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TITLE:

Measurement of Chitinase Activity in Biological Samples

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KEYWORDS:

Chitinase activity, Chitotriosidase, biological fluids, BAL, bronchoalveolar lavage, serum

SUMMARY:

Presented here is a simple method to measure chitinase activity in biological fluids such as bronchoalveolar lavage or serum.

ABSTRACT:

Chitinases are the enzymes that cleave chitin. Even in the absence of chitin, mammalians have significant amounts of chitinases present in the body including at baseline. The precise role of chitinase is not known, however it was believed to play important role in digestion and host defense against chitin-containing food and pathogens, respectively. Recent work, including ours, has shown an important role of chitinase and chitinase-like proteins in host immunity and allergic diseases. Importantly, chitinase activities serve as important biomarkers of disease severity in a wide-range of diseases including type 2 inflammatory diseases such as asthma and pulmonary fibrosis. Similarly, patients with genetic disorders like Gaucher disease have significantly elevated chitinase levels, which not only correlate with disease severity but also serve as a reliable biomarker for therapeutic effectiveness. The protocol outlined here describes a simple, quick, and straightforward way to measure chitinase activity in BAL or serum samples of mice and can be widely adapted to human subjects and other model organisms due to the highly conserved nature of the enzymes.

INTRODUCTION:

Chitin is the second most abundant polysaccharide on the earth after cellulose, serving as a major structural component of a variety of organisms including the exoskeleton of insects, fungi, yeast, and algae; some vertebrates also have chitin[1]. Chitinases are a family of enzymes that are capable of breaking down chitin and are highly conserved throughout evolution in species ranging from bacteria to mammals[2,3]. In addition to chitinases, mammals also have chitinase-like proteins which are similar to chitinases in their ability to bind chitin but differ in that they lack enzymatic ability to cleave the chitin[4].

Although chitin and chitinases have long been studied—with investigations dating back to the

early 1900's—the main focus has been on their role in insects and other invertebrates. In fact, it was not until the 1960s when vertebrates were found to have chitinases at all. Using a chitobiase-based assay, chitinases were shown to be found in the digestive tract of a number of vertebrates including lizards and blackbirds—an observation hypothesized to be due to the consumption of chitin-containing organisms such as insects[5].

Mammals have two enzymatically active forms of chitinase: acidic mammalian chitinase (AMCase; also known as CHIT2 and CHIA) and chitotriosidase (CHIT1)[4]. Both of these proteins are capable of hydrolyzing chitin. However, CHIT1 is more enzymatically active in humans, where almost all of chitinase activity is derived from CHIT1.[4] In mice, both Chit1 and AMCase almost equally contribute to the overall chitinase activity[6]. On the other hand, chitinase-like proteins lack chitinase activity.

Chit1 is mainly secreted by macrophages and is often considered to be an immune response to chitin-containing pathogens[7]. The enzyme has also been shown to be involved in the maturation of monocytes into both M1 and M2 macrophage subtypes, even without the presence of the substrate chitin[8]. Furthermore, it may also be involved in the maturation of other immune cells including T helper type 2 (Th2) cells and eosinophils—as was shown to be the case in cryptococcal lung infection[9]. These studies point to a complex role of chitinases in the immune system.

In recent years, Chit1 levels have been found to serve as an important biomarker of progression for over 40 different human diseases including lysosomal storage diseases, infectious diseases, respiratory diseases, endocrinological diseases, cardiovascular diseases, neurological diseases, and others (reviewed[10]). In many of these diseases, the levels of Chit1 are a strong predictor of disease severity and therapeutic effectiveness[10].

As chitinases have gained a reputation as a biomarker for numerous medical conditions including Gaucher Disease, tools and assays have been developed to facilitate testing for chitinase presence. Older methods include Schales' procedure, a protocol adapted from a blood glucose test, and the 3,5 dinitrosalicylic acid (DNS) method. However, these methods are often time sensitive and technically difficult[11]. The procedure for both of these tests requires the reduction of inorganic oxidants, ferricyanide in the case of the Schales' procedure for example, producing a color change that can only be measured spectrophotometrically. Additionally, both tests involve a heating or boiling step that is both time consuming and necessary for the color to develop[12,13].

Described here is a quick and simple fluorimetric assay to determine chitinase levels in mammalian samples[14,15]. Two of the samples used here include serum and bronchoalveolar lavage fluids (BAL); chitinase activity has also been measured in breast milk and urine samples, and the technique can be performed in those type of samples as well as in any other biological fluid[16,17].

PROTOCOL:

All animal procedures were performed under an IACUC-approved protocol at Yale University

School of Medicine.

