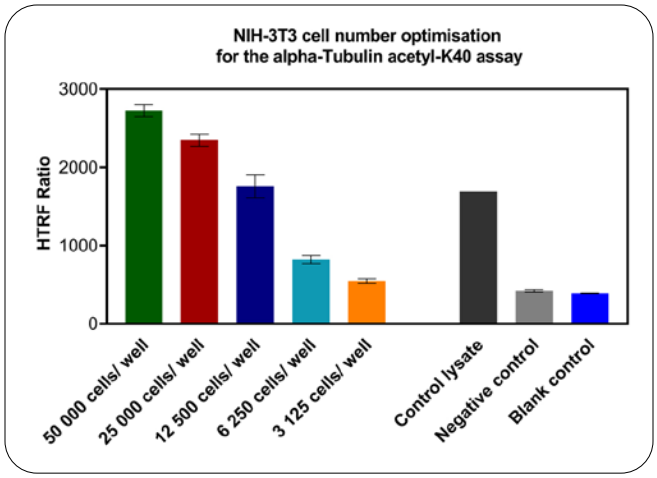


Figure 3: Results of the NIH-3T3 cell density optimization. Cell densities in a range of 50 000 down to 3 125 cells/well were tested in the alpha-Tubulin acetyl-K40 HTRF assay after 2 days of incubation. For each sample apart from the control lysate, the mean and standard deviation of three wells are shown. Of the control lysate, one well was tested due to the limited volume available.

In a second experiment, two compounds were tested in dose response. The experiment was repeated once to check reproducibility of results. Since the test compounds were dissolved in DMSO, this solvent was used as vehicle control (Figure 4, A). From the dose response curve fit, the following EC_{50} values were determined: EC_{50} (Trichostatin A)= 40.5 ± 0.2 nM and EC_{50} (Tubacin)= 4.95 ± 0.84 uM. For comparison, the assay manual reports an EC_{50} value of 16.71 nM for Trichostatin, and 2.723 uM for Tubacin, respectively, which is in the same order of magnitude.[5]

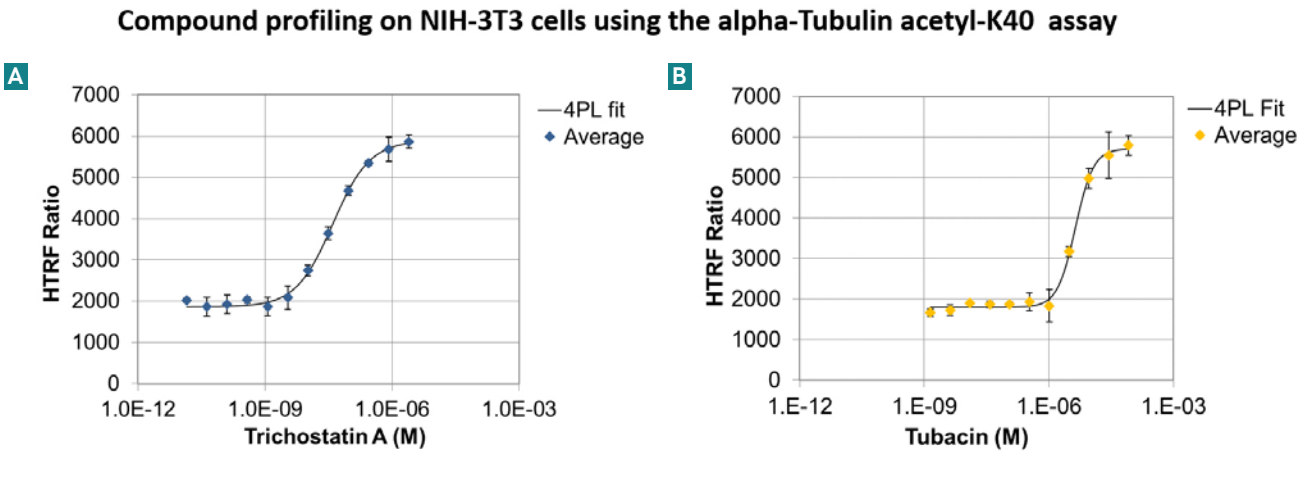


Figure 4: The compounds Trichostatin A (A) and Tubacin (B) were tested in 12-point dose response experiments in NIH-3T3 cells. The highest Tubacin concentration precipitated in media, therefore this sample was excluded from analysis. For each data point, the mean and standard deviation of three wells are shown.

