# Monitoring Enzymatic Glucose Production from Cellulosic Feedstock

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**Research into producing inexpensive cellulosic ethanol is on-going and still provides hope for a practical replacement of ethanol from corn. Here we detail cuvette and microplate-based workflows for the quantification of the intermediate product glucose in the conversion of lignocellulosic material to ethanol.**

**Key Words:**

Miniaturization

Cellulose

Ethanol

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## Introduction

While glucose is mainly known as a ubiquitous fuel source in biological systems, it is playing an increasing important role as a precursor to ethanol production in biofuel applications.  Glucose is a monosaccharide and it used in nature as a monomer to create polysaccharides such as starches and cellulose in plants and glycogen in animals.  While starches and glycogen provide a readily accessible source of glucose and energy; cellulosic degradation presents a much more challenging source for efficient utilization of stored energy. Commercial, cost-effective production of biofuels from biomass currently remains elusive.    Considerable effort is currently being expended to design and optimize various processes, such as fermentation of cellulosic feedstock, to this end.   Quantification of intermediate products formed during fermentation is critical to allow optimization of the steps necessary to improve product yields.  The ability to monitor formation of glucose, the intermediate product in the production of ethanol is of critical importance for sustained commercial operations.

One method for quantitative determination of glucose is with the use of enzymatic methods as outlined below (Figure 1).  Glucose oxidase is known to oxidize glucose to gluconic acid with the concomitant release of hydrogen peroxide.  The quantitative release of hydrogen peroxide in this enzymatic reaction can be harnessed by a secondary reaction involving the reduction of the colorless o-dianisidine in the presence of peroxidase resulting in formation of a colored product.  The oxidized o-dianisidine can be further stabilized by reaction with sulfuric acid producing an intense pink color with absorbance measurable at 540 nm.  The final product, and thus absorbance, is directly proportional to the glucose concentration found in the sample.  Here we show the quantitative analysis of glucose production from enzymatic degradation of

carboxymethyl cellulose by cellulase using both a cuvette- and microplate-based workflow.
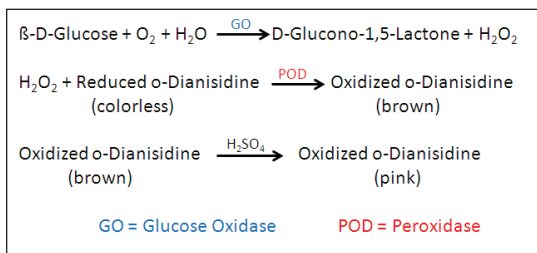


*Figure 1.* Enzymatic method for quantitative determination of glucose concentrations.  Glucose oxidase and peroxidase enzymes are for the sequential oxidation of glucose and oxidation of o-Dianisidine to a stable, colored product capable of detection by microplate or cuvette based absorbance spectroscopy.

## Materials and Methods

### Enzymatic cellulose degradation
Cellulase (Aspergillus niger), sodium carboxymethyl cellulose, and sodium acetate were purchased from Sigma (St. Louis, MO).  Carboxymethyl cellulose (CMC) working solution was prepared at a concentration of 5 mg/mL in 100 mM sodium acetate (NaOAc) buffer, pH=5.0, made fresh daily.  Cellulase working stock was prepared to 10 U/mL in NaOAc buffer.  A 1:2 serial dilution of the cellulase working stock was prepared and mixed in a 1:1 ratio (v/v) with CMC working stock and incubated overnight at 37°C.  The enzymatic reaction was then analyzed for glucose product.

### Glucose analysis

Glucose (GO) Assay Kit and sulfuric acid were purchased from Sigma (Saint Louis, MO) and run in accordance with the kit manufacturers' instruction with the following modifications. Briefly, glucose oxidase/peroxidase reagent was diluted in 39.2 mL $ddH_2O$ and o-dianisidine reagent was reconstituted in 1 mL $ddH_2O$. 0.8 mL of o-dianisidine reagent was added to the glucose oxidase/peroxidase reagent and protected from light. Sulfuric acid ($H_2SO_4$) was used as a concentrated stock (12 N). A glucose standard curve was prepared as indicated in table 1 from a NIST traceable glucose standard supplied in the kit. The assay was scaled to a volume of 500 µL, 10% the standard volume of 5 mL, for analysis using either a microplate or microvolume cuvette. Briefly, 100 µL of glucose standard or sample was added to 200 µL of assay reagent and allowed to react at 37°C for exactly 30 minutes followed by the addition of 200 µL sulfuric acid. Either 100 µL or 500 µL of both standards and samples were measured in a microplate or micro-volume cuvette, respectively, in an Eon Microplate Spectrophotometer controlled by Gen5™ Software (Figure 2). Data analysis was done in Microsoft Excel (Redmond, CA).
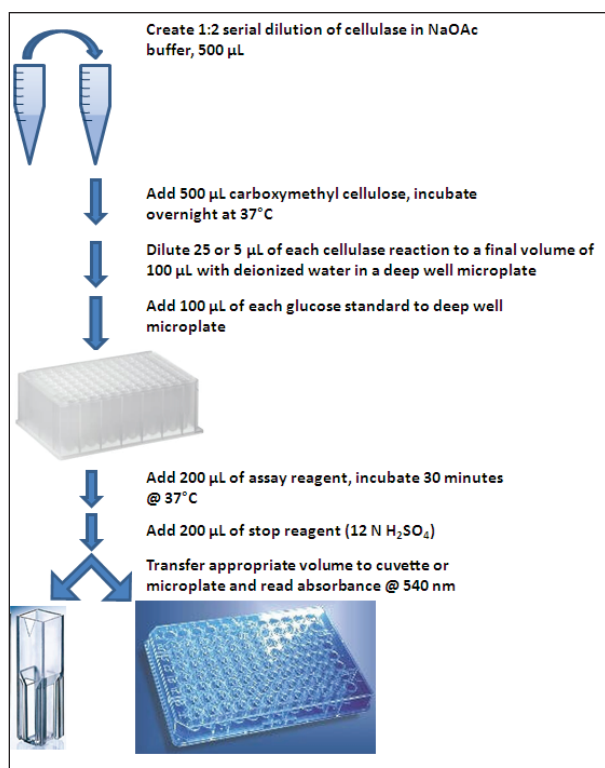