1. Mouse sample collection

1.1. Anesthetization

1.1.1. Anesthetize mice using ketamine and xylazine (100 mg/kg of ketamine and 10 mg/kg of xylazine).

1.2. Blood sample collection

1.2.1. Confirm the depth of anesthesia by non-responsiveness to a toe pinch.

1.2.2. Surgically open the chest cavity to expose the heart.

1.2.3. Insert a 26.5 G needle into the left ventricle and collect blood into a syringe.

NOTE: Typically, about 0.5–1 mL of blood can be harvested from a healthy 20–25 g mouse.

1.2.4. Collect the blood in heparinized tubes, for plasma collection, or non-heparinized tubes for serum collection.

1.3. Collection of bronchoalveolar lavage fluid (BAL)

1.3.1. To collect the BAL, make a vertical cut on the neck to expose the trachea.

1.3.2. Cannulate the trachea with a 22 G catheter and secure it firmly using a string in order to prevent leakage out of the nose of the mouse.

1.3.3. Slowly inject two aliquots of sterile phosphate-buffered saline (PBS, 0.75 mL each) into the lungs via the trachea and retrieve back.

1.3.4. Pool aliquots and keep on ice for further analysis.

1.4. Processing the biological samples.

1.4.1. Blood sample: Either fresh (plasma) or after allowing coagulation for 4 h (serum), centrifuge at 600 x *g* for 10 min. Take the supernatant and either freeze for storage or use for experiment.

1.4.2. BAL: Centrifuge the sample at 350 x *g* for 5 min. Take the supernatant and either freeze for storage or use for experiment.

2. Chitinase assay

NOTE: The science behind the assay is relatively simple. A non-fluorescent substrate is cleaved by enzymatically active chitinase to produce a fluorescent product, which is then measured as an indirect marker of chitinase activity. The chitinases within the samples break down the substrate 4-methylumbelliferyl-D-N, N'-diacetylchitobiose present in the 1x McIlvain buffer. A fluorescent molecule 4-methylumbelliferyl is released. By measuring the total fluorescence of each well, we obtain an accurate measurement of the active chitinase in each sample. Because the breakdown of chitin by chitinase is a hydrolytic reaction, the stop buffer, a mixture of 0.3 M glycine and NaOH (12.0 g/L at pH 10.6), ends the breakdown of chitin by creating an environment that is too basic for the enzyme to function.

2.1. Prepare the substrates, standards, and solutions.

2.1.1. Prepare McIlvain buffer. McIlvian Buffer is comprised of 0.1 M citrate and 0.2 M phosphate at a pH of 5.2. Dilute at a ratio of 1:1 for 1x McIlvian buffer.

2.1.2. Dissolve both 4-methylumbelliferyl-D-N, N'-diacetylchitobiose and 4-methylumbelliferyl ß-D-N, N', N"-triacetylchitotriose in 1x McIlvain Buffer to a concentration of 500 µM each.

NOTE: Stock solution can be stored at -20 °C for further uses; best stored in aliquots.

2.1.3. Dissolve standard, 4-methylumbelliferone, to a concentration of 0.1 mM in stop buffer (mixture of 0.3 M glycine and NaOH (12.0 g/L at pH 10.6)) to create the standard curve stock solution.

2.2. Create and plate the working solution.

2.2.1. Combine 1x McIlvian Buffer and chitin substrate 4-methylumbelliferyl-D-N, N'-diacetylchitobiose to create the working solution. For every 10 samples, mix 100 µL of substrate with 2.17 mL of 1x McIlvian buffer. See **Table 1** for additional calculations based on sample size.

NOTE: Use 4-methylumbelliferyl ß-D-N, N', N"-triacetylchitotriose for human samples. While both substrates can be cleaved by either human or mouse enzymes, they have been assigned for either human or mouse samples based on efficiency of cleavage[6].

2.2.2. Pipette 95 µL of working solution into each well of a 96-well plate.

2.3. Add biospecimen samples to the working solution.

2.3.1. Next, add 5 µL of the test sample (blood/BAL/tissue lysate/other biological fluid) to each well.

2.3.2. Mix the sample into the working solution for the most accurate and reliable results.

NOTE: Samples should be run in duplicates/triplicates with special attention paid to potential

edge effects. All the samples should be pre-warmed to minimize the edge effect. As the assay involves a reaction between chitinases in the sample and substrate in the working solution, it is best to work relatively quickly to minimize the difference in time between when the first sample is plated and the last sample is plated. Multichannel pipette is recommended.

