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Protocol
Quantitative assay to detect bacterial glycan-degrading enzyme activities in mouse and human fecal samples

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SUMMARY
The gut microbiome expresses a multitude of enzymes degrading polysaccharides in dietary plant fibers and in host-secreted mucus. The quantitative detection of these glycan-degrading enzymes in fecal samples is important to elucidate the functional activity of the microbiome in health and disease. We describe a protocol for detection of glycan-degrading enzyme activity in mouse and human fecal samples, namely sulfatase and four carbohydrate-active enzymes. Assessing their activity can inform treatment strategies for diseases linked to the gut microbiome.

For complete details on the use and execution of this protocol, please refer to Desai et al. (2016).

BEFORE YOU BEGIN
The following protocol describes the detection of glycan-degrading enzymatic activity for 4 different carbohydrate-active enzymes (CAZymes) and sulfatases, 2 of which target linkages in plant fiber-derived glycans as substrates and 3 of which use mucus-specific glycosidic linkages. See Table 1 for the names of the bacterial CAZymes, their abbreviations as used in this protocol, and their biological substrates.

This protocol is a further development of a previously published procedure to detect enzyme activity from bacterial cultures (Berteau et al., 2006), in mucus-supplemented fecal cultures (Hoskins and Boulding, 1981) and in cecal samples from mice harboring a 14-member synthetic human gut microbiota in a gnotobiotic setting (Desai et al., 2016). We implemented and evaluated further changes in the required amount of fecal protein and substrate concentrations to make this procedure also reliably suitable for enzyme activity detection in fecal samples from humans or mice harboring a conventional microbiota. The protocol reports detection of five enzymes, but could be adapted to detect activities of several other enzymes using appropriate substrates.

Functionally, the protocol is based on the following principles to allow for the detection of enzyme activities: co-incubation of 4-nitrophenyl-coupled substrates with solubilized fecal protein; enzyme-catalyzed hydrolysis of the glycosidic/sulfate group linkages between the 4-nitrophenyl residue and the respective monosaccharide/sulfate, resulting in free 4-nitrophenol; and detection of the time-dependent increase in free 4-nitrophenol by measuring absorption increase at 405 nm.
We recommend setting up an in-house custom-made activity detection kit consisting of 5 kit components as described below.

**Preparation of kit component 1: Reconstitution and aliquotation of the 4-nitrophenyl-coupled substrates**

- **Timing:** 2 h

  - **Note:** All substrate aliquots can be stored at −20°C for at least 6 months. In case longer storage times are desired, we recommend carefully evaluating whether extended storage time affects experimental outcomes. Repeated freezing and thawing does not reduce substrate solution quality.

1. Reconstitute 4N-S, 4N-FP, 4N-GalP and 4N-GluP in a solvent (as recommended by the supplier) to obtain a 100 mM stock solution.
   a. Filter sterilize solutions with a 0.22 μM filter.
   b. Dispense each solution in 500 μL aliquots.
   c. Store aliquots at −20°C.

2. Reconstitute 4N-NAG in ddH2O to obtain a 20 mM stock solution of this substrate. The lower concentration is necessary due to restricted solubility of 4N-NAG.
   a. Filter sterilize solutions with a 0.22 μM filter.
   b. Dispense 4N-NAG solution in 500 μL aliquots.
   c. Store aliquots at −20°C.

**Preparation of kit component 2: Reconstitution and aliquotation of the 4-nitrophenol standard**

- **Timing:** 1 h

  - **Note:** All standard aliquots can be stored at −20°C for at least 6 months. In case longer storage times are desired, we recommend carefully evaluating whether extended storage time affects experimental outcomes. Repeated freezing and thawing does not reduce standard solution quality.

3. Reconstitute the 4-nitrophenol standard in ddH2O to obtain a 100 mM stock solution.
   a. Filter sterilize 4-nitrophenol standard solution with a 0.22 μM filter.
   b. Dispense solution in 500 μL aliquots.
   c. Store aliquots at −20°C.
Preparation of kit component 3: Detection buffer (DB buffer)

- **Timing:** 1 h

4. Prepare a buffer containing 50 mM Tris-HCl, 100 mM KCl and 10 mM MgCl$_2$ in ddH$_2$O (Table 2).
5. Adjust pH to 7.25.
6. Filter sterilize DB buffer.
7. Aliquot the DB buffer in 15 mL or 50 mL aliquots.
8. Store aliquots at 4°C.

