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Determination of enzyme kinetics in the Eppendorf BioSpectrometer® kinetic using a hexokinase and glucose-6-phosphate-dehydrogenase from *Saccharomyces cerevisiae*

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Abstract

This Userguide will demonstrate measurement of enzyme activity using the Eppendorf BioSpectrometer. In order to optimize the measurement process, preliminary measurements were performed using the method "single λ continuous". The actual activity measurements were then performed based on these results. Enzyme activities were determined via linear regression.

For the measurements, a coupled reaction of a hexokinase and a glucose-6-phosphate-dehydrogenase from the baker's yeast , was used. Since the Eppendorf BioSpectrometer kinetic is equipped with a temperature controlled cuvette chamber, temperature dependence of this reaction could also be demonstrated. The highest activity was detected at 37 °C.

Introduction

Metabolic significance of the hexokinase reaction

Glycolysis

One central area within the metabolism of nearly all living beings is the enzymatic degradation of glucose in the cytosol, or cytoplasma, respectively, in their cells. This process is called glycolysis. Glucose is converted to 2 molecules of pyruvate in a stepwise fashion. One molecule of glucose yields 2 ATP and 2 redox equivalents in the form of $NADPH_2$. Pyruvate then enters a further elementary metabolic pathway, the citrate cycle, yielding further reduction equivalents for the respiratory chain, which are ultimately transferred to oxygen. The final degradation products are H_2O and CO_2.

Activation of glucose via the hexokinase reaction

Glucose degradation thus sustains respiration of all living beings. Prior to degradation, glucose needs to be rendered susceptible for degradation, i.e., activated. This process involves phosphorylation by a hexokinase; with the expenditure of ATP, glucose-6-phosphate is produced. Thus, the latter is the actual initial substrate of glycolysis (fig.1).

In addition, glucose-6-phosphate is the initial substrate of a further important metabolic pathway, the pentose phosphate pathway, which serves to provide important redox equivalents, such as $NADPH_2$, as well as various carbohydrates deemed for further catabolic and biosynthesis pathways.

Detection of enzymatic degradation of metabolic enzymes

Due to the critical significance of these metabolic pathways and the enzymes involved, they are subject to great interest within the areas of biochemical research and education. One important aspect is the determination of enzyme activities, as these constitute the specific hallmark of an enzyme. The primary method for measurement of the respective enzyme activities is photometry, using a spectrophotometer.
Since NADP (NAD) and NADPH2 (NADH2), respectively, are involved in nearly all enzymatic reactions within the central metabolism, enzyme activity is mainly determined via the increase or decrease of NADPH2. Both NADP and NADPH2 show an absorbance maximum at 260 nm; however, NADPH2 shows an additional peak at 340 nm (fig.1), which allows for photometric distinction between NADPH2 and NADP.
In addition to enzyme characterization, measurements of enzyme activity may serve the detection of certain substrates or substrate concentrations, respectively.
Glucose or ATP concentrations can be determined using the above-mentioned hexokinase reaction. Detection occurs indirectly via generation of NADPH2 in a coupled reaction with a glucose-6-phosphate-dehydrogenase. The amount of ATP generated is proportional to the amount of glucose or ATP (fig.2).

The detection reaction depicted in figure 2 served as the example reaction in this Userguide. The purpose was to show two applications of measurements of enzyme kinetics using the Eppendorf BioSpectrometer kinetic:

1) Enzymatic determination of ATP concentration
Substrate concentration is to be determined via two-point calibration using defined amounts of ATP at 37 °C. During this process, two measurements are taken within the linear range of substrate conversion. The linear range of conversion needs to be determined in preliminary measurements.

2) Determination of enzyme activities.
The temperature dependence of the hexokinase reaction shown in figure 2 is to be tested.
The Eppendorf BioSpectrometer kinetic offers the option of sample temperature regulation in a heatable cuvette chamber: a range of temperatures between 20 °C and 42 °C may be selected.
Since enzyme activity is also dependent on incubation temperature, temperature regulation is crucial for accurate determination of activity. Therefore, hexokinase enzyme activity was measured at 22, 30 and 37 °C. Exact determination was performed using linear regression of the curve.

