

# Short Technical Reports

## Microplate Diffusion Assay for Screening of $\beta$ -Glucanase-Producing Microorganisms

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### ABSTRACT

A method is described to screen fungal strains rapidly for overexpression of extracellular  $\beta$ -1,4-endoglucanase in the presence of high levels of sugar compounds. The semi-quantitative assay utilizes microplates in a 96-well format and an azurine dye covalently cross-linked (AZCL) chromogenic substrate. The digestion of AZCL-hydroxyethyl- $\beta$ -1,4-endoglucanase results in the release of a blue dye directly proportional to the amount of enzyme activity present in the sample. Sample absorbance was read at 590 nm, and the enzyme activity was determined by reference to a standard curve. The results from the microplate diffusion assay were similar to the results derived from the Ostazin Brilliant Red-hydroxyethyl cellulose assay. The technique described allowed the rapid comparison and screening  $\beta$ -1,4-glucanase activity directly in spent fungal supernatant, from cultures grown in potato dextrose broth. The method could also be easily adapted for the screening of the presence of other activities such as  $\beta$ -1,3-glucanase activity by using either AZCL- $\beta$ -glucan or AZCL-pachyman in place of the AZCL-hydroxyethyl-cellulose. This assay could be used to measure supernatant within an activity range of 0.1–2 U/mL.

### INTRODUCTION

Cellulose ( $\beta$ -1,4-D-glucan) and  $\beta$ -glucans ( $\beta$ -1,3(4)-D-glucan) are among the most abundant groups of naturally occurring polysaccharides (12). Many microorganisms are capable of degrading cellulose and  $\beta$ -glucans by producing enzymes such as cellulases/ $\beta$ -1,4-endoglucanase and  $\beta$ -glucanases/ $\beta$ -1,3(4)-endoglucanases. These enzymes are commercially produced for various industrial applications by the mass fermentation of high enzyme yielding microorganisms generated through mutation, screening, and selection. The cost

associated with the development of high enzyme yielding microorganisms is dependent on the efficiency of the screening step.

$\beta$ -endoglucanase activities are determined by the extent the natural polysaccharide substrate is hydrolyzed. Microorganisms can be screened for  $\beta$ -endoglucanase production by measuring their ability to grow on defined or semi-defined media (9), by growing cultures in conjunction with an indicator of enzyme activity or by measuring the extracellular enzyme activity in spent fungal cultures. Growing cultures on specific media capable of enzyme detection is the simplest screening method, but it may take days to visualize enzyme activity. Alternatively, measuring cell-free enzyme activity in spent culture filtrates is more rapid, but it may also require more processing.

One of the most widely used methods to measure  $\beta$ -endoglucanase activity in liquid samples is the reducing sugar assay (3,8,11). The reducing sugar assay measures the amount of reduced sugars, such as glucose, present after a standard incubation of substrate with an enzyme sample. The reducing sugar assay can be adapted to a microplate format (15); however, its application is limited to samples with low reducing sugar levels. Samples already high in reducing sugars, such as spent culture filtrates grown in potato dextrose broth, or the use of poor quality assay substrate will result in spectrophotometric background values too high for analysis.

Assays based on chromogenic substrates such as Ostazin Brilliant Red hydroxyethyl cellulose (OBR-HEC) (1), Remazol Brilliant Blue-dyed substrates (Azo-substrates) (13), and azurine blue-dyed and cross-linked substrates (AZCL-substrates) are unaffected by background reducing sugar levels (7). Soluble dyed substrates such as Azo-substrates and OBR-HEC are dissolved in a reaction buffer; after a standard incubation of substrate with an enzyme sample, the amount the dye molecules that have been enzymatically cleaved from the substrate are quantified by spectrophotometric assessment. Chromogenic substrates can also be incorporated into solid assay media. A specific volume of spent culture supernatant is spotted onto the assay medium and al-

lowed to diffuse over a standard period of time. Enzyme activity is visualized as a clearing or a precipitate, and this zone is measured and referenced to a standard curve. More recently, additional assay substrates have been reported, such as amorphous, dyed beads (10), a bifunctionalized cellohexaoside derivative for cellulase assays (2), and an enzyme-linked sorbent assay for estimating  $\beta$ -glucanase activity (14).

The method we describe in this paper uses the same principles and materials described above, but in an unconventional way, thereby allowing one to apply many samples to one microplate and quickly measure their relative enzyme activities on an automatic plate reader. We demonstrate the microplate diffusion assay in this paper by screening isolates of *Coniothyrium minitans* grown in potato dextrose broth for enhanced  $\beta$ -1,4-endoglucanase activity after UV mutagenesis (5).