**Note:** Prepare double the volume of DB buffer since DB buffer is also the basis for the LB buffer (see description of kit component 4 below).

| Reagent      | MW         | Final concentration | Amount  |
|--------------|------------|---------------------|---------|
| Tris-HCl     | 157.6 g mol$^{-1}$ | 50 mM             | 7.87 g  |
| KCl          | 74.5 g mol$^{-1}$  | 100 mM            | 7.45 g  |
| MgCl$_2$·6H$_2$O | 203.3 g mol$^{-1}$ | 10 mM            | 2.03 g  |
| ddH$_2$O     | ad         | 1x                  | 1,000 mL|
| **Total**    | n/a        |                     | **1,000 mL**|

Table 2. Recipe for 1 L of Detection buffer (DB)

Preparation of kit component 4: Lysis buffer pre-mix (LB-PM buffer)

- **Timing:** 1 h

9. Prepare a 50 mL solution of 12% (v/v) Triton X-100 in ddH$_2$O (6 mL Triton X-100 + 44 mL ddH$_2$O) (Table 3).
10. Dilute the 12% Triton X-100 solution by factor of 1:1,000 in DB buffer to obtain the 1x LB-PM buffer.
11. Aliquot the LB-PM buffer in 15 mL or 50 mL aliquots.
12. Store aliquots at 4°C.

| Reagent          | Concentration | Final concentration | Amount  |
|------------------|---------------|---------------------|---------|
| Triton X-100     | 12% (v/v)     | 0.012% (v/v)        | 1 mL    |
| DB buffer        | 1x            | 1x                  | 999 mL  |
| **Total**        | n/a           | n/a                 | 1,000 mL|

Table 3. Recipe for 1 L of lysis buffer pre-mix (LB-PM)

Preparation of kit component 5: Enzyme mix

- **Timing:** 1 h

**Note:** Enzyme mix aliquots can be stored at −20°C for at least 6 months. In case longer storage times are desired, we recommend carefully evaluating whether extended storage time affects experimental outcomes. Repeated freezing and thawing is not recommended! After solubilizing an aliquot, discard any remaining enzyme mix.

13. Prepare enzyme mix aliquots by adding a tiny amount (roughly the amount attached to a tip of a sharp tweezer corresponding to a 3-digit μg amount) of both DNase I and Lysozyme into a sterile 1.5 mL tube.
14. Store aliquots at −20°C.
KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Biological samples  |        |            |
| Stool from C57BL/6J mice, housed under SPF conditions | This paper | N/A |
| Stool from healthy adult human volunteers | This paper | N/A |
| Chemicals, peptides, and recombinant proteins |        |            |
| DNase I, grade II, from bovine pancreas | Roche | Cat#10104159001 |
| Lysozyme | Thermo Fisher | Cat#89833 |
| Critical commercial assays |        |            |
| Pierce 660nm Protein Assay Reagent | Thermo Scientific | Cat#22660 |
| 4-Nitrophenyl α-L-fucopyranoside | Sigma-Aldrich | Cat#N3628 |
| 4-Nitrophenyl α-D-galactopyranoside | Sigma-Aldrich | Cat#N0877 |
| 4-Nitrophenyl β-D-glucopyranoside | Sigma-Aldrich | Cat#N7006 |
| Potassium 4-nitrophenyl sulfate | Sigma-Aldrich | Cat#N3877 |
| 4-Nitrophenyl N-acetyl-β-D-glucosaminide | Sigma-Aldrich | Cat#N9376 |
| 4-Nitrophenol | Carl Roth | Cat#6524.1 |
| Roche cOmplete, EDTA-free Protease Inhibitor Cocktail | Sigma-Aldrich | Cat#11873580001 |
| Experimental models: organisms/strains |        |            |
| Mouse: C57BL/6J | The Jackson Laboratory | Cat# JAX.000664, RRID:IMSR_JAX.000664 |
| Software and algorithms |        |            |
| GraphPad Prism v8.0.1 | GraphPad Software | [https://www.graphpad.com/scientific-software/prism/; RRID:SCR_002798](https://www.graphpad.com/scientific-software/prism/; RRID:SCR_002798) |

MATERIALS AND EQUIPMENT

To perform this protocol, the following technical equipment is necessary:

1. A refrigerated centrifuge compatible with 1.5 mL tubes.
2. A spectrophotometer suitable for continuous kinetic detection of absorption at 405 nm and endpoint detection at 660 nm. The reader must be equipped with both shaking and incubation for readings at 405 nm.
3. A sonication device.