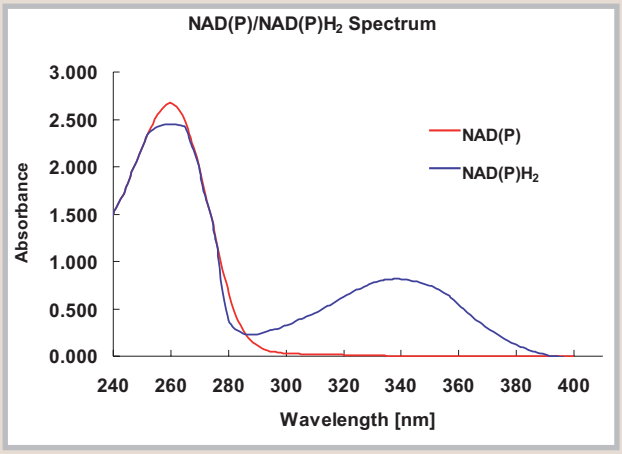


Figure 1: Absorbance spectrum of NADP/ NADPH2.
Both components can be easily distinguished by the absorbance maximum of NADPH2 at 340 nm. Thus, the activities of NADP/ NADPH2-dependent enzymes are determined via a change in absorbance at 340 nm.

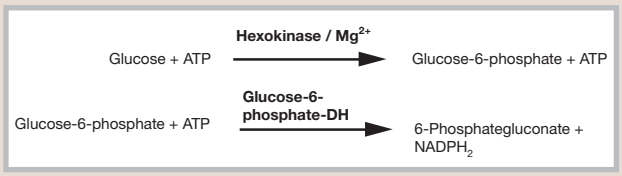


Figure 2: Indirect enzymatic detection of glucose or ATP.
Both substances may be determined via a coupled reaction of hexokinase and glucose-6-phosphate-dehydrogenase (coupled test system [1]). The respective amount is calculated from the amount of NADPH2 generated.

Materials

> BioSpectrometer kinetic, IsoPack with IsoRack (Eppendorf)
> Ultra-Mikro cuvette 105.202-QS (Hellma, 105-202-85-40)
> ATP (Roche Applied Science, 10127523001)
> Hexokinase from Saccharomyces cerevisiae (Roche Applied Science, 11426362001)
> Glucose-6-phosphate-dehydrogenase from Saccharomyces cerevisiae (Roche Applied Science, 10127671001)
> NADP (Sigma-Aldrich, 93205-50MG)
> α-D-Glucose (Sigma-Aldrich,158968-25G)
> MgCl2 (Sigma-Aldrich, 63069-100ML),
> Tris buffer pH 7.0 (AppliChem, A5247, 0500),
> Water for molecular biology (AppliChem, A7398,1000)

Methods

Hexokinase activity measurements

All measurements were performed in the Eppendorf BioSpectrometer. The following solutions were prepared for the enzyme test:

> 10	mL	0.1	M	Tris buffer pH 7.0
> 1	mL	0.1	M	MgCl2
> 1	mL	5	mM	Glucose
> 0.5	mL	10	mM	NADP+
> 1	mL	1	mM	ATP
> 1	mL			Hexokinase
> 2	mL			Glucose-6-phosphate-dehydrogenase

The solutions were prepared using water for molecular biology and were kept cool at 4 °C in the Eppendorf IsoPack. The Tris buffer was kept at ambient temperature.
The enzymes were returned to the refrigerator immediately after use.

The following amounts of the above-mentioned components were used for one measuring reaction:

5	µL	MgCl2
5	µL	NADP
5	µL	Glucose
12.5	µL	ATP
121.5	µL	Tris buffer
0.5	µL	Glucose-6-phosphate-dehydrogenase
∑=149.5	µL	

The solutions were immediately pipetted into an ultra micro cuvette which was then placed into the cuvette chamber. For the purpose of adaptation to the reaction temperature, the measuring parameters were chosen to include a pre-incubation period for the reactions of at least 6 minutes. The measurements were started, and changes in absorbance at 340 nm were recorded. When no further changes in absorbance could be detected, the enzymatic reaction was initiated by the addition of 0.5 µL hexokinase solution (after approx. 1 min).
Following addition of the glucose-6-phosphate-dehydrogenase, as well as the hexokinase, the solutions were thoroughly mixed. Measurements were performed at 22, 30 and 37 °C.