Conclusion

The VICTOR Nivo is a robust plate reader of small footprint equipped with all major detection technologies: Luminescence, Fluorescence Intensity, Absorbance, Fluorescence Polarization, Alpha and Time-Resolved Fluorescence like HTRF, LANCE® or DELFIA®. We found the cell-based alpha-Tubulin acetyl-K40 HTRF assay kit easy-to-use and it was highly suitable to demonstrate the HTRF detection mode on the VICTOR Nivo. The plate reader proved to be robust across the set of experiments. The available setup recommendations facilitated the setup of a new HTRF measurement protocol with no further adjustment required by the user.[6] In conclusion, we could show that the HTRF certified VICTOR Nivo optimally supports the application of HTRF kits.

References

1. Li L, Yang XJ. Tubulin acetylation: responsible enzymes, biological functions and human diseases. Cell. Mol. Life Sci. 72, 4237–4255 (2015). https://doi.org/10.1007/s00018-015-2000-5.

2. Eshun-Wilson L, Zhang R, Portran D, Nachury MV, Toso DB, Löhr T, Vendruscolo M, Bonomi M, Fraser JS, Nogales E. Effects of α-tubulin acetylation on microtubule structure and stability. Proc Natl Acad Sci U S A. 2019 May 21;116(21):10366-10371. doi: 10.1073/pnas.1900441116. Epub 2019 May 9. PMID: 31072936; PMCID: PMC6535015.

3. Zhang Y, Li N, Caron C, et al. HDAC-6 interacts with and deacetylates tubulin and microtubules *in vivo*. EMBO J. 2003;22(5):1168-1179. doi:10.1093/emboj/cdg115

4. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A. 2003 Apr 15;100(8):4389-94. doi: 10.1073/pnas.0430973100. Epub 2003 Apr 3. PMID: 12677000; PMCID: PMC153564.

5. Revvity Bioassays' alpha-Tubulin acetyl-K40 assay protocol: https://www.cisbio.eu/media/asset/c/i/cisbio_dd_pi_63adk072peg-63adk072peh.pdf (web access 8th Dec 2020).

6. Revvity Setup recommendations for VICTOR Nivo: https://www.revvity.com.

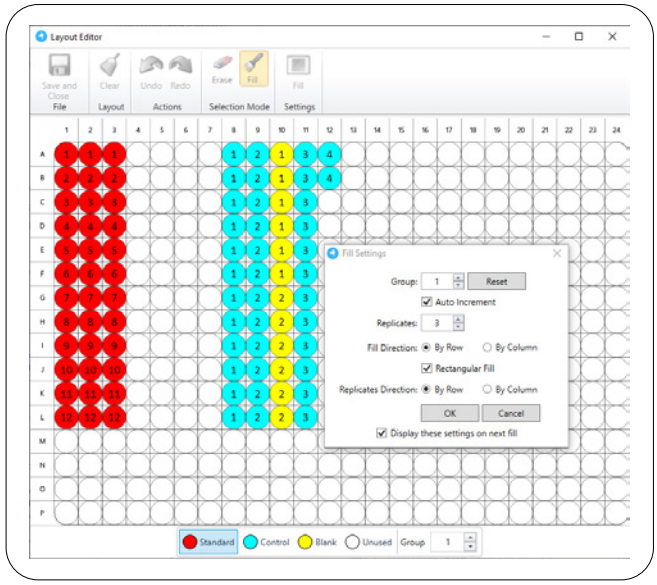
7. https://www.myassays.com.