STEP-BY-STEP METHOD DETAILS

Step A: Preparation of the lysis buffer (LB buffer)

© Timing: 30 min

**Important:** LB buffer should be prepared freshly right before fecal protein solubilization (step B; see below). Store LB buffer on ice throughout the solubilization procedure. LB buffer is not stable and cannot be stored.

1. The kit is designed to yield 100 mL of ready-to-use LB buffer. If less than 100 mL are needed, the required volumes can be easily adapted (500 μL of LB buffer are needed per sample to be lysed).
2. Prepare 100 mL LB-PM buffer (kit component 4).
3. Remove 1 mL from the 100 mL LB-PM buffer and use this volume to resuspend 1 aliquot of the enzyme mix (kit component 5) then transfer it back to the LB-PM buffer. In case less than 100 mL are needed to lyse your batch of samples, adapt the volume of the enzyme solution accordingly (example: For 20 mL of LB buffer, resuspend enzyme mix in 1 mL and add only 200 μL of this suspension to 20 mL of LB-PM buffer).
4. Remove 1 mL of the enzyme mix-supplemented LB-PM buffer and use this volume to resuspend 1 tablet of EDTA-free Protease inhibitor then transfer it back to the 100 mL of enzyme mix-supplemented LB-PM buffer. In case less than 100 mL are needed to lyse your batch of samples, adapt the volume of the protease inhibitor solution accordingly (example: For 20 mL of LB buffer, resuspend protease inhibitor tablet in 1 mL and add only 200 µL of this suspension to 20 mL of the enzyme-supplemented LB-PM buffer).

5. Store LB buffer on ice until further use (see step 2).

**Step B: Solubilization of glycan-degrading enzymes from mouse and human fecal samples**

ettings: 1–2 h (depending on the amount of processed samples)

This step describes the solubilization of glycan-degrading enzymes present in mouse or human fecal samples. Solubilized enzymes, as obtained by the end of step 2, can be either (1) subjected to immediate activity detection or (2) be stored at \(-20°C\).

6. For enzyme detection in mouse fecal samples, start with step 6a. For enzyme detection in human fecal samples, start with step 6b. (See Troubleshooting 1). All subsequent procedures apply for both mouse and human fecal samples.

   a. Preparation of mouse fecal samples:
      Transfer 1 mouse fecal pellet into a sonication device-compatible 1.5 mL tube and keep stored on ice or at \(-20°C\) until lysis.

      **Note:** If needed, more than one fecal pellet can be used for enzyme solubilization. Approximately 10 mg of feces is usually sufficient for detection of all 5 enzymes described in this protocol. As enzyme activity will be normalized on the protein content only, determination of the weight of the fecal samples is not necessary.

   b. Preparation of human fecal samples:
      Fecal samples from human donors can either be prepared right after donation or, more conveniently, stored at \(-20°C\) until preparation. Do not supplement the fecal matter with any additives, such as preservation buffers, glycerol, etc.
      On the day of sample preparation, remove a small portion of frozen (or freshly donated) human feces (approximately 20–100 mg) and transfer the piece of feces into a sonication device-compatible 1.5 mL tube and store on ice. Remaining feces can be used for other readouts that are compatible with the storage conditions.

      **Note:** We do not recommend comparing freshly donated fecal samples with frozen fecal samples.

7. Add 500 µL of ice-cold LB buffer to the fecal sample, irrespective of the weight.

8. Homogenize the feces in the LB buffer.

   **Tip:** Use a pipette tip or an inoculation loop to break up large clumps.

9. Vortex sample for 10 s.

10. Perform pulsed sonication of the samples at the highest available frequency for 15 s at a time, pausing for at least 10 s between every sonication step. Apply a total sonication time of 45 s.