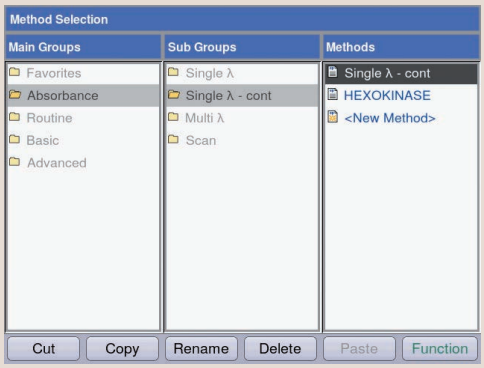
Substrate determination via the hexokinase test

For substrate determination, the same components were used which were listed for the hexokinase activity test.
In order to determine an unknown concentration of ATP within a sample, 1 mM and 0.1 mM ATP solutions served as standards.

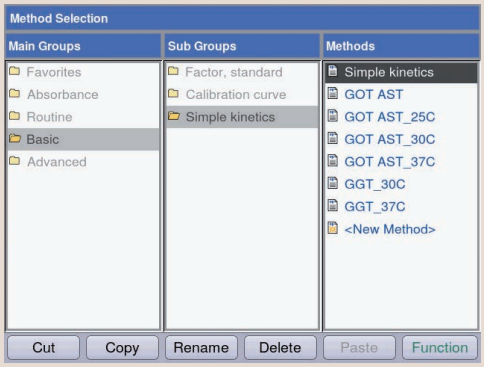
Settings on the BioSpectrometer kinetic.

The BioSpectrometer kinetic offers three methods for activity measurements (fig.3):
a) "Single λ-continuous (Single λ-cont)": Simple measurement of changes in absorbance at a defined wavelength, at defined time intervals, over a defined period of time.

3A



3B



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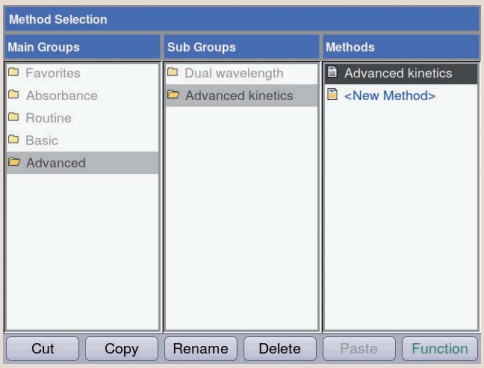

Figure 3
Activity measurement options.
A) Single λ - cont
B) Simple kinetics
C) Advanced kinetics.

Optional temperature control is possible. This method is especially well suited for preliminary experiments, i.e. determination of the kinetic of a reaction (speed, range of linearity).
b) "Simple kinetics": as in a); additionally, units and conversion factors for direct conversion of the absorbance values may be defined. Endpoint measurement, two-point measurement, or linear regression are available. "Delay" offers the option of programming a delayed course of measurement for the purpose of, for example, ensuring sufficient adaptation of the reaction mixture to the actual measurement temperature.
c) "Advanced kinetics": as in b), with the additional option of programming a "reagent blank" and a measurement including a standard. "Reagent Blank" allows for verification that no apparent changes in absorbance have occurred prior to reaction initiation. In order to test the progress of the hexokinase reaction, a preliminary reaction was first performed using the method "Single λ cont". The parameters were chosen in such a fashion that changes in absorbance within the reaction mix could be tracked over an extended period of time (10 min). Since reaction speed may change rapidly during enzymatic measurements, data were collected every 10 s (fig. 4)The parameters for activity measurements (fig. 5A) and substrate determination (fig. 5B and 5C) were set based on the results obtained from the preliminary n experiments.

As shown in figure 5, different methods were chosen for determination of activity vs. substrate concentration. Hexokinase activity was determined using linear regression, where a measurement was taken every 5 s.
Total measurement time was 6 min; the reaction was started by the addition of hexokinase following a warm-up period of 6 min (parameter "Delay"). The experimental parameters were selected based on the preliminary experiments using "single λ cont" (see above). The following formulae were used to calculate enzyme activity or substrate concentration, respectively:

Direct calculation of enzyme activity

The factor shown in figure 5A is calculated from the molar absorption coefficient for NADPH (6.32 * 103 L / (mol * cm)).

