Please cite this article as: Marie-Paule *et. al.*, (2017). Protease Activity Assay in Fly Intestines, *Bio-protocol* 7 (18): e2560. DOI: 10.21769/BioProtoc.2560.

bio-protocol www.bio-protocol.org/e2560 Vol 7, Iss 18, Sep 20, 2017 DOI:10.21769/BioProtoc.2560

Protease Activity Assay in Fly Intestines

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[Abstract] The intestine is a central organ required for the digestion of food, the absorption of nutrients and for fighting against aggressors ingested along with the food. Impairment of gut physiology following mucosal damages impacts its digestive capacities that consequently will affect growth, wellbeing or even survival of the individual. Hence, the assessment of intestinal functions encompasses, among others, the monitoring of its integrity, its cellular renewing, its immune defenses, the production of enteroendocrine hormones and its digestive capacities. Here, we describe in detail how to assess the activity of the proteases secreted in the intestinal lumen of adult *Drosophila melanogaster* flies. This method can also be used for larval intestines. The present protocol is adapted and improved from the Sigma-Aldrich's protocol proposed in the 'Protease Fluorescent Detection Kit' (Product code PF0100).
Keywords: *Drosophila melanogaster*, Intestine, Opportunistic bacteria, Protease activity, Protein metabolism

[Background] The intestine is subjected to many stresses such as feasting, fasting, chemicals, pathogens, injuries *etc.* The gut is able to overcome such stresses by maintaining its physiological equilibrium named homeostasis. To perceive the incoming stress and to yield an adapted answer to maintain gut functions, the intestine has developed robust and conserved mechanisms such as local innate immune defenses and tissue regeneration (Royet and Charroux, 2013; Bonfini *et al.*, 2016). However, the maintenance of gut homeostasis can be compromised in certain cases. For example, during aging, there is an overall decline in tissue homeostasis maintenance with the presence of numerous immature or misdifferentiated cells (Jasper, 2015; Hu and Jasper, 2017). Another case where homeostasis can also be disrupted is upon exposure to xenobiotic or pathogens (such as opportunistic bacteria) that damage or kill cells impairing their functions (Bonfini *et al*., 2016). Hence, during the above cited examples, the digestive capacities of the gut are reduced. Moreover, during the process of tissue regeneration itself that produces many precursor cells the digestive capacities are also reduced (Loudhaief *et al*., 2017). Therefore, the assessment of the digestive capacities of the gut are of prime importance to evaluate the potential impact that can have an aggression on the gut physiology. Importantly, gut digestive function disruption may have both local and systemic metabolic consequences that will affect growth, immune defenses, reproduction, wellbeing, longevity…. Dietary proteins are essential for many (if not all) physiological functions (Soultoukis and Partridge, 2016). Imbalanced amino-acid absorption by the intestine can have dramatic consequences on growth for example. Protein digestion being essential to generate absorbable amino-acids by the enterocytes, the measurement of

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luminal protease activity appears a good readout to evaluate the physiological state of the intestine and its capacity to fulfill its digestive functions.

Materials and Reagents

A. *Drosophila* rearing
1. 6oz *Drosophila* stock bottles (Genesee Scientific, catalog number: 32-130)
2. Cotton balls for stock bottles (Genesee Scientific, catalog number: 51-102B)
3. *Canton*^{S} flies (Bloomington *Drosophila* Stock Center, catalog number: 64349) (flystocks.bio.indiana.edu)
4. Agar (VWR, BDH®, catalog number: 20768-361)
5. Sugar (Carrefour or any other supermarket)
6. Cornflour (AB, Celnat - NaturDis)
7. Yeast (Biospringer, catalog number: BA10/0-PW)
8. Tegosept (Apex, Fly Food preservative, Genesee Scientific, catalog number: 20-258)
9. Standard nutrient medium for *Drosophila* (see Recipes)