   **CRITICAL:** Keep samples on ice during sonication and between sonication steps!

   **Note:** All types of sonication devices work for this purpose.
11. Centrifuge the sonicated samples for 10 min at 10,000 \( \times \) g and 4°C.

12. Transfer 350 \( \mu \)L of the supernatant into a new 1.5 mL tube.

\[ \text{CRITICAL: Do not touch the pellet during supernatant removal to avoid contamination of the clear supernatant with solid, unsolubilized material.} \]

\[ \text{Pause point: Removed supernatants can be stored at -20°C. If immediate activity detection is preferred, store samples on wet ice.} \]

**Step C: Detection of substrate turnover**

\[ \text{Timing: 4–6 h} \]

After determination of the protein concentrations in the supernatants (step 15, see below) obtained from step B, protein amounts should be equalized among all samples to be detected. We recommend using the Pierce 660nm Protein Assay Reagent as it works well with the detergent-containing LB buffer. DB buffer and substrates are added afterward, followed by immediate start of absorption detection (step 20, see below).

13. When frozen supernatant samples are used, thaw samples on ice.

14. While samples are thawing, create a standard curve using 4-nitrophenol (kit component 2):

\[ \text{Thaw 1 aliquot of the 4-nitrophenol standard (kit component 2) and perform serial 1:2 dilutions down to } 1 \mu M. \]

\[ \text{Tip: The detection limit of the highest and lowest concentrations of the 4-nitrophenol standards depends on the spectrophotometer being used. Thus, we recommend to detect one complete dilution series from 100 \mu M down to a 3-digit nanomolar range.} \]

See Figure 1 for an example standard curve. In this example, we detected 4-nitrophenol concentrations ranging from 4.8 \mu M to 2.5 mM.

\[ \text{Tip: Despite only minor differences between standard curves detected on different days or settings, we recommend running a standard curve with every sample detection.} \]

15. Detect fecal protein concentrations in the supernatants using any commercially available protein concentration detection kit. We recommend the Pierce 660nm Protein Assay Reagent.

\[ \text{Attention: Always use a blank of LB buffer only and subtract the detected absorption of the blank from the sample absorption.} \]
16. Calculate the volume needed from each sample to achieve the required fecal protein amounts for the detection of each of the 5 enzymes.

Attention: The required protein amounts to be used are specific for each of the 5 enzymes (Table 4).

| Bacterial enzyme                      | Chemical substrate used for activity detection | Fecal protein amount needed per sample |
|---------------------------------------|-----------------------------------------------|---------------------------------------|
| Sulfatase                             | 4N-S                                          | 25 μg                                 |
| β-N-Acetyl-glucosaminidase            | 4N-NAG                                        | 1 μg                                  |
| α-Fucosidase                         | 4N-FP                                         | 5 μg                                  |
| α-Galactosidase                       | 4N-GalP                                       | 1 μg                                  |
| β-Glucosidase                        | 4N-GluP                                       | 5 μg                                  |

Note: Detection of sulfatase requires relatively high amounts of protein. In case protein concentration in a given sample is low, use the maximum volume possible (75 μL). Be sure to input the protein amount that was actually used for activity calculation (step D).

17. Add DB buffer into the respective wells of a 96-well plate to obtain a final volume of 150 μL per well. We recommend using flat-bottom transparent plates. See Table 5 for a summary of the pipetting scheme.

| Bacterial enzyme                      | Chemical substrate used for activity detection | Fecal protein | Volume of substrate stock per sample | DB buffer |
|---------------------------------------|-----------------------------------------------|---------------|-------------------------------------|-----------|
| Sulfatase                             | 4N-S                                          | 25 μg in x μL | 15 μL                              | 135 μL-x μL|
| β-N-Acetyl-glucosaminidase            | 4N-NAG                                        | 1 μg in x μL  | 75 μL                              | 75 μL-x μL|
| α-Fucosidase                         | 4N-FP                                         | 5 μg in x μL  | 15 μL                              | 135 μL-x μL|
| α-Galactosidase                       | 4N-GalP                                       | 1 μg in x μL  | 30 μL                              | 120 μL-x μL|
| β-Glucosidase                        | 4N-GluP                                       | 5 μg in x μL  | 30 μL                              | 120 μL-x μL|

