Application note

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Monitoring enzyme kinetics with the NanoDrop One^C Spectrophotometer

Introduction

One of the most well-studied genetic regulatory models is the *Escherichia coli* (*E. coli*) *lacZ* gene and the β-galactosidase (β-gal) enzyme model.[1] β-gal serves to breakdown lactose into galactose and glucose for energy production.[2] In the presence of a β-galactoside substrate, such as lactose, the *lac* repressor undergoes a conformational change, and β-gal is synthesized to hydrolyze the substrate.[3] Frequently used for monitoring eukaryotic transfection efficiency, the *lacZ* gene product, β-gal, is easily assayed via absorbance spectroscopy. To monitor β-gal activity, the substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) is hydrolyzed by β-gal to form the yellow chromophore ortho-nitrophenol (ONP), and endpoint absorbance is determined at 420 nm (Figure 1).[4]

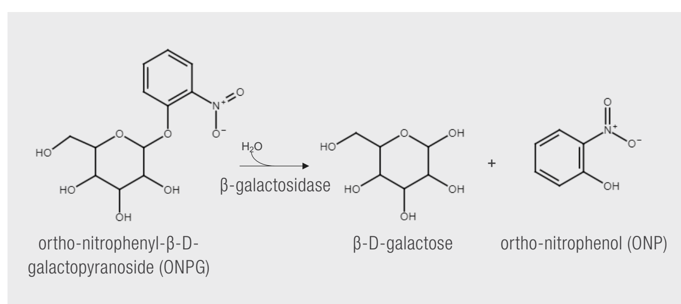

Figure 1: Hydrolysis of ortho-nitrophenyl-β-D-galactopyranoside (ONPG) by β-gal to form the yellow chromophore ortho-nitrophenol (ONP).

To monitor the reaction in real time, a kinetics experiment was performed on the Thermo Scientific™ NanoDrop™ One^C UV-Vis Spectrophotometer, which includes a cuvette port and a microvolume pedestal. The Kinetics application is available for the NanoDrop One^C model only, as a cuvette is required for the experiment. The cuvette port built into the NanoDrop One^C instrument can incubate the sample at 37 °C and various stir speeds can be applied. The hydrolysis of ONPG results in a broad peak at 400 nm and the addition of a high pH stop buffer results in the denaturation of β-gal, a rapid increase in absorbance, and a peak shift to 420 nm (Figure 2). The absorbance results at 420 nm from this experiment were used to calculate the β-gal specific activity, a useful measure of enzyme purity. Upstream of the kinetics experiments, protein concentration was quantified for calculating the enzyme's specific activity.

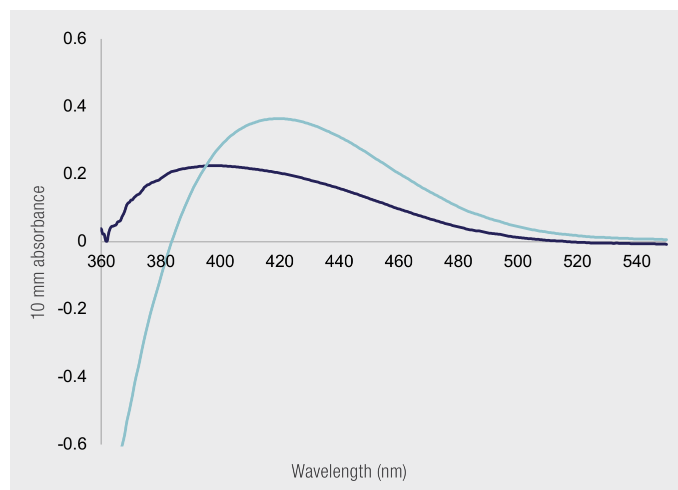

Figure 2: The addition of a high pH stop buffer results in an absorbance increase and a peak shift from 400 nm to 420 nm.

Experimental procedures

β-Gal Assay

The Invitrogen™ β-Gal Assay Kit (Invitrogen, K145501) was modified to meet the proper sample volume requirement for cuvette measurements. For the NanoDrop One^C instrument, the sample volume should be sufficient to satisfy the Z-height of 8.5 mm. From the β-gal assay kit, 10X Cleavage Buffer was diluted to 1X to create a 10 mL stock. DL-1,4-dithiothreitol (DTT) (Thermo Scientific, 426380100) was used as a reducing agent in the 1X Cleavage Buffer in place of β-mercaptoethanol.

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In lieu of cells transfected with plasmids expressing the *lacZ* gene, β-galactosidase from *E. coli* (Sigma-Aldrich, G4155) was diluted 1:100 and served as the cell lysate. The reaction buffer consisted of 490 µL ONPG and 1.4 mL 1X Cleavage Buffer with DTT. Three different volumes of "cell lysate" were tested to assess assay and instrument linearity: 140 µL, 70 µL, and 35 µL; distilled, deionized water was added to a final volume of 210 µL. The reaction was performed at 37 °C for 30 minutes and 1.0 mL Stop Buffer (1 M sodium carbonate) was added to stop the reaction.