B. Bacterial culture
1. Petri dishes
2. Sterile tip
3. 15 ml tubes (Corning, Falcon®, catalog number: 352096)
4. Graduated test tube
5. *Bacillus thuringiensis* var. *kurstaki* (*Btk*) strain identified under the code *4D22* at the *Bacillus* Genetic Stock Center (http://www.bgsc.org/) and described by (Gonzalez *et al.*, 1982)
6. *Erwinia carotovora carotovora* (*Ecc*) was kindly provided by Bruno Lemaitre's laboratory (École Polytechnique Fédérale, Lausanne, Switzerland)
7. *Escherichia coli* (*Ec*) (One Shot™ TOP10 Chemically Competent *E. coli*) (Thermo Fisher Scientific, Invitrogen™, catalog number: C404003)
8. Luria broth powder (Conda, catalog number: 1551)
9. Agar bacteriological (Euromedex, catalog number: 1330)
10. LB medium (see Recipes)
11. LB-agar medium (see Recipes)

C. Intoxication
1. Cotton balls for narrow vials 25 mm (Genesee Scientific, catalog number: 51-101)
2. Spectrophotometry cuvettes (Ratiolab, catalog number: 2712120)
3. 2 ml microtubes (Paul Bottger, catalog number: 02-043)
4. 20 mm filter disks (Chromatography paper 3MM Chr) (GE Healthcare, catalog number: 3030-917)

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5. 50 ml tube
6. *Drosophila* narrow vials 25 mm (Genesee Scientific, catalog number: 32-109RL)
7. Sucrose (Euromedex, catalog number: 200-301-B)
8. 10% sucrose (see Recipes)

D. Dissection

1. 1.5 ml microtubes (Paul Bottger, catalog number: 02-063)
2. Graduated test tube
3. Watch glass (Steriplan Petri dishes, DWK Life Sciences, catalog number: 237554008)
4. Ice
5. Ethanol 70% (VWR, catalog number: 83801.360)
6. 10x PBS (Euromedex, catalog number: ET330)
7. 1x phosphate-buffered saline (PBS) (see Recipes)

E. Sample preparation

1. Microtube pestle 1.5 ml (Argos Technologies, catalog number: P7339-901)
2. 0.5 ml microtubes (Paul Bottger, catalog number: 02-053)
3. 1x phosphate-buffered saline (PBS) (see Recipes)

F. Assay

1. 1.5 ml microtubes (Paul Bottger, catalog number: 02-063)
2. 96-well black microplates (Greiner Bio One International, catalog number: 655076)
3. 2 ml microtube
4. 15 ml tube
5. Aluminum foil
6. Trypsin from bovine pancreas (Sigma-Aldrich, catalog number: T1005)
7. 1 mM HCl
8. Casein Fluorescein IsoThioCyanate from bovine milk (Sigma-Aldrich, catalog number: C0528)
9. Distilled water
10. cOmplete tablets EDTA-free (Roche Diagnostics, catalog number: 04693132001)
11. Trichloroacetic acid (TCA) (Sigma-Aldrich, catalog number: T6399)
12. Tris base
13. Trypsin solution (see Recipes)
14. Casein-FITC (see Recipes)
15. 25x cOmplete (see Recipes)
16. 10% TCA (see Recipes)
17. 0.5 M Tris/HCl pH 8.5 (see Recipes)

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Equipment

A. *Drosophila* rearing

Refrigerated oven at constant temperature of 25 °C and with a 12 h/12 h light/dark cycle (Fisher Scientific, catalog number: 11857552). Humidity has to be maintained between 40% and 70% *Manufacturer: LMS, model: Model 240.*

B. Bacterial culture
1. 100 ml flasks (Fisher Scientific, catalog number: 15409103)
2. 30 °C/37 °C shaking incubator (Infors, model: AK 82)