18. Transfer the required volumes of the fecal protein solutions into the respective wells.

19. Add the substrate solutions (kit component 1) to the wells.

| Bacterial enzyme                      | Chemical substrate used for activity detection | Concentration of the substrate stocks | Volume of substrate stock per sample | Final substrate concentration |
|---------------------------------------|-----------------------------------------------|--------------------------------------|-------------------------------------|-------------------------------|
| Sulfatase                             | 4N-S                                          | 100 mM                               | 15 μL                               | 10 mM                         |
| β-N-Acetyl-glucosaminidase            | 4N-NAG                                        | 20 mM                                | 75 μL                               | 10 mM                         |
| α-Fucosidase                         | 4N-FP                                         | 100 mM                               | 15 μL                               | 10 mM                         |
| α-Galactosidase                       | 4N-GalP                                       | 100 mM                               | 30 μL                               | 20 mM                         |
| β-Glucosidase                        | 4N-GluP                                       | 100 mM                               | 30 μL                               | 20 mM                         |

Attention: The required substrate solution volumes to be used are specific for each of the 5 enzymes (Table 6).

20. Detect absorption at 405 nm for 2–4 h at 37°C with 1 to 2 min detection intervals and a shaking step before each detection.

We have verified the optimal concentrations for each substrate as demonstrated in Figure 2.
Note: See Figure 3 for example substrate turnover curves as obtained by following the protocol above. According to these curves, we recommend the detection times listed in Table 7. See also Troubleshooting 2

Table 7. Recommended detection time

| Bacterial enzyme | Chemical substrate used for activity detection | Recommended detection time |
|------------------|-----------------------------------------------|-----------------------------|
| Sulfatase        | 4N-S                                          | 4 h                         |
| β-N-Acetyl-glucosaminidase | 4N-NAG                                       | 2 h                         |
| α-Fucosidase     | 4N-FP                                         | 2 h                         |
| α-Galactosidase  | 4N-GalP                                       | 2 h                         |
| β-Glucosidase    | 4N-GluP                                       | 2 h                         |
EXPECTED OUTCOMES

Expected concentrations of solubilized fecal protein and total amount of fecal protein obtained using the procedure described in step A.

The protein concentration of the supernatants derived from human and mouse fecal samples typically range between 1–20 μg per mg feces (Figure 4), for both mouse and human samples.

Expected range of CAZyme activity in mouse and fecal samples

CAZyme activities detected in fecal samples are dependent on microbiome composition and dietary conditions in the respective donor. Thus, considerable differences in detected CAZyme activities can occur between different groups of mice or human individuals. However, Table 8 is designed to give the reader an impression of which range of activities to typically expect in wildtype Specific Pathogen Free (SPF) mice fed a standard diet as well as in healthy human stool donors.

QUANTIFICATION AND STATISTICAL ANALYSIS

A critical factor for successful characterization of enzyme activity under certain conditions is the final calculation of enzyme activity normalized on the concentration of solubilized fecal protein. Here, we provide step-by-step instructions to reliably calculate enzyme activities.
We recommend reporting enzyme activities in the unit nkat mg⁻¹. This corresponds to the number of cleaved substrate molecules (mol) per time unit (s), normalized on the amount of solubilized fecal protein used for the assay (kat is equal to mol/s).

1. Creating a proper standard curve
   We recommend to use log values for both absorption and 4-nitrophenol serial dilution concentrations to avoid under-estimating the lower concentrations when linearization is performed. Additionally, we recommend converting the concentrations of the serial dilutions into corresponding number of molecules (mol) by taking into account the reaction volume of 150 µL.
   Table 9 illustrates how to compute the log values used for the standard curve.
   Using the calculated values in the last 2 columns results in the standard curve depicted in Figure 5. Check the goodness of the linear fit and note the equation of the linear function.

2. Plot the detected absorption of a given sample as a function of time (see Figure 6A for an example of the absorption using a mouse fecal sample and 4N-NAG as a substrate).