The Lambert-Beer law postulates:

$A = \varepsilon * c * l$ or $c = A / \varepsilon * l$
Where c = concentration, A = absorption, ε = molar absorption coefficient, l =optical pathway (path length of the cuvette).

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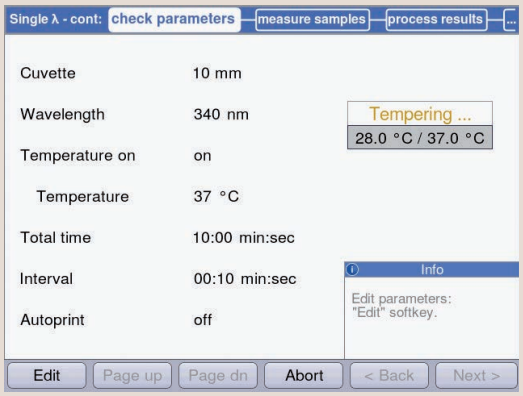


5A

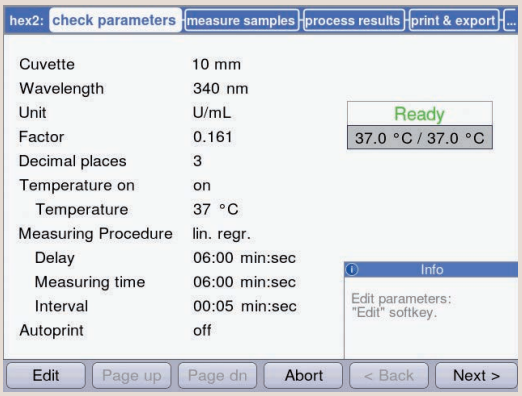
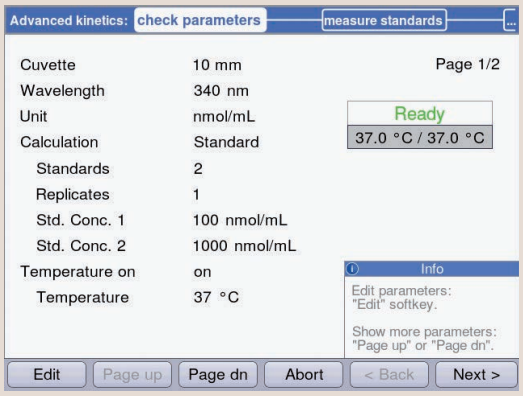


Figure 4: Measurement parameters of preliminary experiments for hexokinase measurements within the method "Single λ cont".

5B



5C

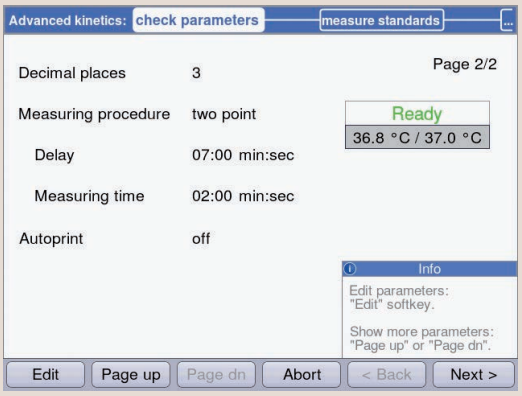


Figure 5: Parameters for determination of activity and substrate concentration.
A) Parameters for determination of hexokinase via linear regression in the method "simple kinetics".
B) + C) Parameters for determination of substrate concentration in the method "advanced kinetics" via two point calibration.

For a cuvette with a path length of 1 cm the following formulae apply: c= A/ε or c= A * 1/ε or c=A * F, respectively. Thus, the factor F is the reciprocal value of the molar extinction coefficient and it bears the unit mol/L or µmol/µL, respectively (for NADPH2 1.61 * 10-4).