2.4. Incubate at 37 °C.

2.4.1. Cover the plate and shake briefly (5 s).

2.4.2. Incubate the plate at 37 °C for 15 min. This allows the enzymatic reaction to take place.

2.5. Prepare the standard curve.

2.5.1 While the samples are incubating, prepare a serial dilution of 4-methylumbelliferone, a standard often used in the fluorimetric determination of enzyme activity.

2.5.2. Dilute the stock of the standard solution in stop buffer to a concentration of 5 µM.

2.5.3. Perform a series of dilutions as indicated in **Table 2**. Add the components in the amount indicated and mix well.

NOTE: The standard curve helps to ensure that measured samples fall in the linear range of the standard curve.

2.6. Stop the reaction with stop buffer.

2.6.1. Add 200 µL of stop buffer to each well to stop the reaction.

2.7. Plate the standards.

2.7.1. Add 300 µL of each standard per well in duplicates on the same plate.

2.8. Read the plate using a fluorometric reader.

2.8.1. Read the plate at an excitation of 360 nm, and an emission of 455 nm.

3. Data analysis

3.1. Subtract the blanks

3.1.1. Average the values of the duplicate standard dilutions that have no 4-methylumbelliferone. Subtract this value from all of the other recorded values.

3.2. Take average of technical replicates and plot the standards.

3.2.1. Average the two readings for each concentration and graph the average reading by concentration.

NOTE: The resulting plot should look linear and have an R^2 value close to 1. If it does not, it may be necessary to redo the standards and reread the plate to ensure quality of data analysis.

3.3. Standardize the read-out values.

3.3.1 Create a best-fit line for the plotted standards data. Divide the read-outs of the samples by the slope of the best-fit line.

3.4. Compare values from control group and variable group.

3.4.1. Use a t-test or ANOVA, for more than two groups, to determine if the difference between groups is statistically significant. Values will take the units nmol/mL·h.

REPRESENTATIVE RESULTS:

The results shown here are from a study measuring the chitinase activity in serum and BAL samples of wild type (C57BL/6) and Chit1 transgenic mice (on C57BL/6 background) that overexpress the Chit1 gene on a doxycycline promoter. Our data show that both serum and BAL samples have detectable chitinase activity at baseline 698.2 ± 189.9 nmol/mL·h and 485.7 ± 114 nmol/ml·h, respectively. Overexpression of the Chit1 gene using doxycycline in drinking water for 4 weeks resulted in the elevation of chitinase activity that was observed in both BAL (4,575 ± 673.8 nmol/mL·h, $p = 0.003$) and serum 1556 ± 251.2 nmol/mL·h, $p = 0.0272$, when compared to the baseline) samples.

Using the protocol described here, the assay confirms that overexpression of the Chit1 gene in mice results in increased chitinase activity. A t-test was used to compare the means of the two groups. Each group contained 5 mice.

FIGURE AND TABLE LEGENDS:

Figure 1: Chitinase activity. Chitinase activity was measured in the (**A**) serum and the (**B**) bronchoalveolar lavage fluid of wild type (WT) and chitotriosidase (ChitTg) over-expressing transgenic mice. Each dot represents an individual mouse sample. (**C**) Values were standardized using the linearity of the standard curve as described in the protocol. Fluorescence levels were measured using a plate reader at wavelength of 360 nm for excitation and 455 nm for emission and presented as AU. AU = arbitrary unit *$p < 0.05$, ***$p < 0.001$.

Table 1: Sample calculations for creating the working solution.

Table 2: Dilutions for standard curve measurements.

DISCUSSION:

Chitinase activity has emerged as an important biomarker for predicting disease severity, disease progression, therapeutic effectiveness and the presence of specific pathogens[18]. Although many of the long-postulated theories about the role of chitinases have not been experimentally proven[19], new studies have provided important insights into the role of chitinases and chitinase like proteins in various diseases[2,9,20].

The protocol described here is a simple, quick and effective way to measure chitinase activity in biological samples. Little to no modification is required to measure the chitinase activity in a wide variety of biological samples and species due to the highly conserved nature of chitinases. Listed in this protocol are two substrates, one of which as been designated for mouse samples and one for human samples; however, both substrates can be used on either species, as they have been designated as such based on previous papers' demonstration of the efficiency with which the enzymes can cleave the substrate.

Compared to previously existing techniques, such as the Schales' procedure and DNS method, the assay outlined here removes the need for time intensive steps such as boiling or heating that were necessary in older methods. Additionally, the assay is less technically difficult and requires fewer complicated steps than preceding measurement techniques. For best results both biological and technical replicates should be used.