C. Intoxication
1. Spectrophotometer (Aqualabo, Secomam, model: Prim Light & Aduanced)
2. Droso-sleeper (Inject-Matic)

D. Dissection
1. Stereomicroscope (Leica Microsystems, model: Leica M60)
2. Dumont forceps #5 (Fine Science Tools, catalog numbers: 11251-20 and 11252-20)

E. Sample preparation
1. Pestle motor (Heidolph Instruments, model: RZR 2100)
2. Refrigerated microfuge (Eppendorf, model: 5430 R)

F. Assay
1. Vortex (Scientific Industries, model: Vortex-Genie 2)
2. Automated pipette (Eppendorf, model: Multipette® plus)
3. 37 °C solid-door incubator (Jouan)
4. Fluorimeter (Agilent Technologies, model: Cary Eclipse)

Software

1. *Kyplot*
2. Excel

Procedure

A. *Drosophila* rearing
1. *Canton*^{S} flies are reared on standard medium for *Drosophila melanogaster* (see Recipes) at 25 °C.

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2. Only virgin five-days-old females are used in our experiments. To obtain synchronized five-day-old females, we empty bottles from adult flies, then we wait for the emergence of new flies from pupae. Among the emergent flies, males are rapidly discarded and females are transferred in new bottles for five days at 25 °C.

B. Bacterial culture

In the experiments presented below, we use three different bacterial strains (*Btk*, *Ecc* and *Ec*, see Materials and Reagents) and water as negative control (Ctrl). Spread bacteria from the stocks on LB agar Petri dishes and let grow overnight at the required temperature (30 °C for *4D22* and *Ecc* and 37 °C for *Ec*).

1. Pick a single colony using a sterile tip and initiate a 10 ml starter culture of LB (see Recipes) in a 15 ml tube. Let grow for 8 h shaking (220 rpm) at 30 °C for *4D22* and *Ecc* and at 37 °C for *Ec*.
2. Next, use the small starter culture to inoculate a second, larger culture: 50 µl of starter culture + 50 ml LB in a 100 ml flask and allow to grow overnight at the required temperature.

C. Intoxication

1. On the day of infection, measure the optic density (OD) of the culture at a dilution of 1/5 (200 µl culture + 800 µl LB). A reliable OD must be between 0.2 and 0.8.
2. Depending on the cultured bacteria, the required ODs to intoxicate flies with 10^6 Colony Forming Unit (CFU) per *Drosophila* are presented in the Table 1:

Table 1. Required bacterial concentrations

Bacterium	Volume to put in the media	Required OD	CFU/tube (for 10 flies)	CFU/fly
4D22	50 µl	1.8	1×10^7	1×10^6
Ecc	50 µl	5.2	1×10^7	1×10^6
Ec	50 µl	33.6	1×10^7	1×10^6

3. Dilute the overnight bacterial culture with LB to obtain the required OD. For example: to obtain an OD 1.8 of *4D22* bacteria from an overnight culture at OD 9, you need to dilute the overnight culture by 5 (9/1.8 = 5). Use LB medium to make the dilution.
4. Then mix at 1:1 the diluted culture with 10% sucrose (see Recipes). For practical reasons mix 1 ml of diluted culture with 1 ml of 10% sucrose. This mixture will constitute the intoxication solution.
5. 5-day-old virgin females are allowed to fast for 2 h at 25 °C: flies are placed in empty *Drosophila* bottles at 25 °C for 2 h.
6. Starved flies are then transferred onto a *Drosophila* narrow vials containing fly medium covered with filter disks soaked with 50 µl of the intoxication solution (Figure 1).

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Note: Before to deposit your flies onto the soaked filter disk in the vials, wait for a few minutes until the filter disk becomes dry, otherwise the flies will be wet and will probably remain stuck on the filter disk.

