# Pyruvate kinase inhibitor measurements and effortless data analysis using the MARS data analysis software

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- **Pyruvate kinase enzyme activity assay using real-time detection of fluorogenic substrate conversion**
- **MARS data analysis software enables simple evaluation of $IC_{50}$, slope and $R^2$**
- **EDR feature of the VANTAstar™ avoids signal saturation during the entire measurement period**

## Introduction

Enzymes are proteins which can catalyse biochemical reactions. They are particularly important due to their role in the synthesis, modification, and degradation of organic matter. Enzymatic reactions can range from the cleavage of intra-molecular bonds, transfer of electrons, ligation of molecules to the transfer of functional groups. In some cases, enzymatic activity can even be used as biomarker for diseases such as pancreatitis.

Pyruvate kinase is an enzyme involved in energy-supplying glycolysis and ATP synthesis. It catalyses the transfer of a phosphate group from phosphoenolpyruvate to adenosine diphosphate (ADP), yielding pyruvate and ATP. Pyruvate kinase thus is pivotal for the eucaryotic energy metabolism.

In addition, overexpression of pyruvate kinase is believed to play a role in cancer. Here, elevated levels of the PKM2 isoform of pyruvate kinase are used as a cancer marker and are associated with unfavourable prognosis. Accordingly, the development of pyruvate kinase inhibitors may offer new treatments for various cancer diseases.

This application note highlights the benefits of BMG LABTECH's VANTAstar™ microplate reader and the MARS data analysis software for the effortless application of a pyruvate kinase activity assay and its kinetic evaluation. This assay can be employed for the screening and identification of pyruvate kinase inhibitors.

## Assay Principle

To test for pyruvate kinase inhibitors, kinetic measurements in the presence of increasing concentrations of pyruvate kinase inhibitor were performed. For this purpose, a pyruvate kinase activity assay kit and human recombinant pyruvate kinase were used as sources of pyruvate kinase substrate and activity. This assay is a coupled enzyme assay using two enzymatic reactions (fig. 1). First, pyruvate kinase transfers the phosphate group from phosphoenolpyruvate to ADP, yielding pyruvate and ATP. Pyruvate is then used in a second enzymatic reaction by the enzyme peroxidase. This results in a fluorometric product, proportional to the pyruvate present. If a pyruvate kinase inhibitor is added, the amount of produced pyruvate and accordingly the amount of fluorometric product is reduced, resulting in lower fluorescence signals.

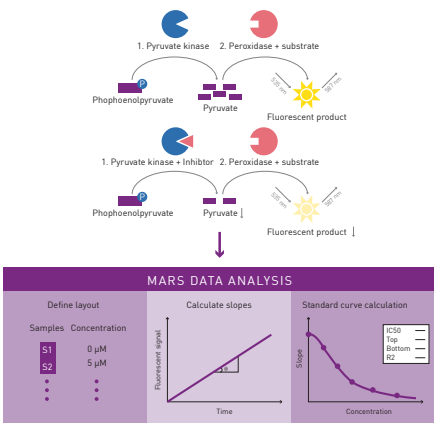


**Fig. 1:** Pyruvate kinase inhibitor assay principle and evaluation.

## Materials & Methods

- Black half area 96-well plate (#675076, Greiner)
- Human recombinant pyruvate kinase isoform M2 (#NATE-0938, Creative Enzymes)
- Pyruvate kinase activity assay kit (#MAK072, Sigma-Aldrich)
- Scutellarin (#DML0014-5MG, Sigma-Aldrich)
- VANTAstar microplate reader (BMG LABTECH)

*Experimental procedure*

Pyruvate kinase was employed at a concentration of 100 pU/mL, the enzyme activity kit was used according to the manufacturer's instructions.

1. Assay components were brought to room temperature.
2. 44 µL of 1x assay buffer, 5 µL pyruvate kinase inhibitor solution/DMSO and 1 µL of 10 µU/mL pyruvate kinase were added to wells in a black half area 96-well plate.
3. A reaction master mix, containing 44 µL 1x assay buffer, 2 µL pyruvate kinase substrate mix, 2 µL pyruvate kinase enzyme mix and 2 µL fluorescent peroxidase substrate per sample well, was freshly prepared.
4. 50 µL of the reaction mix were added to each sample well to start the enzymatic reactions.
5. Plates were immediately transferred to the VANTAstar and measured for 13 min.
6. Kinetic data evaluation for pyruvate kinase inhibitors was performed with the MARS data analysis software.