If the chitinase activity is too high, dilutions of the biological sample are sufficient to reduce the chitinase activity for measurement, and the true values can be calculated based on the dilution. When performing this experiment on new biological samples, it may be helpful to perform repeat measurements on the same plate over time until saturation to ensure a proper incubation time for best results. Additionally, the protocol can be scaled up or down as long as consistency between the groups remains. The future applications of this protocol are far reaching as research is just picking up in the role of chitinase activity in a number of diseases.

One of the first category of diseases that chitinase activity was found to be relevant in was lysosomal storage diseases including Gaucher's disease, Niemann-Pick A/B and C, GM-1 gangliosidosis, and many others[21]. Gaucher disease is a lysosomal storage disease caused by insufficient activity of glucocerebrosidase and is characterized by the buildup of glucosylceramide, the substrate of glucocerebrosidase, in the lysosome of macrophages[15]. It is a genetic disease, with clinical symptoms that include spleen and liver enlargement, low platelet count, anemia, fatigue, and bone problems[22]. Clinical research has demonstrated that a majority of people suffering from Gaucher disease have a median chitinase activity that is over 600 times the median value of normal control volunteers; additionally, when people with Gaucher disease were put on enzyme supplementation therapy—the current treatment for the disease—their chitinase activity levels dropped significantly lending chitinase activity to be an excellent marker of both disease progression and treatment monitoring[15]. No role has yet been found for Chit1 in the disease however. Similar research has led to Chit1 activity monitoring for numerous other lysosomal storage diseases[10].

Additionally, further research has indicated a potential role for Chit1 activity during various pathogen infection including fungal infection, malaria, tuberculosis and *K. pneumoniae* to name a few[2,3,10]. A 2012 study found that chitinase activity was elevated in patients with active tuberculosis (TB) infection even when the sputum smear was negative, indicating that chitinase activity could be used as an effective biomarker in TB diagnosis, especially during the long wait time that often is necessary to accurately diagnosis the disease[23]. Similar findings were seen during *Plasmodium falciparum* infection, with chitotriosidase serum levels significantly increased in those with acute infection[24]. While many of these diseases take days to diagnose, quick measurements of chitinase activity levels in the blood can be provide beneficial insights in the diagnosis. Additionally, as malaria parasites and tuberculosis bacterium become increasingly resistant to treatments, measuring chitinase activity levels in the serum may help with treatment monitoring for its effectiveness in real time.

More and more research has begun to focus on the role of chitinase and chitinase-like proteins in the immune response, particularly its role in inflammatory pathways. As this research continues, this protocol will allow for quick and accurate measurement of this chitinase activity to further research. The protocol is easily adjusted for any number of species samples including both mouse and human, making it widely applicable.

In addition to the research benefit, better methods for detecting and measuring chitinase activity in samples will be beneficial in the clinical realm. Recent research has demonstrated the role of chitinase as a biomarker in the pathology of numerous chronic diseases including Gaucher disease, diabetes mellitus, sarcoidosis, atherosclerosis, inflammatory bowel disease and cancers[3,21,25,26].

Because of this role as a biomarker, quick and accurate measurement of chitinase activity as shown here is incredibly relevant to the field of medicine, allowing healthcare providers to have more information for a proper diagnosis and treatment plan.

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DISCLOSURES:

None.