APPENDIX – How to set Up a protocol in MyAssays® desktop for the analysis of a HTRF dose response curve

1. This protocol was created with MyAssays® Desktop version 8.2.28.751. For more information **visit www.myassays.com.**

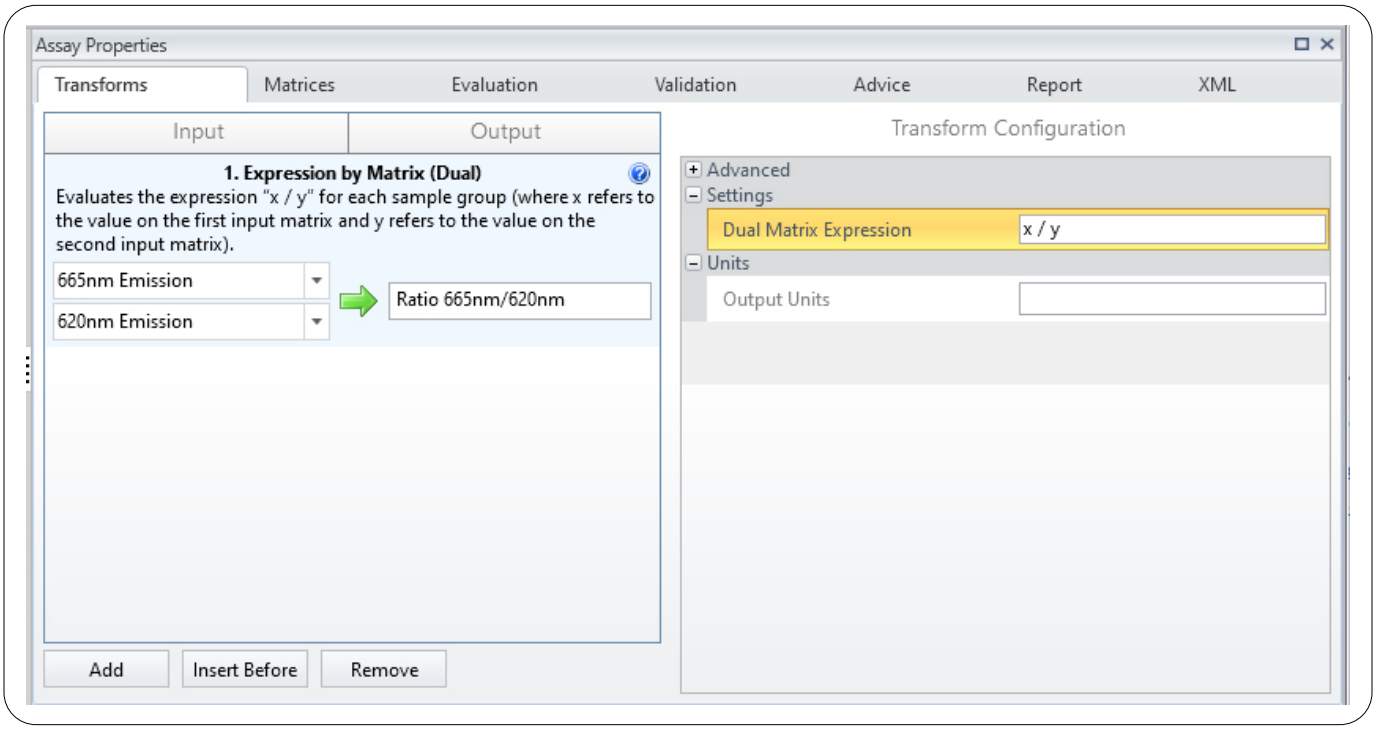
2. In the MyAssays® Explorer, choose **"New Protocol…"**. When the New Protocol Wizard opens, select the option "My data is in a file or spread across multiple files" and select the file containing your measurement data on the next page. The data will be recognized by MyAssays® Desktop including information about the measurement details.

3. In the **"Layout Editor"**, you can select what kind of samples are present on the plate. Select "Standard", "Control" and "Blank" and edit the plate layout. The inhibitor dilution series was defined as the standard in our analysis with 12 groups representing the triplicates of the dilutions. The four control groups and the blank were also defined on the plate map.