Figure 2. Workflow diagram depicting two alternate methods for a low-volume assay for glucose production from enzymatic degradation of cellulosic feedstock by cellulase.

| Standard Curve | | |
|---|---|---|
| Glucose (µg) | ddH$_2$O (µL) | Glucose (1 mg/mL) (µL) |
| 0 | 1000 | 0 |
| 5 | 995 | 5 |
| 10 | 990 | 10 |
| 20 | 980 | 20 |
| 30 | 970 | 30 |
| 40 | 960 | 40 |
| 50 | 950 | 50 |
| 60 | 940 | 60 |
| 70 | 930 | 70 |
| 80 | 920 | 80 |
| 90 | 910 | 90 |
| 100 | 900 | 100 |

Table 1. Glucose standard curve, 0-100 µg/mL, was generated using a NIST traceable glucose standard for analysis of glucose production from enzymatic degradation of cellulosic feedstock by cellulase.

### Results and Discussion

The aliquots of the cellulosic degradation reactions from the dilution series were diluted 5- or 20-fold, using 25 or 5 µL of reaction volume, to a final volume of 100 µL in deionized water for analysis by a glucose oxidase assay. Solution absorbance was read at 540 nm in either a microplate or low-volume cuvette and the linear portion of each measurement series plotted (Figure 3). The glucose concentration was then calculated from the standard curve (Figure 4).
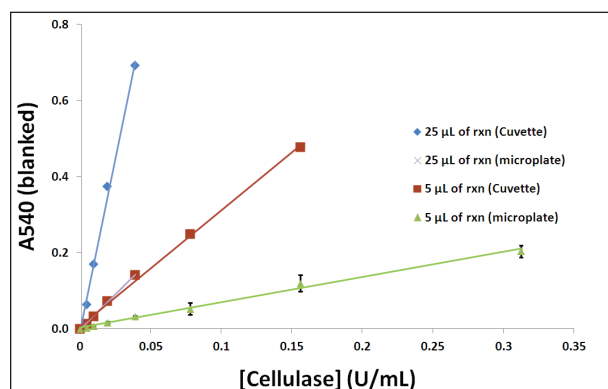


Figure 3. Absorbance measurements representative of the linear portions of a cellulase dilution series. Either 5 or 25 µL of a 1:2 serial dilution series of cellulosic degradation reactions catalyzed by cellulase were analyzed by a glucose oxidase assay. Absorbance measurements were performed in both a microplate and low-volume cuvette format for comparison.
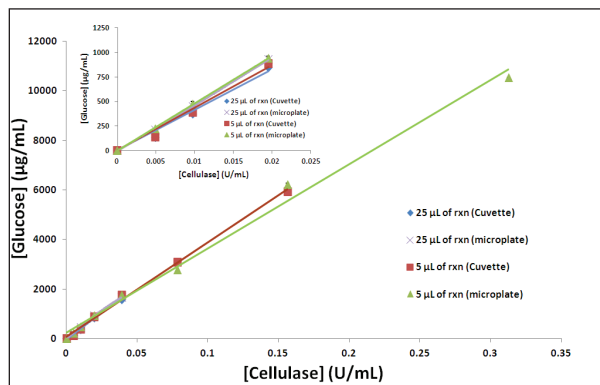
*Figure 4.* Glucose concentration in each reaction was calculated from a standard curve and plotted versus cellulase concentration in each degradation reaction.

As seen in figure 4, excellent correlation between the calculated glucose concentrations is apparent regardless of the volume of cellulose degradation reaction analyzed or measurement vessel used for absorbance readings. The ability to easily vary sample volume during analysis allows the experimenter to insure sample detection falls within the linear response range of the standard curve.

## Conclusions

The enzymatic determination of glucose provides a specific, sensitive and rapid means to quantify glucose from a variety of sources with minimal sample preparation. In the field of biofuels research, the use of cellulosic feedstock for generation of glucose, and ultimately ethanol, has the potential to overcome some of the costs associated with the use of other sources of biomass such as corn. Reliably monitoring glucose production during bioprocessing is a critical step required during research and development of novel fermentation methods. The ability to miniaturize the analysis provides further benefits such as the ability to screen large numbers of processes, under a variety of conditions, in parallel. The Eon™ Microplate Spectrophotometer with cuvette port provides a flexible platform to perform both standard and low-volume sample analysis within a single instrument.