

Video Article

A High Throughput Screen for Biomining Cellulase Activity from Metagenomic Libraries

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Abstract

Cellulose, the most abundant source of organic carbon on the planet, has wide-ranging industrial applications with increasing emphasis on biofuel production¹. Chemical methods to modify or degrade cellulose typically require strong acids and high temperatures. As such, enzymatic methods have become prominent in the bioconversion process. While the identification of active cellulases from bacterial and fungal isolates has been somewhat effective, the vast majority of microbes in nature resist laboratory cultivation. Environmental genomics, also known as metagenomic, screening approaches have great promise in bridging the cultivation gap in the search for novel bioconversion enzymes. Metagenomic screening approaches have successfully recovered novel cellulases from environments as varied as soils², buffalo rumen³ and the termite hind-gut⁴ using carboxymethylcellulose (CMC) agar plates stained with congo red dye (based on the method of Teather and Wood⁵). However, the CMC method is limited in throughput, is not quantitative and manifests a low signal to noise ratio⁶. Other methods have been reported^{7,8} but each use an agar plate-based assay, which is undesirable for high-throughput screening of large insert genomic libraries. Here we present a solution-based screen for cellulase activity using a chromogenic dinitrophenol (DNP)-cellobioside substrate⁹. Our library was cloned into the pCC1 copy control fosmid to increase assay sensitivity through copy number induction¹⁰. The method uses one-pot chemistry in 384-well microplates with the final readout provided as an absorbance measurement. This readout is quantitative, sensitive and automated with a throughput of up to 100X 384-well plates per day using a liquid handler and plate reader with attached stacking system.

Protocol

Before starting this protocol, you will need your metagenomic library stored in a 384 well plate format. In our study, we used the pCC1 copy control fosmid vector in combination with phage T1-resistant TransforMax EPI300-T1^R *E. coli* cells as the library host and stored our plates at -80°C¹¹.

1. Replication of the Metagenomic Library Plates

1. Defrost the plates containing your library at 37°C for approximately 20 minutes, or until all wells are thawed.
2. Use the UV light sterilizing feature on the qPix2 to sterilize the robot for 15 minutes.
3. Prepare LB broth with chloramphenicol at a final concentration of 12.5ug/mL and arabinose at 100ug/mL in a 500mL reagent bottle. Each plate will use approximately 20mL, plus make an additional 50mL to allow for dead volume.
4. Set up the qFill3 as per manufacturer's instructions with the media bottle attached to the manifold via the sterile tubing. Program it for the appropriate amount of media, and set it to fill a 45uL volume in each well.
5. Purge the air from the tubing and manifold using the purge feature of the robot until media is visible coming from each pin of the manifold.
6. Fill the desired number of plates with LB media using the qFill3. Each plate takes approximately 20 seconds to fill.
7. Load the library plates and the fresh plates into the appropriate areas of the qPix2 robot. Fill the cleaning baths with the appropriate reagents; 2% Micro90 in the rear bath, autoclaved distilled water in the middle bath, 80% ethanol in the front bath.
8. Use the "Replicating" program of the qPix2. In the software, select the appropriate head, number and types of source plates and destination plates. Also set up the head to clean between replications, usually 6 cycles in each bath is enough. Capacity of the machine is 10 plates at a time, and it takes approximately 15 minutes to replicate them all with a 384 pin head (or approximately 50 minutes with a 96 pin head).
Note: There is an option to "Stir Source" or "Stir Destination". It is recommended to not use these options as problems have previously occurred with our robot using them.
9. Once the plates are replicated, grow the plates at 37°C for 24 hours in a humidity box. Because we are inducing a high copy number of fosmid with addition of arabinose, the clones tend to grow slower than non-induced clones. Return the library plates to -80°C.
Note: Incubation in a humidity box ensures evaporation of media does not occur in the wells on the edge of the plates, keeping a uniform environment for all wells.
10. Clean the qFill3 robot by first purging it with water (~50mL) and then 80% ethanol (~50mL). Disassemble the components, and clean and autoclave the bottle, lid, and tubing wrapped in aluminium foil.

2. Measuring the Growth of *E. coli* Clones

1. Remove plates from 37°C incubator. Remove and set aside the lids, and place plates onto the magazine loading platform provided with the RapidStak, up to a maximum of 25 plates.
Note: The plate at the bottom of the stack will be the first one to be read. Make sure to keep track of the order of the plates, as the software does not record this.
2. Remove one magazine from the Rapid Stak, ensure it is empty, and push it onto the stack of plates to be read. Grab it by the handle, and the plates should all be loaded into the magazine. Load the magazine into the RapidStak.