7. Flies are kept to feed on the contaminated media at 25 °C until dissection.

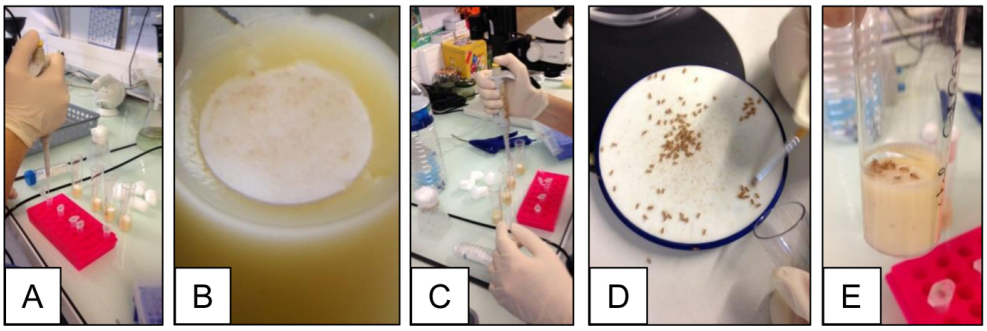

Figure1. Intoxication procedure. A. Annotate the *Drosophila* vials and place the filter disks inside on the top of the medium; B. Zoom up on a filter disk on the top of the medium; C. Add 50 µl of the intoxication solution in the vials; D. Sort by 10 the starved female flies using the Droso-sleeper; E. Deposit carefully 10 flies in each contaminated vial.

D. Dissection

1. Forceps and dissecting watch glasses have to be washed with a 70% ethanol solution.
2. The flies (10 virgin females per condition) are anesthetized using the Droso-sleeper.
3. Place one fly in a watch glass pre-filled with 1 ml 1x PBS (see Recipes) (Figure 2A).
4. Using forceps, hold the fly by the head and pull gently on the posterior part of the abdomen to carefully detach the abdomen from the thorax (Figure 2B).
5. Then, the intestine is carefully stretched (Figure 2C).
6. Cut the head and untie very gently the intestine first from its anterior part and then from its posterior part (Figures 2D-2F).

   Note: Here, we need only the midgut so we cut the foregut and the hindgut. We also removed Malpighian tubules that can still be attached at the midgut/hindgut boundary (Figures 2D-2F).

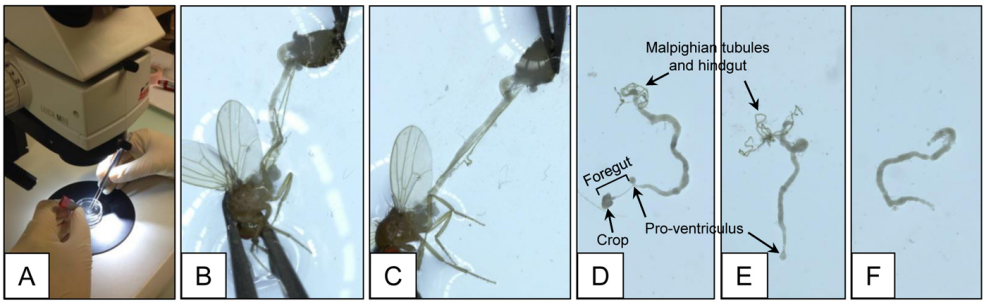

Figure 2. Dissection procedure. A. Place one fly in a watch glass pre-filled with 1 ml 1x PBS under a stereomicroscope; B. Hold the fly by the head and pull gently on the posterior part of the abdomen to carefully detach the abdomen from the thorax; C. Carefully stretch the intestine;

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D. Cut the head and untie very gently the intestine first from its anterior part and then from its posterior part. The whole intestine (foregut/midgut/hindgut) must be separated from the rest of the abdomen. E. Remove the crop; F. Remove the proventriculus, the hindgut and the Malpighian tubules. See also http://www.bio-protocol.org/e2079 (Chen *et al.*, 2016)

7. The dissected midguts are then immediately placed in 1x PBS on ice waiting for the dissection of the other intestines.
8. Only 8 guts (over the 10) are transferred into 50 µl of 1x PBS in a 1.5 ml microtube for sample preparation.