NanoDrop One^C Software setup

Figure 3 outlines the NanoDrop One^C kinetics method parameters. Two stages were set for the experiment: stage one did not include a starting measurement delay and absorbance was monitored at one-minute intervals for 30 intervals for an overall duration of 30 minutes. Stage two included a 30-second measurement delay to add Stop Buffer and absorbance was monitored at 30-second intervals for 3 intervals for an overall duration of 2 minutes. A solution of ONPG and 1X Cleavage Buffer with DTT was made to blank the spectrophotometer. For each "cell lysate" volume (140 µL, 70 µL, and 35 µL), the kinetics experiment was performed in a plastic cuvette at 37 °C, stir speed was set to level 9, and absorbance was monitored at 420 nm.

Measurement Range: 350 - 850 (nm)

Time Unit: Time (minutes)

Stage	Delay	Interval Time	# Interval	Duration
1	0.00	1.00	30	30.00
2	0.50	0.50	3	2.00

Figure 3: Kinetics method parameters displayed on the NanoDrop One/One^C PC Control Software. Not shown in the figure is the Monitored Wavelength set to 420 nm. Delay, interval time, and # interval can be edited in the method setup; duration is automatically populated after inputting the previous parameters.

BCA Protein Assay

The Thermo Scientific Pierce™ BCA Protein Assay (Thermo Scientific, 23227) was performed to quantify the protein in the stock "cell lysate" solution. Bovine serum albumin (BSA) was diluted to create a standard curve consisting of 2.0 mg/mL, 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, and a zero reference. In separate microcentrifuge tubes, 25 µL of each standard or stock "cell lysate" was added. The BCA working reagent was prepared to a total volume of 5.0 mL and 200 µL was added to each standard and sample tube. The mixtures were vortexed and incubated at 37 °C for 30 minutes. Absorbance was measured for each standard and sample in duplicate and triplicate, respectively, at 562 nm using the microvolume pedestal on the NanoDrop One^C Spectrophotometer.

Results

BCA Protein Assay

The absorbance results of the BCA assay are shown in Figure 4. The standard curve exhibited strong linearity with an R^2 of 0.9916. The average calculated protein concentration of the stock "cell lysate" solution was 1.65 mg/mL, averaged from three measurements with high reproducibility (*SD* = 0.003).

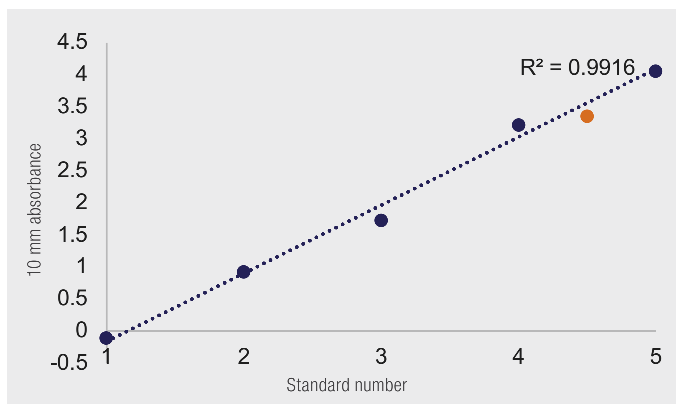

Figure 4: BCA assay absorbance results at 562 nm. The standards are in dark blue and the β-gal stock is shown in orange. Standard 1: zero reference. Standard 2: 0.5 mg/mL. Standard 3: 1.0 mg/mL. Standard 4: 1.5 mg/mL. Standard 5: 2.0 mg/mL.

NanoDrop One^C kinetics data

The endpoint absorbance of ONP at 420 nm when graphed against several enzyme dilutions displayed signal linearity (R^2 = 0.9968) shown in Figure 5. The results indicate the measured enzyme concentrations were within the assay and instrument linear range. This also confirms the enzyme's calculated specific activity was not skewed by incorporating non-linear enzyme concentrations.[5]

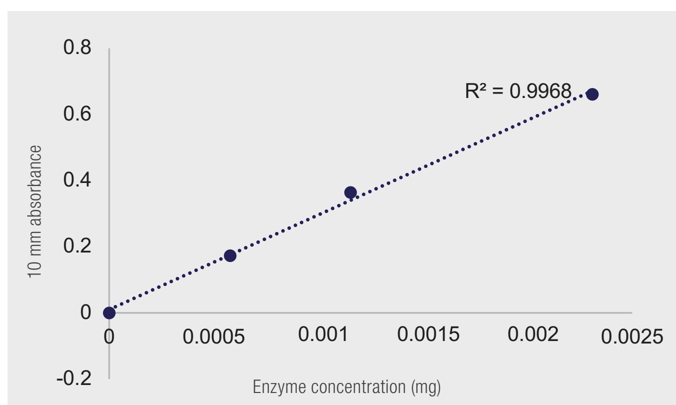

Figure 5: ONP end point absorbance at 420 nm graphed against enzyme concentration (R^2 = 0.9968).