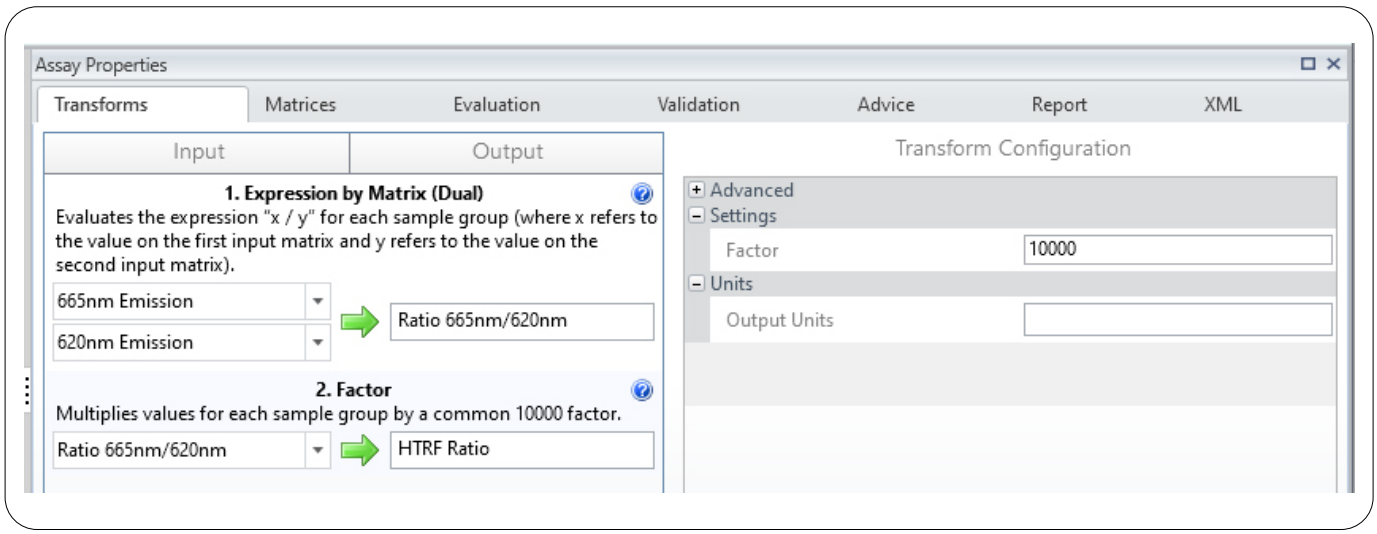


4. Finish the New Protocol Wizard and **"launch"** the protocol.

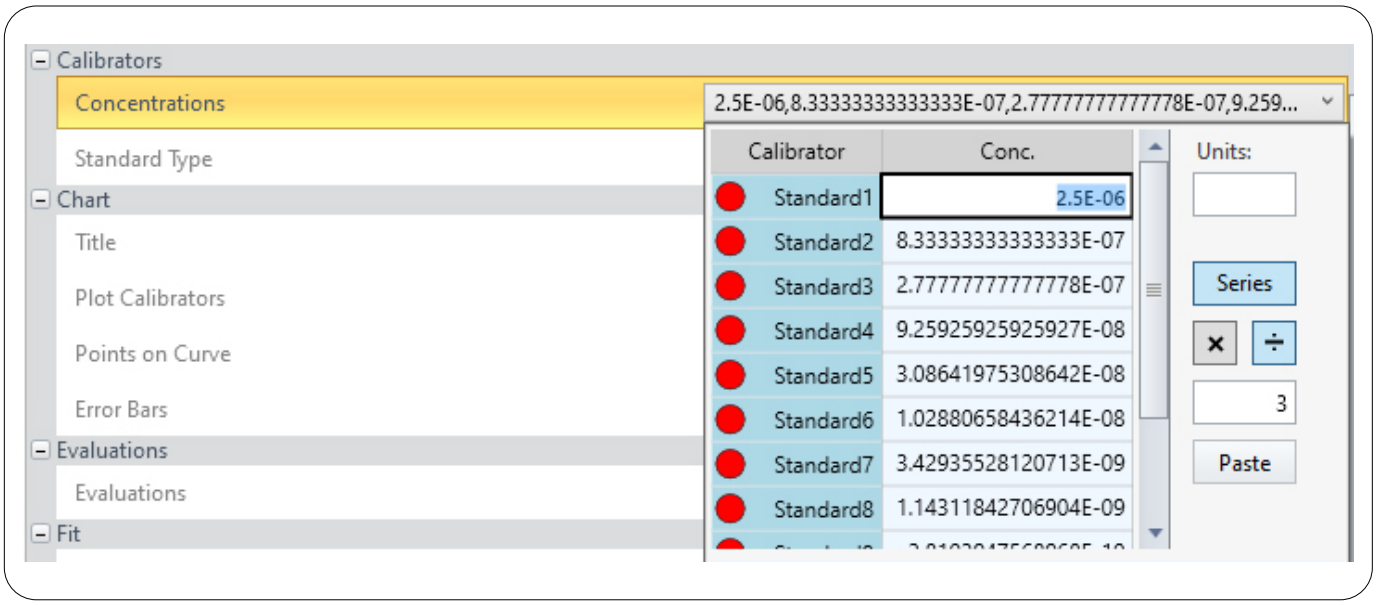
5. Select assay properties to define your analysis in the **"Transforms"** tab. To calculate the ratio of the acceptor and donor emission, add the "Expression by Matrix (Dual)" transform. Select the raw data of the emission at 665 nm as input matrix "x" and at 620 nm as input matrix "y" and define a name for the output matrix (e.g.: "Ratio 665nm/620nm"). Under "Transform Configuration/ settings", define the "Dual Matrix Expression" as "x / y".