E. Sample preparation

1. Recover gut content by pressing them with a microtube pestle fixed on a motor set to 1,000 rpm: put the motor on and perform 10 up and down movements with the microtube to allow the pestle to reach the bottom of the microtube (Video 1).
   Note: Keep the tubes on ice to avoid any protein degradation. The goal is to recover most of lumen contents, so do not strongly crush the guts and do not perform more than 10 up and down movements.

   Video 1. Crushing intestines. The video describes the procedure to gently crush intestines using a microtube pestle. Note at the end of the video that to avoid to waste gut content, we take off the pestle and press it against the edge of the microtube

2. Centrifuge for 5 min at 10,000 *x g* and 4 °C.
3. Dilute at 1:10 a part of the supernatant: take 20 µl of the supernatant and transfer it in a 1.5 ml microtube containing 180 µl of 1x PBS.
4. Take another 20 µl of the supernatant in a 0.5 ml microtube and place it at 4 °C waiting for protein assay if one wants to normalize the results per mg of protein.

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F. Assay

Note: For each sample, we assay 3 volumes to be sure to be in the fluorimeter's measurement range and to have reproducible results. As negative control, we use the maximal sample volume (i.e., 20 µl) complemented with a cocktail of protease inhibitors (25x cOmplete). As positive control, we use trypsin. The Table 2 gives an example of a 96-well plate's scheme according to these rules. This is a plane of an independent experiment with two technical replicates (one from columns 1 to 4 lanes B to E and one from columns 6 to 9 lanes B to E).

Table 2. 96-well plate's plane. Volume (µl) for:10x PBS/Distilled water/Sample/Trypsin/25x cOmplete.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 10/70	0 10/70		**Trypsin** 10/50/20	**Trypsin** 10/50/20		**Trypsin** 1:10[a] 10/50/20	**Trypsin** 1:10[a] 10/50/20				
B	Ctrl 5 10/65/5	Ctrl 10 10/60/10	Ctrl 20 10/50/20	Ctrl 20C 10/40/10/20		Ctrl' 5 10/65/5	Ctrl' 10 10/60/10	Ctrl' 20 10/50/20	Ctrl' 20C 10/40/10/20			
C	*4D22* 5 10/65/5	*4D22* 10 10/60/10	*4D22* 20 10/50/20	*4D22* 20C 10/40/10/20		*4D22'* 5 10/65/5	*4D22'* 10 10/60/10	*4D22'* 20 10/50/20	*4D22'* 20C 10/40/10/20			
D	*Ec* 5 10/65/5	*Ec* 10 10/60/10	*Ec* 20 10/50/20	*Ec* 20C 10/40/10/20		*Ec'* 5 10/65/5	*Ec'* 10 10/60/10	*Ec'* 20 10/50/20	*Ec'* 20C 10/40/10/20			
E	*Ecc* 5 10/65/5	*Ecc* 10 10/60/10	*Ecc* 20 10/50/20	*Ecc* 20C 10/40/10/20		*Ecc'* 5 10/65/5	*Ecc'* 10 10/60/10	*Ecc'* 20 10/50/20	*Ecc'* 20C 10/40/10/20			
F												
G												
H												

[a]*To be sure to stay in the reading range of the spectrophotometer, carry out two assays with a dilution of trypsin to 1:1*