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Figure

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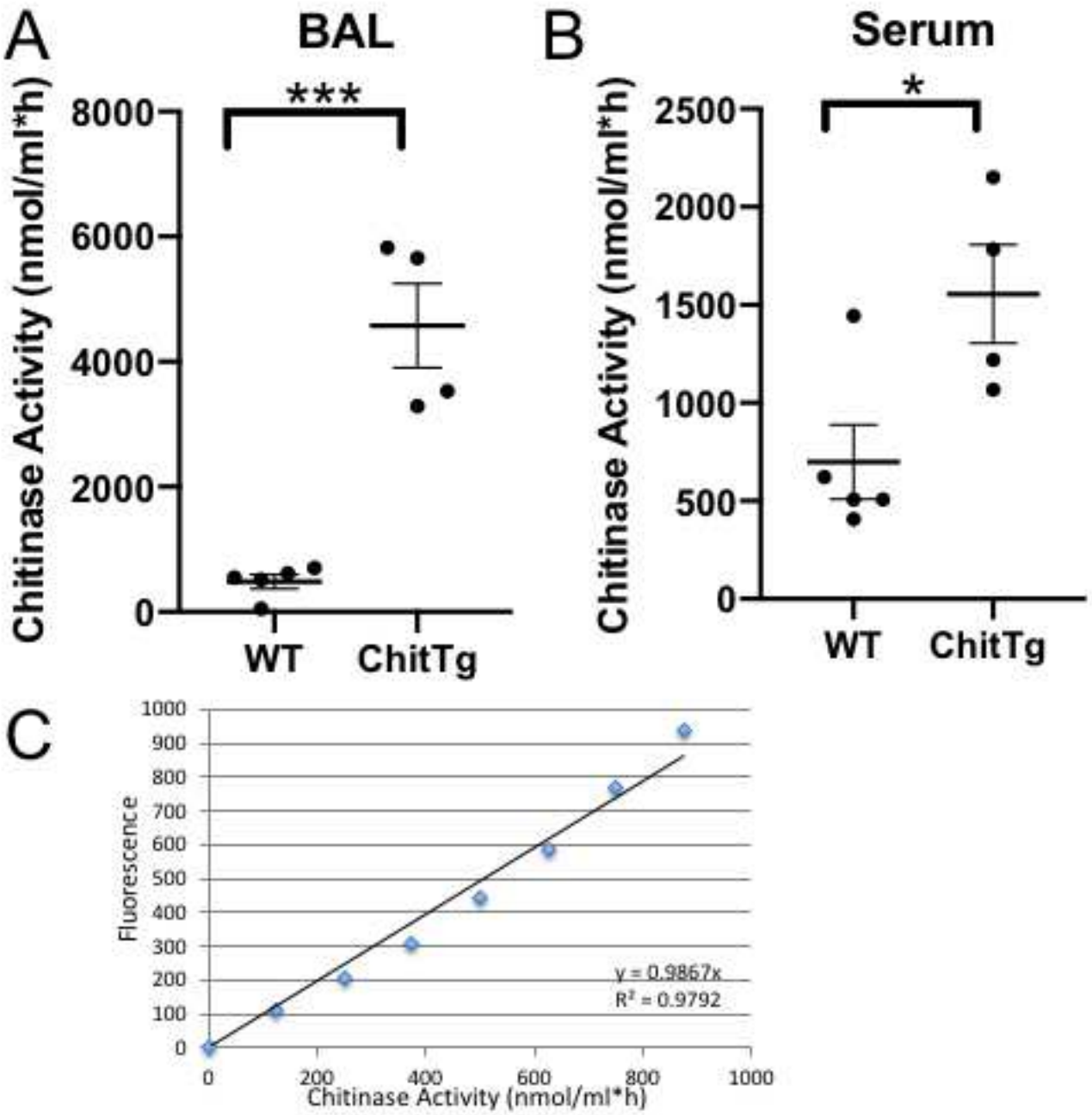

Table

Number of Sample Wells	Substrate (µL)	McIlvain Buffer (mL)
20	100	2.17
50	250	5.425
100	500	10.85

Table

(µL)	1	2	3	4	5	6	7	8
Conc. (nM)	**0**	**125**	**250**	**375**	**500**	**625**	**750**	**875**
Standard	0	20	40	60	80	100	120	140
2x McIlvian	200	200	200	200	200	200	200	200
Distilled H_2O	200	180	160	140	120	100	80	60
Stop Buffer	400	400	400	400	400	400	400	400

Name of Material/Equipment	Company	Catalog Number	Comments/Description
4-methylumbelliferone	Sigma	M1381	Standard: commonly used in flourimetric assays for determination of enzyme activity
4MU-GlcNAc2	Sigma	M9763	fluorescent chitinase substrate for use in mouse samples
4MU-GlcNAc3	Sigma	M5639	fluorescent chitinase substrate for use in human samples
Citric Acid-monohydrate			for use in McIlvain Buffer
Glycine			for use in Stop Buffer at a concentration of 0.3 M
Na2HPO4xH2O			for use in McIlvain Buffer
NaOH			for use in Stop Buffer at a concentration of 12 g/L
Vision Plate: Non-sterile, untreated black 96 well plate	4titude	4ti-0224	

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Date:	04/24/2019

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April 24, 2019

Dear Editor(s):

Enclosed please find a copy of our revised manuscript entitled " Measurement of Chitinase Activity in Biological Samples". The manuscript has been updated based on the suggestions provided by reviewers and editors.

A point by point response to each of the reviewer's comments has been given below. The updates on the manuscript are track changed.

We hope that this article will be of interest to your readers.

We thank you in advance for your efforts on our behalf.

Sincerely,

Lokesh Sharma, PhD

Yale University

Dear Dr. SHARMA,

Your manuscript, JoVE60159 "Measurement of Chitinase Activity in Biological Samples.," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by **May 24, 2019**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Alisha DSouza, Ph.D.
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_____

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NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Language:** The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative voice/tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief

“Note” at the end of the step (please limit notes). Please re-write your ENTIRE protocol section accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.
1) For example: " To collect the BAL, a vertical cut on the neck is made to expose the trachea" should be " To collect the BAL, make a vertical cut on the neck to expose the trachea."
2) Long descriptive sections need to be fully re-written as numbered steps.
Protocol has been edited to be short steps, written in the imperative voice.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. We really want to thank the editors for this comment. We have added the details specifically to ensure that it can be replicated by others.

• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.
We want to thank the editors for the comment. Numbering was adjusted to be in line with JoVE specifications.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting. Changes were made to reflect the comment here.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next. The changes have been made according to the instructions here.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length. We have ensured

that only full sentences are highlighted and sub-headings and spacing have been taken into account when highlighting.
4) Notes cannot be filmed and should be excluded from highlighting.
Highlighting has been adjusted to include necessary steps while leaving out notes.

• **Results:**
1) Please add at least one paragraph of results text that explains your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures.
2) Mention statistical tests used and sample sizes.
An additional paragraph was added to the results to include the information requested here. Additionally, information was added in the representative results regarding statistical tests and sample sizes.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.
The discussion section has been modified to feature a discussion on the points raised here.

• **Figures:** Please remove the figure legends from the figure files and place them directly below the Representative Results text. The figure legend has been removed from the figure file and placed below the Representative Results text.

• **Tables:** Please remove the embedded Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.
Tables have been removed from the manuscript and will be uploaded as an Excel file. Descriptions of the tables can be found with the Figure legends.

• **References:**Please edit your references to comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, doi:DOI (YEAR).]

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Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
This manuscript aims to describe in detail the method of using 4-methylumbelliferone labeled chitin oligomers to rapidly screen for chitinase activity in biological samples. It presents the general approach that has been commercialized previously as a kit provided by Sigma-Aldrich effectively, but omits significant information in the preparation of substrates and standards (including, most critically, their concentration), which means that the method is not sufficiently described in the manuscript to be reproduced without directly the commercial kit and its associated instructions. The major details that prove challenging in associating this method with

With sufficient additional information regarding preparing the working stocks, and more concrete discussion of converting fluorescence measurement into activities, this manuscript and its associated video may serve as an effective introduction to screening complex samples for chitinase activity using this very sensitive fluorogenic assay.

We review non-anonymously, Benjamin Barad and Roberto Efrain Diaz (UCSF). We have used adapted versions of this assay extensively for measuring activity of purified enzymes, and would like to note that this manuscript and video may additionally prove useful to others who are interested in doing so, beyond measurement of complex biological samples.

Major Concerns:
1. The first time the substrate (the product number provided lists a powder) is mentioned, it is in the context of dilution of a liquid stock. The preparation of this liquid stock should be detailed and substrate concentrations should be listed throughout the method to allow for better reproducibility and calculation of absolute activity.
Response: Substrate preparation and concentrations have been added throughout the manuscript. We agree with the reviewer that adding these concentrations will allow for better reproducibility and ease in the calculation of absolute activity.

2. Similarly, the preparation of a concentrated stock of 4MU standard is not described, and the product number lists a powder. Furthermore, the final presented concentrations of the standard curve are incorrect if I calculate using the stock of standard solution (50 mg/ml) provided in the Sigma-Aldrich kit (CS1030). There is additional preparation work needed that is not described in the manuscript, and which should be fully described and included in the video.
Response: As with the substrate preparation, standard preparation and concentrations were added throughout the manuscript for clarity and ease of reproducibility, as well as to facilitate appropriate calculations of activity.

3. Assuming the final concentrations of standards are correctly calculated, activity is presented in arbitrary units, when enough information is collected to present activity in U/ml or [S]/ml*s, either of which would provide better absolute reference points.
Response: Thank you to the readers for pointing this out to us. Activity for the protocol explained here can be measured in the unit nmol/ml.h. We have added these units into the manuscript for greater clarity to readers and viewers of the protocol.

Minor Concerns:
1. The pH of the McIlvaine buffer used throughout the experiment is not mentioned. Since McIlvaine buffer is effective over a large pH range, discussion of what pH range is appropriate for this experiment would be useful.
Response: Thank you to the reviewer for pointing this out to us. The appropriate pH of the McIlvain buffer for this protocol was added to the manuscript.

2. Different substrates (diacetylchitobiose vs triacetylchitotriose) are recommended for mouse vs human samples, but this difference is not explained in the text and the difference in activity between the two substrates means that this limits comparisons of mouse and human enzyme activity. In our experience both substrates yield satisfactory results and measurement of both is useful.
Response: We would like to thank the reviewer for pointing this out to us. While it is true that both substrates can be used for either species, they have been designated as one or the other based on the efficiency with which the enzyme can cleave the substrate. This was shown previously in Boot et al, 2001. We have added this to our document in discussion and references.