6. To calculate the final HTRF ratio, add a **"Factor"** transform step. Select the matrix "Ratio 665/620" and define the Factor (Transform Configuration/settings) as 10 000 to multiply each value in the matrix by 10 000 and define a name for the output matrix (e.g. "HTRF Ratio").



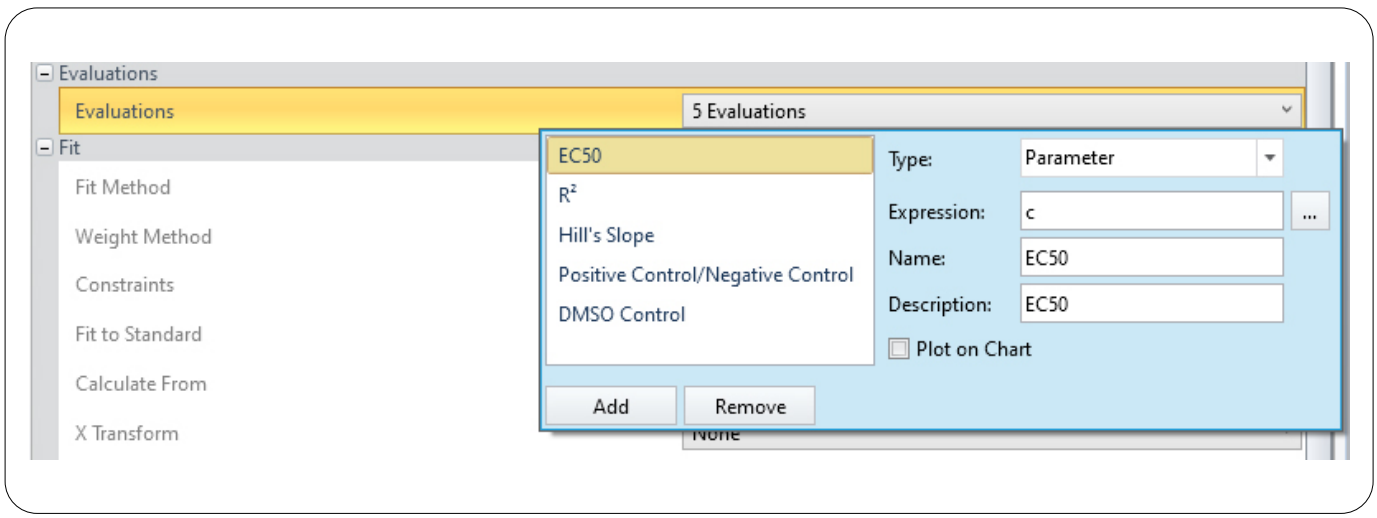
7. To plot the dose-response curve, add the transform “Standard Curve Fit”. Under “Transform Configurations”, many properties of the plot can be customized. Under **“Calibrators -Concentration”,** the dilution series can be defined by setting the concentration for the first group present in row A and the dilution factor.