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1. Prepare the trypsin solution (see Recipes) as described below.
2. Annotate 1.5 ml microtubes with the letter and the number corresponding to their position in the 96-well plate.
3. Add the indicated volume of distilled water in each tube.
4. Add the 10x PBS.
5. Finally add the indicated volume of sample or trypsin.
6. When necessary add the protease inhibitors (25x cOmplete) (see Recipes).
   Note: From this point, it is important to work with the least of light as possible.
7. Prepare the Casein-FITC (see Recipes) as described below.
8. Add 20 µl Casein-FITC in each tube and vortex few seconds.
   Note: Be careful, do not vortex too strongly to avoid fluorescence background.
9. Incubate for 1.5 h in a 37 °C solid-door incubator (samples have to be in the dark).
   Note: Samples can be incubated until 24 h to increase the signal. Beyond one day, Casein-FITC can be degraded leading to high fluorescence background.
10. Add 300 µl 10% TCA (see Recipes) and vortex for a few seconds.
11. Incubate for 0.5 h in a 37 °C solid-door incubator.
12. Centrifuge for 10 min at 10,000 *x g*, 4 °C.
    Note: Whole proteins and big fragments, which underwent a few or no proteolytic cleavage, precipitate with the TCA and are centrifuged down in the pellet. Casein-FITC small fragments that underwent a lot of proteolytic cleavage are in the supernatant. That's what will be measured.
13. In a black 96-well plate, put 50 µl of supernatant according to the Table 2.
14. Add 150 µl 0.5 M Tris/HCl (see Recipes) pH 8.5.
15. Read the fluorescence on a spectrofluorimeter set on 485 nm excitation wavelength and 535 nm emission wavelength.

Data analysis

A. FITC-intensity measurement analysis
   1. We systematically perform at least 3 independent experiments. We defined 'independent experiments' when they are performed on different days.
   2. Each experiment is performed in duplicate meaning that for a given day for one condition of intoxication, there are two different pool of 10 *Drosophila* contaminated by the same batch bacteria.
   3. Below we present results for 4 conditions of intoxication (Ctrl, *4D22*, *Ecc* and *Ec*). For each condition there are 3 independent experiments and for each experiment two replicates.
   4. For each replicate, we have assayed 3 different volumes and a negative control (5 µl, 10 µl or 20 µl of samples and 20 µl of samples + 25x cOmplete).
   5. In each replicate, 8 intestines were used, therefore we have in total 48 intestines/each condition.

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6. We have calculated the mean of results obtained in the 3 independent experiments (*i.e.*, six replicates) for each condition and for each volume.
7. Table 3 and the Figure 3 give an example of raw data obtained after FITC fluorescence measurement.

   Note: Below are presented all the raw data without excluding any value. However, the experimenter can remove some replicates if he has performed more than 3 independent experiments and if he judges that one of the replicates failed.

Table 3. FITC intensity measurements

Ctrl							
	1	2	3	4	5	6	**Mean**
5 µl	557.9304	1318.2874	903.7938	1299.4419	751.5250	1052.3835	980.5603
10 µl	1001.2176	1933.9919	1473.9985	2119.2616	1023.6289		1510.4197
20 µl	1768.0535	2542.1417	2051.9611	2950.2352	1648.8200	2184.4931	2190.9508
20 µl + C	129.9290	351.2914	294.7997	423.8523	275.3432	414.5753	314.9652
4D22							
	1	2	3	4	5	6	**Mean**
5 µl	489.7209	1413.9899	946.9993	965.1006	673.8947	820.4096	885.0192
10 µl	484.3394	2348.1190	1328.7739	1354.1726	1509.3014	858.2746	1313.8302
20 µl	613.4965	3282.2480	1581.6377	1611.8697	2199.9043	967.6626	1709.4698
20 µl + C	177.5911	486.2589	356.9840	363.8075	568.0765	353.4072	384.3542
Ecc							
	1	2	3	4	5	6	**Mean**
5 µl	1158.3686	1427.4310	652.4954	422.6512	575.0048	831.2063	844.5262
10 µl	2115.0953	2182.4358	956.3973	619.5025	654.7500	1149.0205	1279.5335
20 µl	2707.1504	3167.4812	1175.3855	761.3512	860.4086	1809.0962	1746.8122
20 µl + C	531.9915	524.9643	272.6179	176.5872	306.3894	213.0403	337.5984
Ec							
	1	2	3	4	5	6	**Mean**
5 µl	1004.7891	1091.5762	1390.8680	1280.9451	955.7488	691.9489	1069.3127
10 µl	1523.7917	1529.0905	2045.8946	1884.2037	1183.3081	1170.2435	1556.0887
20 µl	2271.1555	2152.2170	2751.9621	2534.4695	2064.5829	1582.9849	2226.2287
20 µl + C	365.3778	428.6756	374.30088	344.7192	318.5829	206.3707	339.6712