3. No mention is made of controlling the temperature of working stocks before mixing - in our hands, without prewarming of substrate and sample solutions we see significant edge effects, in which outer wells warm up faster and report higher 4MU fluorescence than inner wells after a 15 minute incubation. This should be addressed in the manuscript, either by ensuring that duplicate/triplicate measurements account for these edge effects or by prewarming the solution.
Response: The reviewers suggestion was taken and the manuscript adjusted accordingly.

4. There should be some discussion of incubation time - a useful control is measuring total fluorescence at multiple timepoints to ensure that 15 minutes remains in the linear range for the samples and substrate concentrations presented in the manuscript.
Response: We would like to thank the reviewer for mentioning this point. We have included in the discussion mention of the incubation time and control assays to verify appropriate incubation time for modifications in biological sample and chitinase activity level.

5. For preparing the working solution, a table similar to Table 1 would be helpful for the reader.
Response: Per the reviewer's suggestion, a table was made for varying numbers of samples.

6. On line 132, the authors write "Samples should be run in duplicates/triplicates." In context, this appear to indicate technical replicates. However, the example data presented comes from 4-5 mice per measurement, which suggests biological replicates. It is worthwhile to discuss more specifically what technical and biological replicates are recommended to achieve high quality data.
Response: The protocol has been edited to clarify this point. We recommend that both technical and biological replicates are used to achieve the highest quality data.

7. There are no units associated with chitinase activity reported on lines 178, 180, 181. Response: Units have been added to the representative results, as well as to the protocol section.

8. In Figure 1, it is unclear whether the y-axis values presented have been standardized using a standard curve. If they have not, it should be. If they have, the values above 875 (the highest concentration, in nM, used in the standard curve) presented suggest that the standard concentrations selected did not sufficiently cover the range of concentrations produced in the assay. In our experience, much higher standard concentrations can be measured while linearity is maintained. As in minor point 3), presenting more descriptive units than AU will assist in interpreting this issue.
Response: It has been clarified that the y-axis of Figure 1 has been standardized using the standard curve. The concentrations used for the standard curve can be adjusted depending on the concentrations being measured – higher concentrations can be used if necessary. For every assay, the test samples should fall within the range of the standards.

9. In the introduction, it is claimed that chitin is the second most abundant biomolecule on earth - however, the associated citation makes the more reasonable claim that it is the second most abundant polysaccharide.

Response: Thank you to the reviewer for noticing this. The introduction has been amended to more accurately reflect the associated citation.

10. The limitations mentioned for the Schales' procedure and DNS method are time sensitivity and technical difficulty. However, how the 4MU assay overcomes these limitations is not explicitly stated.
Response: The discussion has been amended to explicitly state how the assay outlined in this paper does overcome the limitations of older methods.

11. There is no comparison of the dynamic range of 4MU assay to that of either the Schales' procedure or the DNS method. This is worth mentioning, especially if either of the other two methods are comparable or superior to the 4MU assay.
Response: The dynamic range of the protocol described here is comparable to the Schales' procedure as well as the DNS method. Additionally, if samples have activity level outside of the dynamic range, dilutions of the samples will allow for the assay to still be performed. This has been reflected in the manuscript.

12. On lines 204-205, chitinase activity in samples from patients with Gaucher disease was reported to be 600-fold higher than in control samples. Since for the example data only a 10-fold activity difference was reported, some discussion is warranted of what range of chitinase activity can be effectively quantified with the reported incubation time and standard/substrate concentrations.
Response: We thank the reviewer's for their comment. We have added mention of the dynamic range within the discussion, and further clarified that diluting biological samples out of the dynamic range can make the assay accessible to highly concentrated chitinase activity.

13. There are numerous grammatical and spelling errors throughout the manuscript that need to be addressed.
Response: The protocol has been reviewed for grammatical and spelling errors.

Reviewer #2:
Manuscript Summary:
This is a manuscript that is detailing a protocol to assay chitinases in biological fluids using two substrates. The protocol is simple and straight-forward; however, few issues should be addressed.

Major Concerns:
-Regarding Ref 14 in introduction, the methodology was never mentioned in this manuscript nor the substrate they used. Please elaborate if it is a personal communication with the authors.
Response: We would like to thank the reviewers for pointing out this error, we have corrected it by citing the correct paper that detailed the DNS method in question.

-The protocol is also very similar to the protocol of Hollak et al, 1994 for the substrate 4-MU-triacetylchitotrioside which was used for human plasma. Please add this reference to the mammalian reference upon which this protocol was based in the introduction section.
Response: We appreciate the reviewer mentioning this, and added an additional reference to the paper in the suggested section of the introduction.