In order to calculate the enzyme activity U/L or µmol/min * L, respectively, (relative enzymatic activity based on 1 L) directly from the absorption measurement, the factor needs to be adapted from mol/L for the unit µmol/L, i.e. it needs to be multiplied by 1 * 106. Therefore, the factor would be 161. In figure 5A the conversion factor for the enzyme activity is given for 1 mL (U/mL), and it is therefore 0.161. Enzyme activity is then directly derived from the calculated slope, and it is displayed after the measurement.

Determination of substrate concentration

During determination of substrate concentration, a measurement was performed following a pre-incubation period of 7 minutes, followed by addition of hexokinase. The reaction mixture was then incubated for 2 minutes; according to the two-point method a measurement is taken at the start and another measurement is taken at the end of the incubation. For calculation of sample concentration, two preliminary standard measurements were performed with defined substrate concentrations. The concentration of the unknown sample is calculated from the standards.

Implementation of a linear regression on the BioSpectrometer kinetic for the purpose of activity measurements

Only enzyme activities within the linear range of the curve are analyzed, as inhibitory factors such as declining substrate concentration or inhibition from the final product can only be excluded within the linear range. The Eppendorf BioSpectrometer kinetic offers the option of retroactively adapting the regression curve to the linear curve for activity measurements which are to be analyzed using linear regression. To this end, the function "linear regression" within the area "process results" is activated following the measurement process. Thus, the start and end points of the regression curve may be reset (fig. 6)