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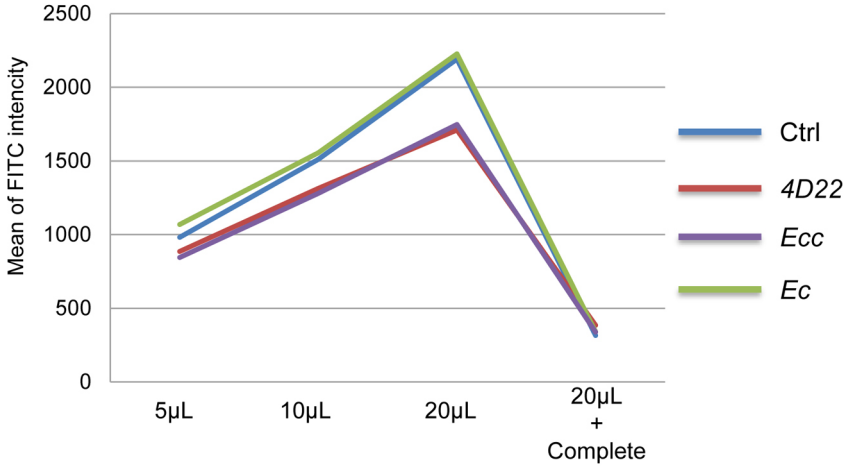

Figure 3. Graph of average FITC-intensity. The data of the Table 3 are reported in this graph. Only the means were used to draw the graph.

8. For result presentation and statistical analyses, we selected the data obtained with the volume 20 µl of samples (more reliable and reproducible).
   a. Results are presented in % of FITC intensity (reflecting protease activity) where Ctrl is considered at 100% (Table 4).
   b. We used SEM (Standard Error of the Mean) to present error bars in the graph (Figure 4).

Table 4. Mean and percentage of FITC-intensity reflecting the protease activity

	Ctrl	*4D22*	*Ecc*	*Ec*
sample 20 µl	2190.9508	1709.4698	1746.8122	2226.2287
% to the Ctrl	100.000	78.0241	78.4651	98.4154
SEM 20 µl	199.3285	386.9306	409.3817	165.2065
% SEM 20 µl	9.0978	17.6604	18.6851	7.5404
sample 20 µl + C	314.9652	384.3543	337.5984	339.6712
% to the Ctrl	14.3757	17.5428	15.4088	15.5034
SEM sample 20 µl + C	44.4646	54.4728	63.1217	30.5620
% SEM sample 20 µl + C	2.0295	2.4863	2.8810	1.3949

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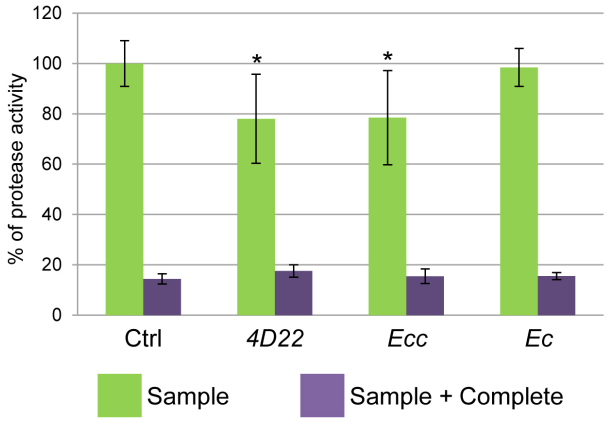

Figure 4. Percentage of protease activity. Protease activity (proportional to FITC intensity) shows a reduction of about 20% following *4D22* or *Ecc* treatment. No difference is observed between the Ctrl and *Ec* ingestion. **P* ≤ 0.05.