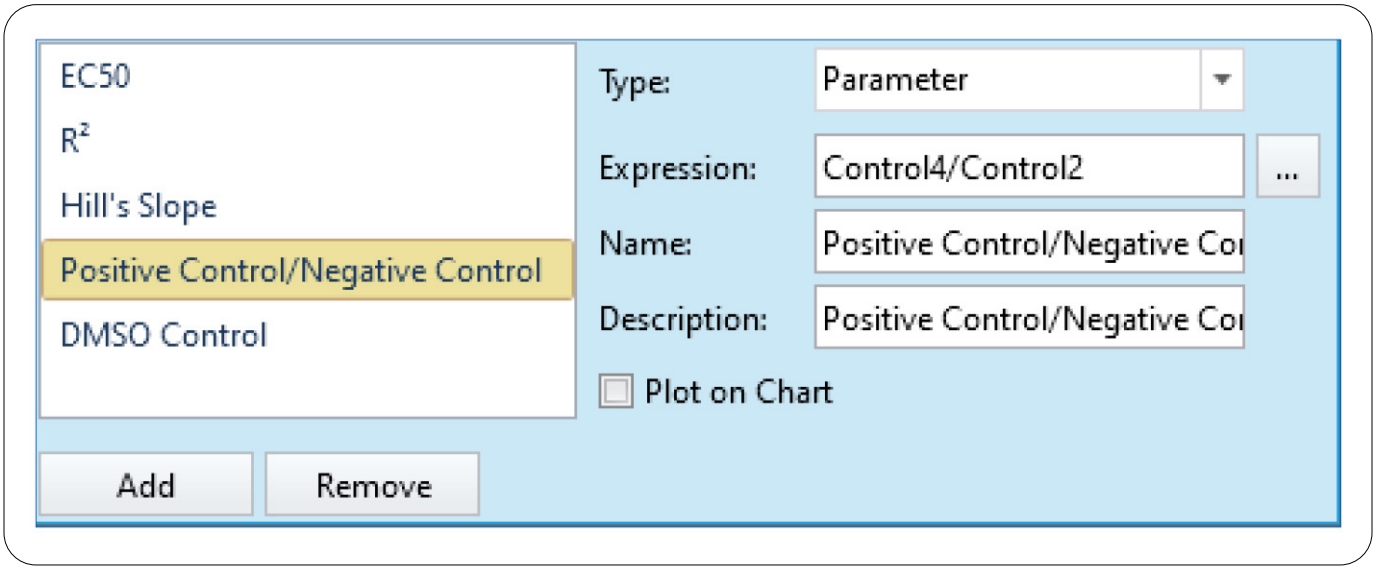
8. Select 4PL as Fit Method which will numerically solve the following equation: $y = d + \frac{a - d}{1 + (\frac{x}{c})^b}$

   Parameter “c” of this equation is also known as EC_{50} and **“b”** is equivalent to the Hill’s Slope of a sigmoidal curve.

9. In the final report, the parameters of the equation used for the respective fit method will be displayed in a table. We recommend adding the following optional evaluations to the Standard Curve Fit transform since not all users of the protocol may be familiar with the parameters of the equation. Under **“Transform Configuration - Evaluations”,** you can define additional parameters and calculations which will be displayed in the report. MyAssays® Desktop uses “expressions” to access parameters or whole groups of samples and to perform calculations with them. To display the $EC_{50,}$ define an evaluation of the type “Parameter” with the description and name “EC 50” and the expression “c”.



For the Hill's Slope, the expression is "d" and to display the maximum and minimum value of the distribution the expression is "max(a,d)" and "min(a,d)". The expression "R2" will add the coefficient of determination (R^2). To display the ratio between control lysate and negative control, add an evaluation where the expression is "Control4/Control2", and to display the average value of the DMSO controls, the expression "Control3" is used (the numbering depends on your initial definition of the different control groups and has to be adapted accordingly).



This setup will result in a report including the plotted fit and a table which displays EC_{50}, R^2, Hill's Slope, the bottom and top limitation of the distribution, the ratio between control lysate and negative control and the average of the DMSO control.

Several other evaluations and validations can be added (for details, check the MyAssays® Desktop manual and tutorial videos).[7] *Under* ***"Assay Properties - Report",*** *you can also decide which graphs and tables will be displayed in the report and define the print scaling and font.*

10. If the protocol setup is done, the analysis can be started by selecting ***"calculate"*** and a report is created which can be exported as a PDF, Excel or Word file. For consecutive analyses, new experimental data can be imported into this protocol and analyzed with these settings. If required, the plate map or other settings can easily be adapted to new experimental conditions.

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