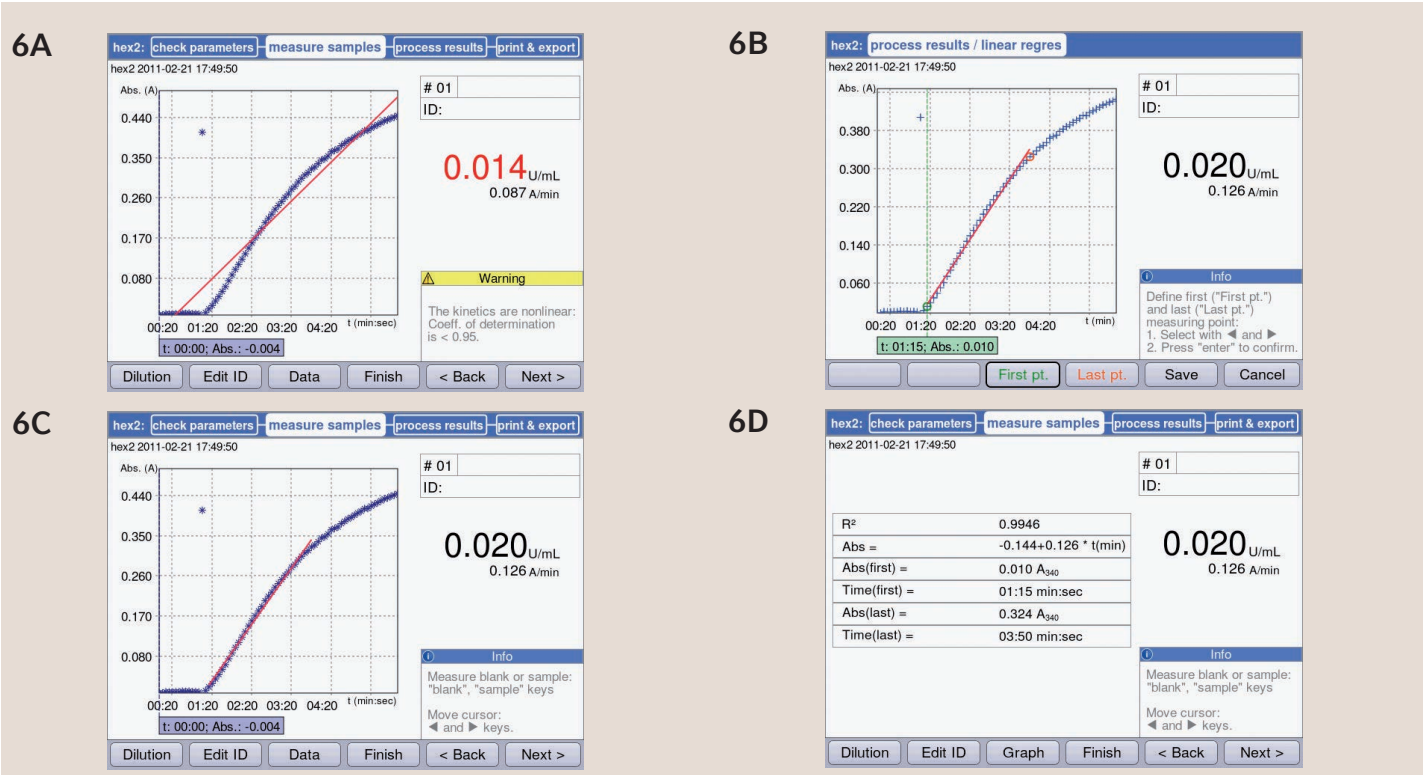


Figure 6: Linear regression analysis following the completion of measurements.
A: Result of a kinetic measurement with linear regression. The regression curve (red) is not congruent with the curve shape. Thus, the coefficient of determination is too low (< 0.95). The result is therefore displayed in red. The soft key "Next>" leads to the area "process results". By activating the function "linear regression" the regression curve may be manipulated (soft key: "lin. regr.").

B: Start and end point of the regression curve may be reset using the soft keys "First pt" and "Last pt". The points are moved using the cursor keys. When a sufficient match of the regression curve with the linear range of the curve has been achieved, the result may be saved.
C: New measurement result by way of adjusted regression curve. The result is now displayed in black; i.e. the regression curve is now congruent with the linear range of the curve; the coefficient of determination equals >0.95.
D: All important information pertaining to the regression curve: coefficient of determination , start and end point of the result, regression curve formula.

Results

Preliminary experiments for activity measurements – "Single λ-cont"

It is often impossible to predict the course of a reaction when a spectrometer is used to determine enzyme activity. This is especially true in cases where one did not perform the measurements previously, or when an activity test for an enzyme is to be developed de novo. In the latter case, neither the necessary substrate, nor the enzyme, or, if necessary, the co-factors or additive concentrations are known. Depending on the components used, the speed at which this particular enzymatic reaction will progress is also unknown. As previously mentioned, the Eppendorf BioSpectrometer kinetic offers the option to test the course of the reaction in a preliminary experiment using the method "Single λ-cont". Over the course of a maximum of 1 h, measurements can be performed as frequently as every 5 s.

For the preliminary measurements of hexokinase activity, data were collected every 10 s over a period of 10 min. Prior to the actual measurement, the sample was pre-incubated for at least 6 min in order to adapt the sample to the reaction temperature. The reaction was initiated by addition of hexokinase after 1 minute.

This reaction had two objectives: to determine the linear range of the curve, and to determine the time at which no further significant substrate conversion occurs. The latter is of importance for the experiment of substrate determination, as in this case, measurement was performed according to the end point method. Preliminary measurements were performed at 37 °C.

The result is shown in figure 7.

Based on the results depicted in figure 7, the parameters for the following experiment were set. Since all substrate appears to be converted after 6 minutes, measurements were limited to this time. The reaction was started after a warm-up period of 1 min. These settings were applied to the activity tests as well as substrate determination.

Determination of an unknown ATP concentration – "advanced kinetics"

As described, an unknown concentration of ATP was to be determined using two-point calibration using the method "advanced kinetics". In order to determine the concentration, two standards were measured initially, a 1 mM and a 0.1 mM ATP solution. The measurement parameters used are shown in figures 5B and 5C. As in the preliminary experiments, the measurements were performed at 37 °C. Figure 8A shows the result of the measurement of the standards. From these standards, an ATP concentration of approximately 0.79 mM was determined for the unknown sample, as shown in figure 8B.

7

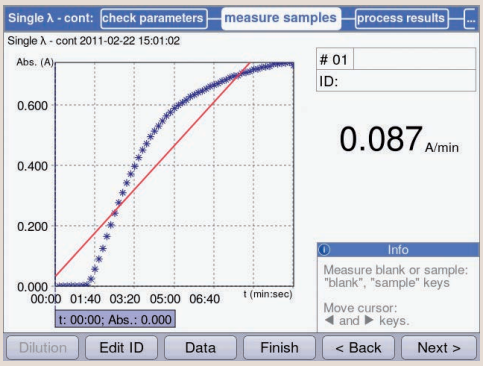
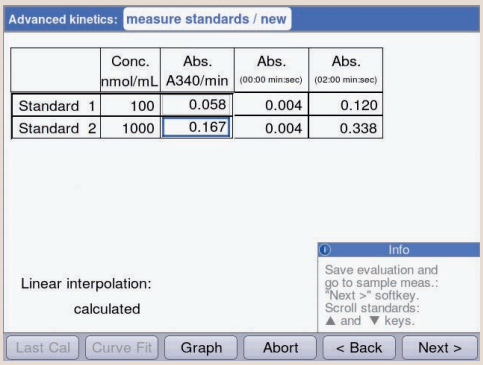