Note: The magazine has a small screw in the corner denoting which corner the A1 wells of the plates should go. The magazine will only mount in the RapidStak in one orientation.

3. Open the SkanIt RE program on the computer connected to the machines. Select "New Session" and name it appropriately. Choose "Corning Flat Bottom 384-well plate" as the plate type.
4. In the "Plate Layout" area, select the "Wizard" button, and choose it to add 384 unknowns to your plate.
5. In the "Protocol" area, select the "Well Loop" option, and enter for 384 wells. A "Well Loop" icon will appear in the flow- tree on the left hand side of the screen. Select the icon and click to add "Photometric Measurement". Set it to read at 600nm.
6. Save your protocol with a unique name, and close the SkanIt RE program.
7. On the computer, open the PolaraRS program.
8. On the main page there will be table listing the instruments connected to the RapidStak device. The VarioSkan should be on the left side of this table. Under the VarioSkan heading, there will be two links; "RunSession" and "Incubate". Click the "RunSession" option.
9. A new window, the assay window, will appear with a flowchart in the middle. Select the "RunSession" item (it should be the only item present). On the right side of the screen a drop-down menu will appear. Find the name of your saved SkanIt RE run in this menu.
Note: There appears to be no rational ordering to this menu. It's not alphabetical, or by date added, or related to where on the computer the protocol is saved. This means you must look through all the protocols before finding your own, which is a pain.
10. Once you have selected your protocol, in the upper right corner click "Run this assay".
11. A box will pop up, asking you to denote which magazine to use as the source, and which magazine is empty. The lower box on the screen corresponds to the front magazine. [Optional: It asks you to enter the number of plates loaded to determine an estimate for the amount of time it will take. This estimate is normally way off.]
12. Ensure both the RapidStak and the VarioSkan are turned on, and press "OK".
13. The RapidStak will automatically load your plates into the plate reader, allowing for continuous measurements of plates. To read 25 plates takes approximately 50 minutes.
Note: It is recommended to observe the first plate loading into the VarioSkan to ensure proper alignment of the machines. If the RapidStak does not load the plate reader properly, use the "Pause this assay" button in the top right corner of the PolaraRS window.
14. Once the VarioSkan is done reading all the plates, remove the full magazine and place it on the magazine loading platform. Lift up the outer rectangle of the platform, and the magazine should slide off, leaving the plates standing in a pile in the middle. Replace the lids to the appropriate plates.

3. Addition of the Assay Mix to Each Plate

1. Prepare assay mix using premade 10x stock of lysis mix (10% Triton X-100, 100mM ris, 10mM EDTA) in 50mM potassium acetate buffer at pH 5.5. Prepare a stock of up to 75mg/mL DNP-cellobioside substrate in DMSO, making sure the substrate is fully dissolved. Add DNP-cellobioside stock solution to assay mix to a final concentration of 0.1mg/mL. Each plate will use approximately 20mL of solution, plus make an additional 50mL to allow for dead volume.
Note: DNP-Cellobioside is not readily soluble in water, so dissolving in DMSO increases the solubility. The presence of DMSO in the final assay solution has no observable effects.
2. Set up the qFill3 as per manufacturer's instructions with the media bottle attached to the manifold via the sterile tubing. Program it for the appropriate amount of media, and set it to fill a 45uL volume in each well.
3. Purge the air from the tubing and manifold using the purge feature of the robot until media is visible coming from each pin of the manifold.
4. Add assay mix to each plate using the qFill3. Each plate takes approximately 20 seconds to fill.
5. Once assay mix is added, incubate the plates at 37°C in a humidity box for 12-16 hours.
Note: Over-incubation of plates once the assay mix is added will cause evaporation in the outer wells of the plate, yielding higher absorbance readings than seen for the inside wells. This can be offset by incubating plates in a humidity chamber.