### MATERIALS AND METHODS

#### Fungus and Culture Conditions

Strains of *C. minitans* collected from Manitoba (4) and Alberta, Canada, were deposited at the type culture collection of Agriculture and Agri-Food Canada. The mutant strains A10-4 and A8-1 were derived from the parental strain LRC2134 (wild-type) after UV irradiation. *C. minitans* spore suspensions were placed 10 cm from a shortwave (254 nm) UV light germicidal lamp (Westinghouse). All stock cultures were stored in 10% glycerol in a liquid nitrogen tank. Working cultures were grown at 20°C for 14 days and then maintained on potato dextrose agar (Difco, Detroit, MI, USA) for up to 45 days at 4°C before use.

#### Production of Extracellular Enzymes in Shake Liquid Cultures

Constitutive extracellular  $\beta$ -1,4-endoglucanase activity was measured in supernatant from *C. minitans* spent cultures grown in potato dextrose broth. Cultures were grown at 20°C under continuous light ( $12.5 \mu\text{E m}^{-2} \text{s}^{-1}$ ) and agitation (shaking at 200 rpm) and sampled at 7 and 14 days.

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## Enzyme Assays

### Microplate diffusion assay (MDA).

The assay medium used to screen high  $\beta$ -1,4-endoglucanase producing fungal isolates contained 0.2% AZCL-hydroxyethyl-cellulose (Megazyme, County Wicklow, Ireland) and 2% agar dissolved in 25 mM sodium acetate, pH 4.5. The assay medium was autoclaved, cooled to 55°C, and stirred, and 20 mL of the assay medium were pipetted into the lid of a 96-well microplate (Falcon 3077; BD Biosciences, Mississauga, Ontario, Canada). Once solidified, a single layer of sterile cheesecloth cut to fit the 96-well microplate lid was carefully placed on the assay medium, thereby lining the lid and reinforcing the assay matrix.

Standard  $\beta$ -1,4-endoglucanase (Megazyme) was diluted in 25 mM sodium acetate (pH 4.5) to final concentrations of 4, 2, 1, 0.75, 0.5, 0.25, and 0.00 U/mL, and 50  $\mu$ L each sample were added into a designated row of wells in the 96-well microplate diffusion assay (in duplicate). Then, 50  $\mu$ L each of the test fungal culture supernatant were transferred into a designated well and repeated in triplicate. All the sample wells in the diffusion assay plate were subsequently filled with 25 mM sodium acetate (pH 4.5) to a final volume of 350  $\mu$ L. The lid lined with the assay medium was placed on top of the sample-filled, 96-well microplate assay and inverted while firmly pressing the lid and plate together. To ensure direct contact between enzyme sample and the assay medium, all air bubbles were removed by firmly pressing the bottom of the plate (forcing any air bubbles to rise to the top of the well) (Figure 1). Assay

plates were incubated for several hours at room temperature (20°C–22°C).

The AZCL-hydroxyethyl-cellulose is insoluble in buffered solutions; however, rapid hydration of the substrate by the assay medium will form a soluble gel particle available for  $\beta$ -1,4-endoglucanase digestion. Digestion by  $\beta$ -1,4-endoglucanase releases blue dye fragments into the media, resulting in a visible blue color. Consequently, the blue dye diffuses from the solid agar assay medium and into the contacting sample/enzyme solution contained within the wells of a 96-well microplate. As a result, a more intense blue color will develop in the sample well containing relatively higher  $\beta$ -1,4-endoglucanase activity. To measure the enzyme activity, the plates were flipped upright, the lids were removed, and the absorbances of samples were read on an automatic microplate reader at 590 nm (Tecan, Durham, NC, USA). Sample activity was predicted from a standard curve produced from the relative absorbances of the standard  $\beta$ -1,4-endoglucanase samples in each individual plate. To minimize the diffusion of dye and enzyme between sample wells, the enzyme reaction was terminated by removing the lid as soon as the blue color became visible in the wells of the assay plate.

**OBR-HEC assay.** The assay was performed as described by Biely et al. (1). OBR-HEC substrate (Sigma, St. Louis, MO, USA) was prepared in 25 mM sodium acetate, pH 4.5. The sample supernatant (enzyme solution) was diluted 1/5 in 25 mM sodium acetate, pH 4.5. Enzyme activities were estimated from a standard curve prepared using  $\beta$ -1,4-endoglucanase (Megazyme) in an activity range of 0.1 to 0.5 U/mL.