Figure 7: Preliminary measurements of hexokinase using the method "Single λ-cont". The increase in extinction occurs immediately following addition of the enzyme. The course of the curve is linear for approximately 2 minutes, followed by a flattening of the curve. After 6 minutes, no further significant increase in absorption can be detected.

8A



8B

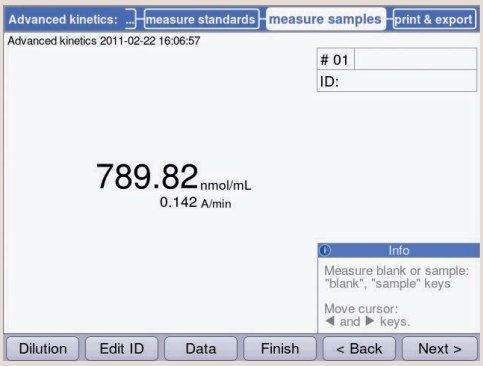


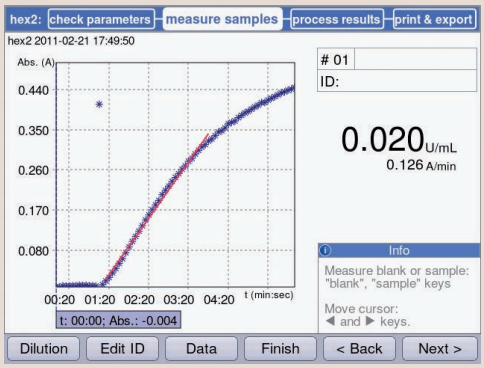
Figure 8: Determination of the ATP concentration using the "Advanced kinetics" methods.
A: Result of the standard measurements.
B: Concentration determined for an unknown sample, calculated from the measured ATP standards.

Determination of hexokinase activity at different temperatures – "simple kinetics"

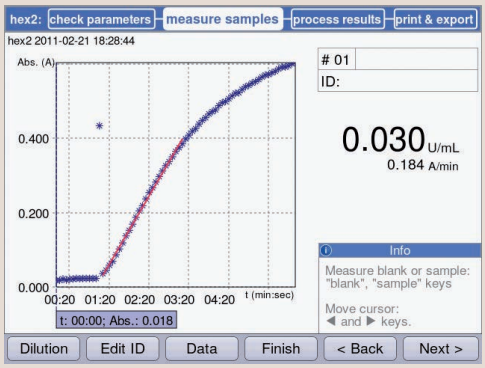
The Eppendorf BioSpectrometer kinetic is equipped with a heatable cuvette chamber, i.e. kinetic measurements may be optimized with regards to the temperature at which they are measured. The influence of the reaction temperature was to be demonstrated by the following experiments. To this end, hexokinase activity was measured at 22, 30 and 37 °C. Measurements were performed in accordance with the preliminary experiment, i.e. 6 minutes of pre-incubation ("delay"), followed by absorption measurements at 340 nm at 5 second intervals (please also refer to fig. 5A).Based on the results depicted in figure 7, the parameters for the following experiment were set. Since all substrate appears to be converted after 6 minutes, measurements were limited to this time. The reaction was started after a warm-up period of 1 min. These settings were applied to the activity tests as well as substrate determination.

After 1 minute warm-up, the reaction was started by addition of the hexokinase. The reaction was tracked for a further 5 minutes.
As shown in figure 6, the regression curve was synchronized with the linear range of the measured curve using linear regression.
The results for the measurements at different temperatures are shown in figures 9A-9C. As evident from figure 9, the highest substrate conversion was achieved at 37 °C; the activity was more than twice that measured at 22 °C. These results are in accordance with the expectations, as the activity maxima of the selected enzymes should lie between 35 °C and 40 °C (1).