-In step 5 of the protocol, line 121, what do you mean by the mouse-specific substrate? The other substrate 4-MU-triacetylchitotrioside can be used for both humans and mice. Please elaborate on this point and explain why.
Response: We have clarified this point within the manuscript. While both substrates can be used for either humans or mice, they have been designated as one or the other based on efficiency with which they are cleaved by the enzymes as found in Boot et al, 2001.

-A comparison between the 2 substrates should be performed even in a few samples of mice serum to compare their performance as long as you use the exact same protocol for both.
Response: A previous paper, Boot et al, 2001, compares these two substrates. It was shown that human and mouse chitinases break down these substrates are different efficiencies and are thus designated as for either mouse samples or human samples accordingly. The manuscript has been updated to reflect and further explain this.

Minor Concerns:
-Introduction line 32: I think it is better to say that chitin is one of the most abundant biomolecules in nature. I think the second most abundant may be debatable.
Response: The sentence has been corrected to accurately reflect chitin as the second most abundant polysaccharide rather than biomolecule.
-The manuscript has some language and style errors:
e.g.
Abstract line 23: The word (inflictions) is not commonly used in this context. Please modify to: a wide range of diseases.
Response: The reviewer's correction has been made.
Abstract line 25: Please modify to: correlate
Response: The reviewer's correction has been made.
Introduction line 34: Please modify to: Fungi
Response: The reviewer's correction has been made.
Introduction line 38: Please change to: lack
Response: The reviewer's correction has been made.

In addition to the corrections identified here, the manuscript has been further proofread to correct for other language and style errors. We thank the reviewer for

pointing these out to us.

Reviewer #3:

Minor Concerns:
Some aspects of the manuscript require clarification:
-How is the substrate solution made? The substrate comes as a powder. What concentration does the substrate solution have and what is it solubilized in? Ideally, provide calculation how many milligrams readers need be purchase to run x samples. Response: Substrate and standard solution preparation has been included. Additionally a table of how much substrate is needed for number of samples has also been added.

-Please provide information about the "McIlvian" buffer. A google search resulted only in "McIlvaine" buffer. This reviewer is not familiar with this buffer. Is this the buffer that was meant? Please provide composition and pH of the used buffer.
Response: The composition and pH of the buffer was added to the manuscript to clarify this point. We would like to thank the reviewer for pointing out to us this lacking detail.

-Please provide more background on the two different substrates that are used and explain to the readers why one is used for human and the other for mice. Also, please clarify "The protocol is easily adjusted for any number of species samples including both mouse and human". Do you mean the protocol can be adjusted to be used for chitinases from species other than human or mouse? If yes, explain possible modifications, e.g. different substrates.
Response: More background has been given on the difference between the two substrates and what they can be used for. Both substrates work for either human or mouse samples, and would work for other mammalian species due to the highly conserved nature of chitinases. This protocol demarcates one for mice and one for humans because previous research has determined that there is a greater efficiency in substrate cleavage when the substrates are paired as is noted here. (Boot RG, Blommaart EFC, Swart E, et al. Identification of a Novel Acidic Mammalian Chitinase Distinct from Chitotriosidase*. 2000. doi:10.1074/jbc.M009886200)

-Line 143: "stock of the standard solution". 4-Methylumbelliferone is a powder. How was this stock solution prepared?
Response: Steps for the preparation of the stock solution were also added to the manuscript based on the suggestions here for easier clarity in reproducing the protocol.

-A standard curve method is described, but apparently not used for Fig. 1? Please explain.

Response: The standard curve was used in calculating the values used in Figure 1 as was explained in the data analysis portion of the protocol. The Figure 1 legend has been updated to further emphasize and reflect this usage of the standard curve.

-Samples are in 105 ul final volume (before stop) and standards 400 ul. What is the reason behind this?
Response: We would like to thank the reader for pointing this out to us. The final volume of the working solution should be 300 µl comprised of 95 µl of working solution 5 µl of sample and 200 µl of stop buffer. This will result in an equal volume to the 300 µl of standard that is plated.

-Can researchers change the 5 ul samples volume? How would this affect the assay?
Response: We thank the reviewer for mentioning this. The protocol can be scaled up or down as long as it remains consistent between groups. This has been added to the discussion as well.

-The discussion focuses on chitinases and their measurement in general. Can the authors also discuss the particular assay they describe? Are there any limitations in the use of this assay that the authors are aware of?

Response: The discussion has been modified to take this reviewers point. Additional information has been added to the discussion that focuses on the assay, its future applications, and potential limitations.