*Instrument settings*

| | | |
|---|---|---|
| Optic settings | Fluorescence intensity, plate mode kinetic | |
| | Filter | Ex 540-20<br>LP 566<br>Em 590-20 |
| General settings | Number of flashes | 20 |
| | Settling time | 0.5 sec |
| Kinetic settings | Number of cycles | 38 |
| | Cycle time | 20 sec |
| Gain | Enhanced Dynamic Range – auto gain | |
| Focal height | 7.6 mm | |

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## Results & Discussion

The pyruvate kinase inhibitor assay was established on the VANTAstar microplate reader.

Measurements were performed employing different known pyruvate kinase inhibitors. Scutellarin, a flavone compound produced by different *Lamiaceae* of the *Scutellaria* genus, was chosen as an example to highlight the kinetic evaluation of pyruvate kinase inhibitors. Adding increasing concentrations of Scutellarin (0-25 µM) resulted in a dose-dependent reduction of pyruvate kinase activity (fig. 2), marking Scutellarin as an effective pyruvate kinase inhibitor.

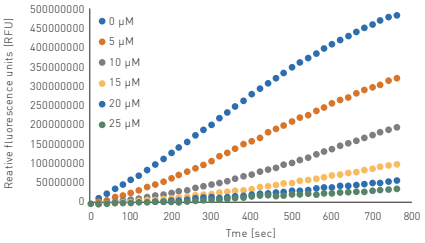


**Fig. 2:** Pyruvate kinase activity curves in the presence of increasing pyruvate kinase inhibitor concentrations. Enzyme activity was measured without pyruvate kinase inhibitor or in the presence of 5-25 µM Scutellarin.

Due to the strong increase in fluorescence signal intensity over time, the Enhanced Dynamic Range (EDR) feature was essential in this process. Where conventional gain adjustment led to signal saturation within seconds, EDR allowed to capture enzyme kinetic curves over the complete measurement range. EDR ensures flexible gain adjustment throughout kinetic measurements without the need of manual adjustment.

BMG LABTECH's MARS data analysis software was used to perform the kinetic evaluation of pyruvate kinase inhibitor measurements. Evaluation can be performed directly with the reader measurement files, requiring no further data export or data formatting. The software comes with a multitude of analysis options and available equations. In this case, a non-linear 4-parameter fit of activity curve slopes over the pyruvate kinase inhibitor concentrations was selected to calculate the $IC_{50}$ values for tested pyruvate kinase inhibitors (fig. 3). To achieve this, pyruvate kinase inhibitor concentrations were assigned to the respective measurement samples, and slopes of the activity curves were used as base for the 4-parameter standard curve calculation. This yielded kinetic parameters like slope, $IC_{50}$ and $R^2$ as highlighted in figure 3.

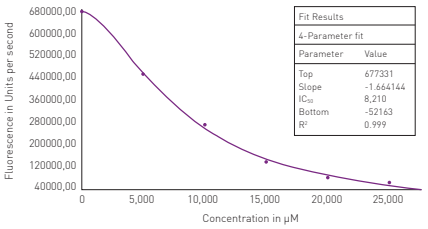


**Fig. 3:** Non-linear 4-parameter fit of the slopes of pyruvate kinase inhibitor measurements against the respective pyruvate kinase inhibitor concentration using the MARS data analysis software.

## Conclusion

The VANTAstar is an excellent platform for the performance of kinetic assays such as the presented pyruvate kinase inhibitor assay. The EDR feature simplifies assay setup and optimisation circumventing pointless repetitions. By using the provided MARS data analysis software, the effort for the assay evaluation process can be substantially streamlined. By assigning the single calculation steps required for evaluation to a template, parameters like top, bottom, slope and $IC_{50}$ can be determined by the click of a button.