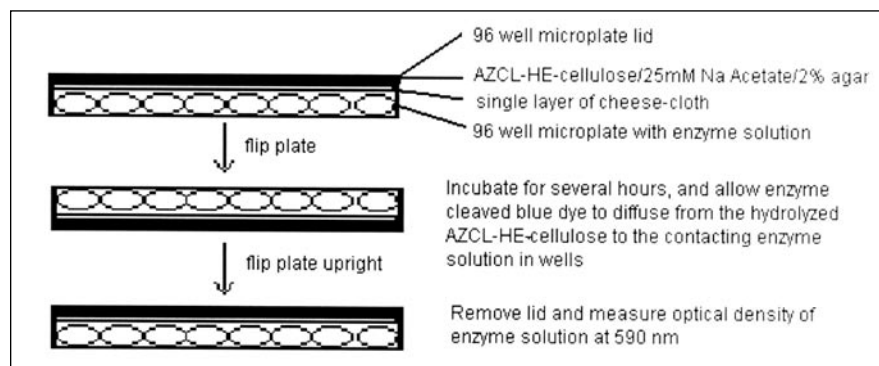


Figure 1. A schematic diagram of the MDA assembly and method. HE, hydroxyethyl.

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## RESULTS AND DISCUSSION

The results of the cellulase MDA on the *C. minitans* culture spent culture supernatants were reproducible. The standard curves derived from the results of the cellulase MDA were linear to a concentration of enzyme activity of 2 U/mL (Figure 1). The variation between individual MDA standard curves resulted from small variations in the distribution of the insoluble AZCL-hydroxyethyl-cellulose. The advantage of dyed, cross-linked insoluble substrates is that they are stable and considerably more sensitive than their soluble counterparts (6). An inherent disadvantage of using an insoluble substrate is that it must be weighed accurately into each assay plate or test tube to ensure that the particles are distributed evenly. Attempts to pipet the assay media directly and accurately into assay wells failed, as the insoluble particles would stick to the plastic pipet tips regardless of bore size. We then decided to pour a set volume into the lid of multi-well plate and use an inverted plate setup. We attempted to minimize plate variation by always allowing the assay media to cool to 55°C, constantly stirring the media before pouring to keep the insoluble particles suspended, adding exactly 20 mL assay medium to each plate lid, pouring plates on a level surface, storing plates in an identical manner, and using plates of the same age. Also, there may be small variations between batches of AZCL-substrates when purchased from the manufacturer. Difficulties associated with achieving very reproducible data between plates can be solved by simply testing a set of standard enzyme samples within each assay plate. If eight wells per plate are used to test standard enzyme samples, then there are still 88 wells left to test unknown samples.

Table 1 demonstrates the similar results obtained when testing the cellulase activities of identical sample supernatants with either the MDA or the OBR-HEC assay. Supernatant samples collected from triplicate cultures (a-c) of UV-mutated strains A10-4 and A8-1, selected for constitutive expression, demonstrated relatively high levels of cellulase expression, while supernatants collected from the non-constitutive ex-

Table 1. Comparison of  $\beta$ -Endoglucanase Activities between the MDA and the OBR-HEC Assays

Fungal Cultures	MDA (U/mL)	OBR-HEC (U/mL)
2134a	0.05 $\pm$ 0.07	ND
2134b	0.02 $\pm$ 0.02	ND
2134c	0.06 $\pm$ 0.02	ND
A10-4a	0.77 $\pm$ 0.12	0.85 $\pm$ 0.21
A10-4b	0.92 $\pm$ 0.14	0.88 $\pm$ 0.16
A10-4c	1.47 $\pm$ 0.44	1.23 $\pm$ 0.21
A8-1a	1.83 $\pm$ 0.23	2.05 $\pm$ 0.18
A8-1b	1.43 $\pm$ 0.40	1.50 $\pm$ 0.05
A8-1c	1.40 $\pm$ 0.28	1.37 $\pm$ 0.41

MDA and OBR-HEC enzyme activity values from cultures of *C. minitans* grown on potato dextrose broth are the average of three replicates.  
ND, not detectable

pressing wild-type strain LRC2134 had very little cellulase expression after seven days of growth on potato dextrose broth. The absorbance readings observed between triplicate samples for each fungal culture were reproducible. To confirm the test results, samples should be tested at least in duplicate on different diffusion plates.