4. Reading Absorbance of Assayed Clones

1. Remove plates from 37°C incubator. Remove and set aside the lids, and place plates onto the magazine loading platform provided with the RapidStak, up to a maximum of 25 plates.
Note: The plate at the bottom of the stack will be the first one to be read. Make sure to keep track of the order of the plates, as the software does not record this.
2. Remove one magazine from the Rapid Stak, ensure it is empty, and push it onto the stack of plates to be read. Grab it by the handle, and the plates should all be loaded into the magazine. Load the magazine into the RapidStak.
Note: The magazine has a small screw in the corner denoting which corner the A1 wells of the plates should go. The magazine will only mount in the RapidStak in one orientation.
3. Open the SkanIt RE program on the computer connected to the machines. Select "New Session" and name it appropriately. Choose "Corning Flat Bottom 384-well plate" as the plate type.
4. In the "Plate Layout" area, select the "Wizard" button, and choose it to add 384 unknowns to your plate.
5. In the "Protocol" area, select the "Well Loop" option, and enter for 384 wells. A "Well Loop" icon will appear in the flow- tree on the left hand side of the screen. Select the icon and click to add "Photometric Measurement". Set it to read at 400nm.
6. Save your protocol with a unique name, and close the SkanIt RE program.
7. On the computer, open the PolaraRS program.
8. On the main page there will be table listing the instruments connected to the RapidStak device. The VarioSkan should be on the left side of this table. Under the VarioSkan heading, there will be two links; "RunSession" and "Incubate". Click the "RunSession" option.
9. A new window, the assay window, will appear with a flowchart in the middle. Select the "RunSession" item (it should be the only item present). On the right side of the screen a drop-down menu will appear. Find the name of your saved SkanIt RE run in this menu.
10. Once you have selected your protocol, in the upper right corner click "Run this assay".
11. A box will pop up, asking you to denote which magazine to use as the source, and which magazine is empty. The lower box on the screen corresponds to the front magazine. [Optional: It asks you to enter the number of plates loaded to determine an estimate for the amount of time it will take. This estimate is normally way off.]
12. Ensure both the RapidStak and the VarioSkan are turned on, and press "OK"
13. The RapidStak will automatically load your plates into the plate reader, allowing for continuous measurements of plates.
Note: It is recommended to observe the first plate loading into the VarioSkan to ensure proper alignment of the machines. If the RapidStak does not load the plate reader properly, use the "Pause this assay" button in the top right corner of the PolaraRS window.

14. Once the VarioSkán is done reading all the plates, remove the full magazine and place it on the magazine loading platform. Lift up the outer rectangle of the platform, and the magazine should slide off, leaving the plates standing in a pile in the middle. The plates can be disposed of according to your lab procedures.
15. To export the absorbance readings, open SkánIt RE software, and choose "Open an existing file".
16. Select the directory where you saved your session, hit the "+" icon to expand the list and under the title heading will be options labeled "Container 1" to "Container N" depending on the number of plates. Select the first plate to be analyzed.
17. In the menu bar at the top of the page, select "Data Processing > Report/Export"
18. Choose the options desired for export.
Note: I typically select "Plate Layout" and "Photometric" data for export.
19. Click "View Report" and then "File > Save". Choose the desired directory. The output format is a Microsoft Excel spreadsheet

5. Representative Results

An example of absorbance readings from a single 384 well plate containing a positive clone is shown in Figure 2. Positive clones show a marked increase in absorbance over those not expressing cellulase activity. Differences in assay time, well location on the plate, or DNP concentration (may be introduced by filtering out un-dissolved DNP) can affect absolute absorbance readings. Relative absorbance readings, such as the difference in absorbance above the plate average or column average, are a more robust method of identifying cellulase positive clones.

Following identification of positive clones from the library plates, it is recommended to replicate all positive clones into a new plate for secondary screening. This eliminates effects arising from well location or plate variation and allows for more direct comparison between positive clones.

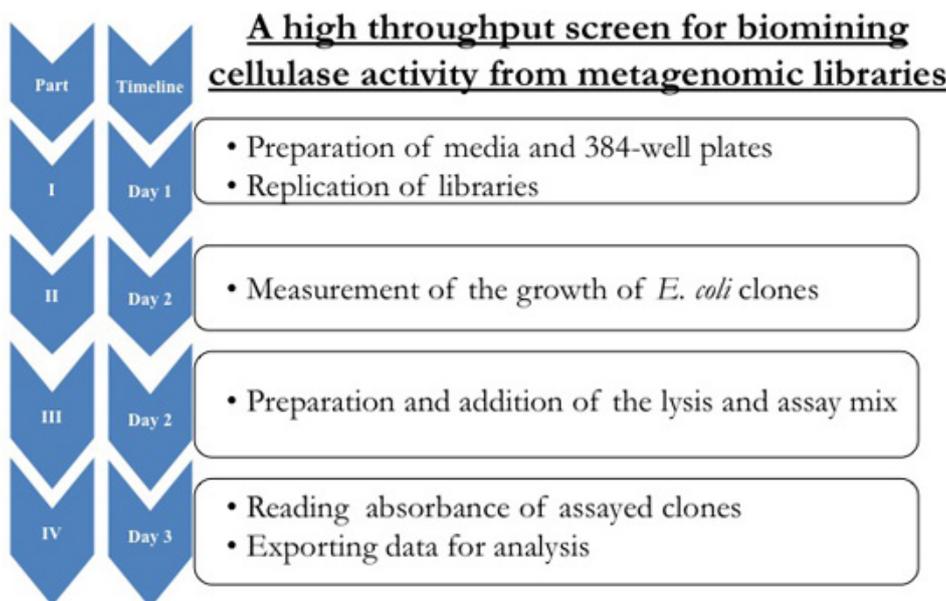


Figure 1. Flow chart of the high-throughput assay for a metagenomic library cloned into *E. coli* and stored at -80°C.