3. Next, choose the linear phase with the highest absorption increase per time unit, which typically occurs at the beginning of an enzymatic reaction (Figure 6B). This phase should contain 10 to 20 data points (in the Figure 5 example, we chose the phase between 30–50 min after start of the enzymatic reaction).

4. Perform a linear regression of the chosen data points and verify the goodness of the fit (Figure 6C). See also Troubleshooting 3.

5. Note the slope of the linear function.
   (Example: 0.004145 ΔAU/min).

6. Perform calculation of the enzymatic activity using the standard curve:
   a. Convert the slope from unit ΔAU min⁻¹ to ΔmAU min⁻¹ by multiplying the slope by factor 10³.
      (Example: 0.004145 ΔAU/min × 10³ = 4.145 ΔmAU/min)
   b. Calculate the log of the slope.
      (Example: log₁₀(4.145) = 0.612752)
   c. Calculate the log of the number of enzymatically released 4-nitrophenol molecules by replacing "x" in the linear function of the standard curve with the logarithmized slope of the enzymatic turnover curve.
      (Example: y = 1.81x – 1.034 = (1.081 × 0.612752) – 1.034 = –0.716455 log nmol/min)

### Table 8. Expected range of CAZyme activity in mouse and fecal samples

| Enzyme                      | Mouse   | Human  |
|-----------------------------|---------|--------|
| Sulfatase [nkat mg⁻¹]       | 0.001–0.1 | 0.01–0.1 |
| β-N-Acetyl-glucosaminidase [nkat mg⁻¹] | 0.5–5.0 | 1.0–10.0 |
| α-Fucosidase [nkat mg⁻¹]   | 0.1–1.0 | 0.1–1.0 |
| α-Galactosidase [nkat mg⁻¹] | 1.0–10.0 | 1.0–10.0 |
| β-Glucosidase [nkat mg⁻¹]  | 0.5–5.0 | 0.5–10.0 |

### Table 9. Example table for the computation of the standard curve

| 4-Nitrophenol  | 4-Nitrophenol | Assay volume µL | 4-Nitrophenol mol | 4-Nitrophenol [nmol] | Detected OD₄₀₅ [mAU] | y-axis: 4-Nitrophenol [log nmol] | x-axis: Detected OD₄₀₅ [log mAU] |
|----------------|---------------|-----------------|------------------|---------------------|---------------------|---------------------------------|---------------------------------|
| 2,500          | 0.0025        | 150             | 0.000000375      | 375                 | 4,516.9916           | 2.5740313                      | 3.6548493                       |
| 1,250          | 0.00125       | 150             | 1.875E-07        | 187.5               | 2,599.1046           | 2.2730013                      | 3.4148238                       |
| 625            | 0.000625      | 150             | 9.375E-08        | 93.75               | 1,325.0502           | 1.9719713                      | 3.1222323                       |
| 312.5          | 0.0003125     | 150             | 4.6875E-08       | 46.875              | 595.60251            | 1.6709413                      | 2.7749565                       |
| 156.25         | 0.00015625    | 150             | 2.34375E-08      | 23.4375             | 349.24686            | 1.3699113                      | 2.5431325                       |
| 78.125         | 0.000078125   | 150             | 1.17188E-08      | 11.71875            | 183.94142            | 1.068813                       | 2.2646795                       |
| 39.0625        | 3.90625E-05   | 150             | 5.85938E-09      | 5.859375            | 90.949791            | 0.7678513                      | 1.9588017                       |
| 19.53125       | 1.953125E-05  | 150             | 2.92969E-09      | 2.929688            | 46.648536            | 0.4668214                      | 1.668838                        |
| 9.765625       | 9.765625E-06  | 150             | 1.46484E-09      | 1.464844            | 38.083682            | 0.1657914                      | 1.5807389                       |
| 4.8828125      | 4.8828125E-06 | 150             | 7.32422E-10      | 0.732422            | 12.849372            | -0.135239                      | 1.1088819                       |
d. Exponentiate this value using base 10 to calculate the number of released 4-nitrophenol molecules per minute. 
(Example: \(10^{(0.716455)} = 0.192 \text{ nmol/min}\))