B. Statistical analysis
   1. Effects of treatments are analyzed using a pair wise comparison test (Tukey's test).
   2. Samples are compared to the control.
   3. Differences are considered significant when $P < 0.05$ (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$) (Figure 4).
   4. The used software is *Kyplot.*

Notes

The protocol described here can easily be transposed to *Drosophila* larval midgut and even to any other insect midguts.

Recipes

1. Standard nutrient medium for *Drosophila melanogaster*
   Note: All the reagents are mixed in distilled water.
   8 g/L agar
   25 g/L sugar
   80 g/L cornflour
   20 g/L yeast
   6 g/L tegosept (stock solution at 100 g/L in 95% ethanol. Store at 4 °C)
2. LB medium
   a. Weigh out 25 g of Luria broth medium powder
   b. Adjust to 1 L with distilled water in a graduated test tube
   c. Adjust pH to 7.2 if necessary
   d. Autoclave

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Please cite this article as: Marie-Paule *et. al.*, (2017). Protease Activity Assay in Fly Intestines, *Bio-protocol* 7 (18): e2560. DOI: 10.21769/BioProtoc.2560.

3. LB-agar medium
    a. Weigh out 25 g of Luria broth medium powder
    b. Adjust to 1 L with distilled water in a graduated test tube
    c. Adjust pH to 7.2 if necessary
    d. Add 15 g agar powder
    e. Autoclave
4. 10% sucrose
    a. Weigh out 2 g of sucrose in a 50 ml tube
    b. Add 20 ml distilled water and vortex

    Note: Do not keep this solution.
5. 1x phosphate-buffered saline (PBS)
    a. Add 100 ml of 10x PBS solution to 900 ml of distilled water in a graduated test tube
    b. Filter and store at 4 °C if you want to keep this solution
6. Trypsin solution

    Note: This solution has to be prepared immediately before use, do not keep it because of trypsin self-digest.
    a. Weigh out 10 mg of trypsin powder in a 2 ml microtube and add 1 ml 1 mM HCl
    b. Vortex to dissolve powder and keep the tube on ice
7. Casein-FITC
    a. Weigh out 10 mg of Casein Fluorescein IsoThioCyanate powder in a 15 ml tube
    b. Add 10 ml of distilled water
    c. Wrap the tube with aluminum foil and vortex thoroughly until total dissolution
    d. Keep Casein-FITC on ice and in the dark until use

    Note: This solution can be stored at -20 °C up to 6 months but do not expose it to the light.
8. 25x cOmplete
    a. Dissolve one tablet of cOmplete tablets EDTA free in 2 ml of distilled water in a 5 ml tube
    b. Vortex strongly
    c. Divide it into two 1.5 ml microtubes and store one at -20 °C

        Note: Keep the other one tube containing cOmplete on ice until use.
9. 10% TCA
    a. Make a stock solution with 100 mg of trichloroacetic acid powder in 100 ml of distilled water
    b. Dilute at 1:10 this stock solution by adding 5 ml of TCA stock solution to 45 ml of distilled water

    Note: Do not keep the diluted solution.
10. 0.5 M Tris/HCl pH 8.5
    a. Weigh out 60.57 g of Tris base powder for 1 L of distilled water
    b. Adjust pH to 8.5 with 12 N HCl
    c. Filter if you want to store this solution

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bio-protocol www.bio-protocol.org/e2560 Vol 7, Iss 18, Sep 20, 2017 DOI:10.21769/BioProtoc.2560

Acknowledgments

We would like thank David Pauron for his help at the beginning of the protocol setup. MPNE was supported by INRA. RL was supported by the Fondation pour la Recherche Médicale (FRM) and Université Côte d'Azur (UCA) and AG was supported by the CNRS.

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