To calculate the specific activity of β-gal, the ONP concentration in mol/L was first determined using the Beer-Lambert Law below where ε is the molar extinction coefficient of ONP, 4,500 M^{-1} cm^{-1}, A is the endpoint absorbance at 420 nm, and l is the pathlength in cm. Absorbance at 420 nm was averaged from four measurements in stage two of the kinetics experiment for each cell lysate volume tested.

$$[\mathrm{ONP}] = \frac{A}{\varepsilon * l}$$

An example of applying the Beer-Lambert Law to calculate β-gal specific activity is shown below where the specific activity was calculated from one of the cell lysate volumes tested:

$$[\text{ONP}] = \frac{0.66}{4500\ \text{M}^{-1}\ \text{cm}^{-1} * 1\ \text{cm}} = 1.47 \times 10^{-4} \frac{\text{mol}}{\text{L}} \text{ or } 0.147\ \mu\text{mol mL}^{-1}$$

Enzyme activity (μmol min^{-1}) was calculated using the total reaction time (30 minutes) and the total assay volume (3.1 mL):

$$\text{Enzyme Activity} = \left(\frac{0.147\ \mu\text{mol mL}^{-1}}{30\ \text{minutes}}\right) * (3.1\ \text{mL}) = 0.0152\ \mu\text{mol min}^{-1}$$

The amount of enzyme in the reaction was calculated to determine the specific activity. The diluted enzyme concentration was calculated below using the stock concentration determined from the BCA assay (1.65 mg/mL) where the 1:100 dilution and the sample volume (140 μL) were applied:

$$\text{Enzyme concentration} = \left(1.65 \frac{\text{mg}}{\text{mL}}\right) * (0.140\ \text{mL}) * \left({}^{1}/_{100}\right) = 0.00231\ \text{mg}$$

Specific activity was then calculated from the enzyme activity and concentration in the reaction:

$$\text{Specific activity} = \frac{(0.0152 \mu\text{mol min}^{-1})}{0.00231\ \text{mg}} = 6.56\ \mu\text{mol min}^{-1}\ \text{mg}^{-1}$$

Using the above calculations for the remaining "cell lysate" dilutions, the specific activity of β-gal is expressed as 6.90 ± 0.34 μmol min^{-1} mg^{-1}.

The amount of ONP formed in μM was calculated at each time point according to the Beer-Lambert Law and the results are displayed in Figure 6. The linear progress curves and the absence of plateaus indicate the proper enzyme concentration was used for all three experiments and the ONPG substrate was not depleted over time.[5]

NanoDrop One^C UV-Vis Spectrophotometer

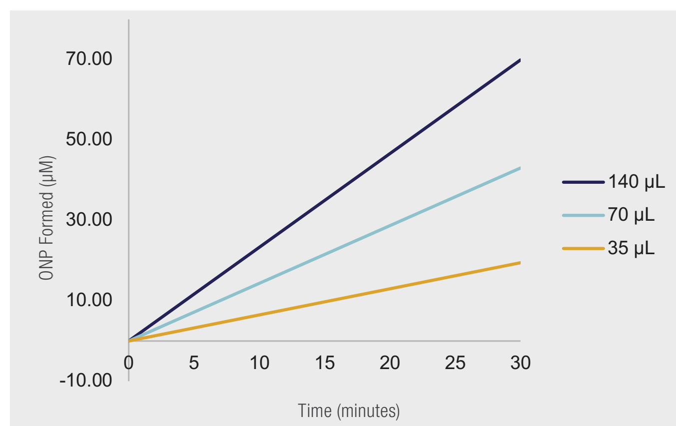

Figure 6: Linear progress curves showing the amount of ONP produced (μM) over time (minutes) for the three tested "cell lysate" volumes.

Conclusions

From start to finish, the NanoDrop One^C instrument can be integrated into a spectrophotometric enzyme kinetic reaction. The colorimetric BCA protein assay used in this method is a crucial component for determining protein concentration in cell lysates prior to the kinetics reaction. Since cell lysates contain many absorbing materials, this interferes with an accurate protein concentration if a standard A280 measurement is made. The NanoDrop One^C instrument includes a preprogrammed BCA assay application, making colorimetric quantification quick and simple. The microvolume pedestal on the NanoDrop One^C instrument also provides the opportunity to scale down the sample volume requirement for the BCA assay if cell lysate volume is limited.

Monitoring the production of ONP over time with the NanoDrop One^C Spectrophotometer tests assay and instrument linearity to ensure an accurate assessment of enzyme specific activity. The acquired absorbance data demonstrates the reliability of the NanoDrop One^C instrument as a means of monitoring enzymatic activity, evidenced by the reported R^2 value.

References

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