e. Normalize the substrate turnover velocity on the amount of fecal protein in the sample. 
(Example: we used 1 \(\mu\)g of protein to detect \(\beta\)-N-acetyl-glucosaminidase activity, therefore: \(\frac{0.192 \text{ nmol/min}}{1 \mu\text{g}} = 0.192 \text{ nmol/(min } \times \mu\text{g)}\))

f. Calculate the final enzymatic activity in nkat mg\(^{-1}\) by multiplying the velocity obtained in the previous step by 1000 (\(\mu\)g to mg conversion) and dividing by 60 (min to s conversion). 
(Example: \(0.192 \text{ nmol/(min } \times \mu\text{g)} \times \frac{1000}{60} = 3.20 \text{ nkat/mg}\))

LIMITATIONS
This protocol describes the activity detection of five different glycan-degrading enzymes in human and mouse fecal samples. It is important to mention that the detection mechanism does not distinguish between enzymes of different origins (microbiota- vs. host-derived). However, using fecal samples as the source of enzyme detection, most of detected differences between certain groups or individuals can be attributed to bacterial enzymes, rooted in functionally distinct microbiota characteristics.

We recommend normalizing the enzymatic activity on the amount of fecal protein. In general, the fecal protein content represents an excellent way to assess the source of the enzymes to be detected (the microbiome) because intestinal microbes are the main source of the stool proteome. Furthermore, normalizing on fecal protein levels confers two major advantages. First, enzymes have to be solubilized for activity detection using the described assay. However, protein solubilization efficiency can differ considerably between distinct samples. Normalizing on solubilized fecal protein eliminates this factor and prevents from over- or underestimation of enzyme activity, which occurs when activities are normalized on pellet weights. Second, fecal pellets harbor different water contents, which greatly influence their weight. Thus, activities in watery pellets are prone to be underestimated when normalized on pellet weights. Normalizing on protein levels eliminates this factor.

In consequence, this approach should only be used for fecal samples obtained from individuals harboring a non-depleted intestinal microbiome, such as human stool samples or samples from mice harboring a conventional, SPF, or gnotobiotic microbiome. Normalization on fecal protein content is not recommended in the comparison of germ-free or antibiotic-treated mice with mice harboring an intact microbiome; in these cases, normalization on fecal pellet weight should be considered.
**TROUBLESHOOTING**

**Problem 1**
Protein concentrations in the fecal supernatants are too low for detection of all five enzymes.

**Potential solution**

**Potential solution 1**
Reduce LB buffer volume (step 7) from 500 μL to 400 μL and incubate sample after homogenization and vortexing (step 9) for an additional 10 min on ice.

**Potential solution 2**
Reduce the amount of fecal protein for sulfatase activity detection. However, we recommend applying no less than 5 μg protein for activity detection of this enzyme.

**Problem 2**
Substrate turnover not detectable.

**Potential solution**

**Potential solution 1**
Check spectrophotometer instrument settings and verify that absorption is detected at 405 nm.

**Potential solution 2**
Verify that pH of DB buffer and LB buffer is at pH 7.25.

**Problem 3**
Regression of the substrate turnover as a function of time (see Figure 6C) does not follow a linear trend (R-squared < 0.99).

**Potential solution**

**Potential solution 1**
Choose a different time frame for linear regression (see Figure 6B) and perform linear regression again.

**Potential solution 2**
In case of very low enzyme activities, performing a proper linear regression is not possible because the time-dependent increase in absorption at 405 nm is too low. Thus, for such samples, determination of enzyme activities is not possible and should be reported as “below detection limit.”

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**Figure 6. Analysis pipeline for the determination of CAZyme activity in nkat mg⁻¹ using 4N-NAG as an example substrate**

(A) Detection of 4N-NAG substrate turnover by plotting absorption at 405 nm against time in 2 min increments. (B) Choosing the linear phase with the highest absorption increase per time unit (chosen phase highlighted in light grey). (C) Performing linear regression (red line) within the phase chosen in (B). Note slope equation and verify proper linearity of the regression line.
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact. Mahesh S. Desai; mahesh.desai@lih.lu

Materials availability
This study did not generate new unique reagents.

Data and code availability
The published article includes all datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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