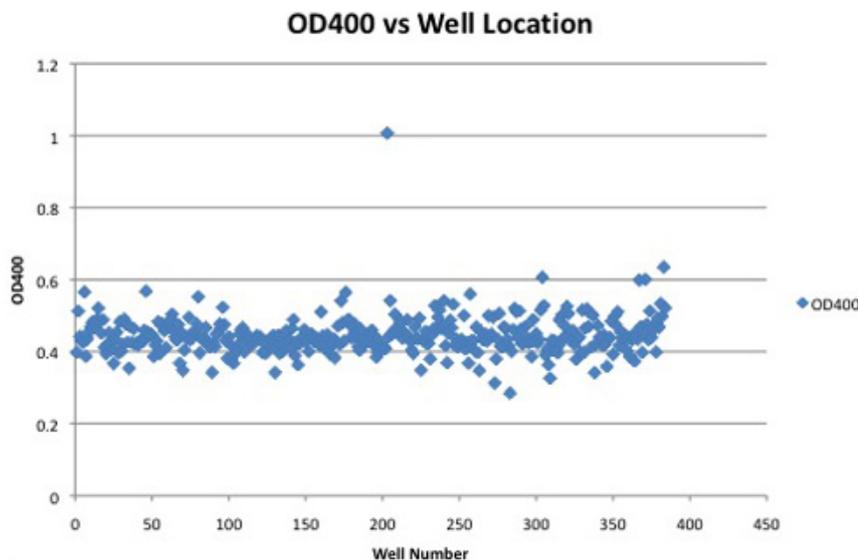


Figure 2. Absorbance readings from one 384-well plate containing a positive clone. Cellulase positive clones can be identified by significantly increased absorbance over negative clones.

Discussion

A high throughput screen for the rapid detection of cellulolytic activity from a large insert genomic DNA metagenomic library expressed in *E. coli* is described in this protocol. This method is an improvement over the CMC/Congo Red assay commonly used in the literature. It is solution

based, and allows for one-pot chemistry screening in 384-well plates, with the final output as absorbance readings from a plate reader allowing for quantitative analysis. The automation of each step of this process allows for the unsupervised screening of more than 25 384-well plates per hour. The data can be easily exported from the software into a Microsoft Excel spreadsheet, allowing for analysis or processing by third party software.

One limitation of this assay exists in the expression potential of exogenous proteins in the *E. coli* host. A metagenomic library contains DNA from many different organisms, only a subset of which can be recognized by the *E. coli* transcription/translation machinery. Even when expressed, exogenous gene products may not achieve functionality due to improper folding, processing, or inadequate expression levels. These limitations can be partially offset through the utilization of copy control systems, as seen in previous functional metagenomic screening studies.¹² The pCC1 copy control fosmid used here allows induction through addition of the inducer L-arabinose, and increases copy number from one, to up to 100 copies per cell¹³. These systems can improve the outcome of activity-based screens by enabling single copy growth for stability with subsequent induction for increased activity.

The substrate 2,4-DNP-Cellobioside used in our screen is not commercially available from suppliers, but similar substrates can be purchased. Sigma-Aldrich offers 2-nitrophenyl (Cat No. N4764) and 4-nitrophenyl (Cat No. N5759) cellobiosides. The general screen as described could be undertaken with these substrates, but some modifications would be required. These mono-substituted phenols have higher pKa values, around 7.2, compared to DNP, which is around 4. Optimum pH for cellulase activity has been reported to range from pH 4.5-6.0^{14, 15}. The use of DNP-C allows for the assay to be carried out at optimal pH conditions, allowing for easier identification of cellulases. In addition, the di-substituted glycoside is much more reactive than the others, allowing for the detection of more reluctant cellulases. Thus, the use of DNP-cellobioside has allowed for a more robust and sensitive screen than would be available with commercial substrates.

It is notable that this screen can be used for detection of any enzymes with an associated colorimetric or fluorometric substrate. Cellulases are a stable and active enzyme, ideal for the initial development and optimization of the screening parameters. The general approach presented here is a powerful tool for the screening of metagenomic libraries for both academic and industrial applications.

Disclosures

No conflicts of interest declared.

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