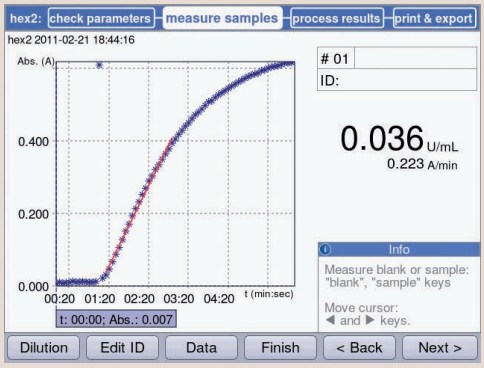
9A



9B



9C



9D

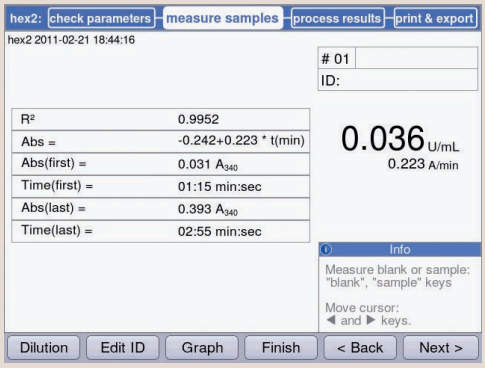


Figure 9: Measurement of hexokinase activity at different temperatures.
The results from the measurements were retroactively adapted to the linear range using the regression curve (red line). Further information about the selected range, such as the coefficient of determination of the regression curve, are stored in the area "Data".

A: Activity at 22 °C
B: Activity at 30 °C
C: Activity at 37 °C
D: Data referring to the selected range, with the example of the measurements at 37 °C. Start and end point of the measurement, as well as the respective absorbance values and the calculated coefficient of determination R^2.

Conclusion

The Eppendorf BioSpectrometer kinetic is ideally suited for determination of enzyme activity, as well as for determination of substrate concentrations. Important parameters, such as optimal enzyme or substrate concentrations, measurement duration, linear range of the curve or reaction temperature, may be assessed in preliminary experiments using the method "Single λ-cont".
Such preliminary measurements are of particular importance in those cases where the course of the reaction is either unknown or not yet optimized. Thus, determination of molecule concentration via activity measurements enables a quick estimate when substrate conversion is complete.

Furthermore, reaction temperature may be selected with high accuracy in the heatable cuvette chamber.

Exact temperature selection is the basis for accurate determination of enzyme activity.

A further unique feature of the Eppendorf BioSpectrometer kinetic is the option to move the time window retroactively, in order to allow for adaptation of the measured curve to the linear range, and thus enable its analysis via linear regression. Thus, through the control of the coefficient of determination "R2", measured curves may be analyzed in a reproducible fashion, and enzyme activities can be determined reliably. The choice between 3 measurement methods, and the optional analysis using standard or blank, enable the determination of enzyme activity as well as substrate concentration.

References

[1] Friedrich Lottspeich, Joachim W. Engels und Angela Simeon, Bioanalytik, 2nd edition (2008)
[2] Abrahão-Neto, J., Infanti, P., Vitolo, M., Hexokinase production from S. cerevisiae. Culture conditions. Appl Biochem Biotechnol. 1996 Spring; 57-58:407-12.

Ordering Information

Product	Description	Order no. international	Order no. North America
Eppendorf BioSpectrometer® kinetic	230 V / 50 - 60 Hz, mains/power plug Europe	6136 000.002	-
	120 V / 50-60 Hz, mains/power plug North America	-	6136000010
Thermo printer DPU 414	Incl. power supply and printer cable 230 V	6131 011.006	-
	115 V/100 V, USA, JP	-	952010140
Thermo paper	5 rolls	0013 021.566	952010409
Cuvette rack	For 16 cuvettes	4308 078.006	940001102
IsoRack + IsoPack 0 °C for IsoTherm-System	1 Rack and 1 cooling pack 1.5 mL/1.7 mL/2.0 mL for 0 °C	5175 220.005	930001091

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