A number of technical variations were performed on the MDA during its development. The use of agarose versus agar in the assay media was compared. Enzyme activity diffused more rapidly into the agarose based assay media than the agar-based media. This caused quicker color development; however, the agarose-based assay media were also more readily degraded by the enzymes present in the spent fungal supernatant than the agar-based assay me-

dia. Consequently, the agarose assay layer would fall apart when the plate and lid were separated from one another, and then pieces of the agarose assay layer would fall into the sample wells; this would interfere with the sample absorbance readings. A single layer of cheesecloth was included into the design of the assay to reinforce and improve the durability of the assay layer. Cheesecloth was chosen because of its availability; however, replacing the cheesecloth with a synthetic material could reduce potential wicking effects and interaction with enzyme.

The length of time the plates were incubated and their orientation during incubation were important. In our experiment, we chose to incubate the MDA overnight at room temperature. However, the length of time required to

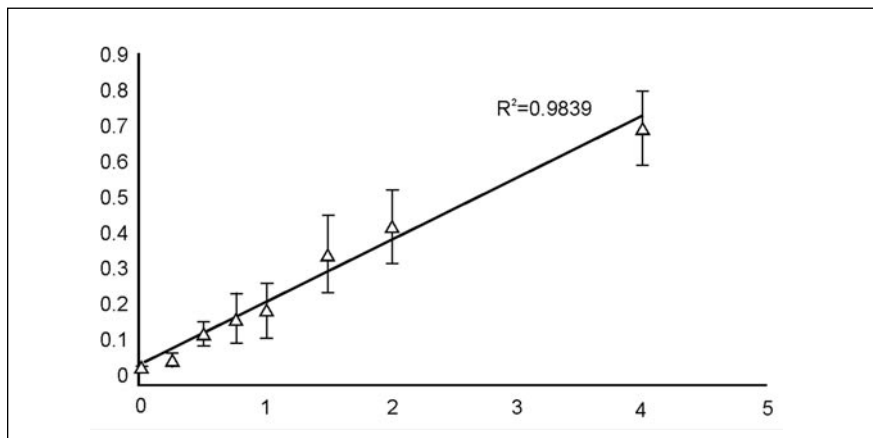


Figure 2. Standard curve derived from the activity of standard  $\beta$ -1,4-endoglucanase. Absorbance activity measurements at 590 nm were estimated at concentrations of 0, 0.25, 0.5, 0.75, 1, 2, and 4 U/mL  $\beta$ -1,4-endoglucanase (x-axis). Each value is the  $\bar{x} \pm$  SEM of five experiments.

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complete the plate assay can be reduced to 4–5 h by increasing the incubation temperature to 37°C (data not shown). However, it is important not to over-incubate the plates, as dye particles from wells containing higher enzyme activities will eventually diffuse into neighboring wells if left too long. This could be minimized by removing the plate immediately upon the appearance of dye in the sample wells. Diffusion of dye particles from wells containing relatively high enzyme activity standards can also be reduced by separating the wells containing the enzyme standards with the wells containing sample unknowns with a single empty row of wells.

Flipping plates upside down was necessary to ensure the substrate was in full contact with the enzyme sample. It was important to firmly press and support plates evenly while flipping over to prevent spillage between wells. This step can be made easier by carefully taping the plate bottom and lid together before flipping.

This novel microplate assay is a relatively easy, rapid, and inexpensive method to screen large numbers of fungal cultures for enhanced enzyme production. Approximately 88 samples could be tested per MDA (after using eight wells for the enzyme standards) while only using 20 mL assay media. In comparison, traditional diffusion plate assays, in which a small amount of enzyme is dropped onto the assay media layer, will only allow the test of 8–10 samples per standard size Petri dish (10 cm diameter) with 20 mL assay media. The traditional diffusion plate assays also requires the measurement of the zone of clearing from each sample while the MDA allows quick measurement of sample absorbance on an automated microplate reader. Unlike the microplate format by Zheng and Wozniak (15) based on the reducing sugar assay, our MDA is unaffected by the presence of reduced sugars and allows the quick semi-quantitative analysis and screening of crude spent fungal culture supernatants. The MDA can also be easily applied to test for other enzyme activities such as  $\beta$ -1,3(4)-endoglucanase activities by changing the AZCL-hydroxyethyl-cellulose substrate to the AZCL- $\beta$ -glucan or AZCL-lichenan substrates.

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## Bacteriophage Gene Targeting Vectors Generated by Transplacement

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### ABSTRACT

A rate-determining step in gene targeting is the generation of the targeting vector. We have developed bacteriophage gene targeting vectorology, which shortens the timeline of targeting vector construction. Using retro-recombination screening, we can rapidly isolate targeting vectors from an embryonic stem cell genomic library via integrative and excisive recombination. We have demonstrated that recombination can be used to introduce specific point mutations or unique restriction sites into gene targeting vectors via transplacement. Using the choline/ethanolamine kinase  $\alpha$  and  $\beta$  genes as models, we demonstrate that transplacement can also be used to introduce specifically a neo resistance cassette into a gene targeting phase. In our experience, the  $\lambda$ TK gene targeting system offers considerable flexibility and efficiency in TV construction, which makes generating multiple vectors in one week's time possible.

### INTRODUCTION

With the estimated discovery of more than 35 000 genes via the genome projects, there is considerable interest in determining the function of these genes in the emerging era of functional genomics. Here there is a need to speed up the process of targeting vector generation and to develop technologies for the generation of subtle mutations beyond the gene knockout. We used bacteriophage  $\lambda$  as an efficient vector for scaffolding and transferring genomic alterations to embryonic stem cells via gene targeting. We have previously developed phage-plasmid recombination to direct modification cassettes to specific sites within phage targeting vectors via double crossover (5). Small regions of homology are used to direct modification cassettes to specific sites within the recombination-proficient gene targeting phages (5,7). A suppressor tRNA gene *supF*, positioned within the homologous region, acts as a positive selectable marker for recombination by suppressing amber mutations in  $\lambda$ TK. A negative marker gene, *gam*, is placed outside the region of homology to enrich for double-crossover recombinants by selectively killing single-crossover recombinants via spi selection (2,4).

We have also established new methods that use the sequential integrative and excisive recombination of plasmids. Retro-recombination screening allows the rapid purification of phage targeting vectors from an embryonic stem cell targeting vector library in  $\lambda$ TK (7), following the homologous integration of a *supF*-bearing recombination plasmid. As the region of homology is duplicated on integration and can revert under relaxed conditions, we incorporated *gam* into the recombination plasmid to spi select phages that have reverted to their original configuration (6,7). Using retro-recombination screening, we isolated specific targeting vector phages in 2–3 days to serve as templates for the rapid completion of targeting vector construction. An alternative method, called transplacement mutagenesis, uses an integrative and excisive recombination strategy to transfer point mutations to target phages using a recombination plasmid interface (6,8). Following the excision

of the plasmid, a mutation is transferred precisely in approximately 50% of condensants. We routinely introduce unique restriction sites into  $\lambda$ TK phages for the subsequent introduction of modification cassettes.

In theory, transplacement can also be used to introduce larger changes, including sequence additions, provided that sufficient regions of homology are included. Accordingly, we have investigated whether transplacement could be used as a strategy for introducing *neo<sup>R</sup>* selection cassettes using integrative and excisive recombination (Figure 1). Using the mouse choline/ethanolamine kinase genes  $\alpha$  and  $\beta$  (CK/EK  $\alpha$  and  $\beta$ ) (1) as a template, we demonstrate that retro-recombination screening and transplacement can be combined to insert unique restriction sites as targeting vectors are plaque-purified. We also show how modification cassettes can be introduced into targeting vectors via transplacement.

### MATERIALS AND METHODS

#### Bacterial Strains, Recombination Plasmid Constructs, and Bacteriophages

The *E. coli* strains,  $\lambda$ TK embryonic stem cell library, and recombination plasmid  $\pi$ AN $\gamma$  have been described previously (6,7). Recombination plasmid  $\alpha$ sx $\pi$ AN $\gamma$  (Figure 2A) was created by ligating two genomic PCR fragments that surround the seventh exon of CK/EK  $\alpha$  into  $\pi$ AN $\gamma$  to introduce two novel restriction sites (*SalI* and *XhoI*) into the recombination probe sequence. A 247-bp (+19 non-homologous region) 5' *EcoRI*-*XhoI* fragment was generated by genomic PCR using forward (5'-AAAGAATTCCAGATAGTAGCTGAG-3') and reverse (5'-AAACTCGAGTCGACCTACAAGAGATTG-3') primers. Similarly, a 213-bp (+16) 3' *XhoI*-*BamHI* fragment was generated by genomic PCR using forward (5'-AAACTCGAGAATATCTTATTGTTGGAAG-3') and reverse (5'-AAAGGATCCTCAGGGACAAACA-3') primers. Its derivative,  $\alpha$ sxneo $\pi$ AN $\gamma$ , was generated by inserting a *XhoI* cassette of *pol2:neo<sup>R</sup>* in opposite transcriptional orientation to the CK/EK  $\alpha$  gene. For  $\beta$ sx $\pi$ AN $\gamma$ , a 194-bp (+18) 5' *EcoRI*-