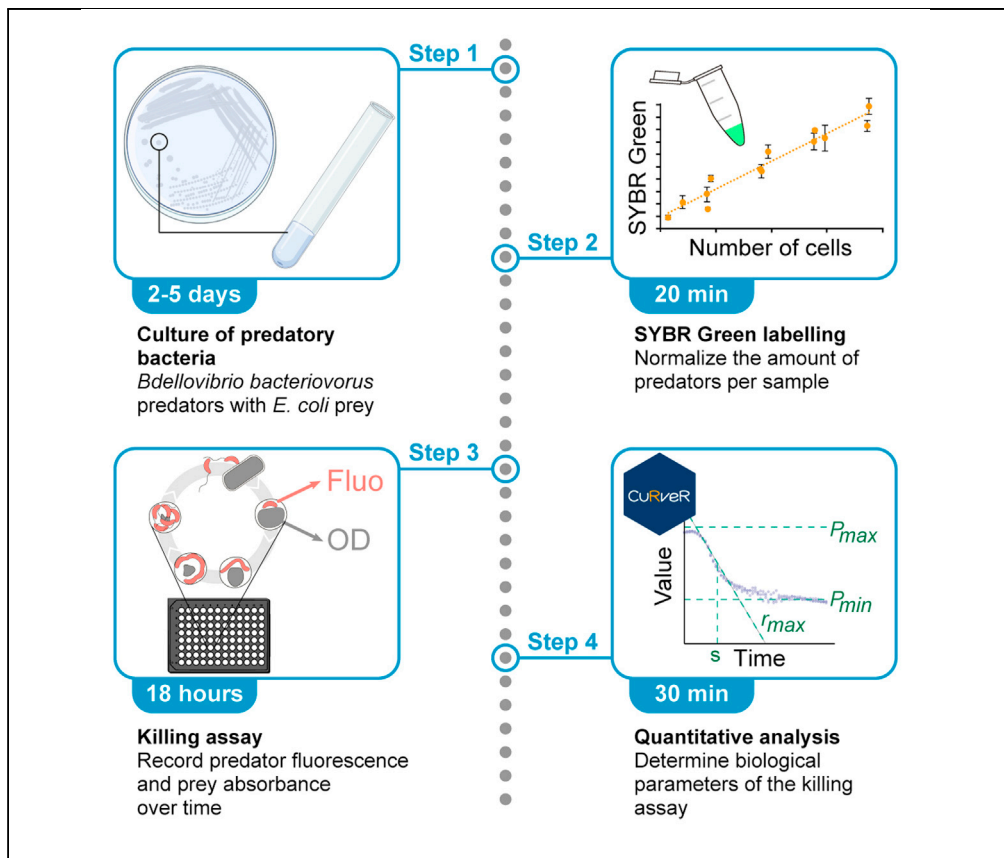


Protocol

An optimized workflow to measure bacterial predation in microplates



The predatory bacterium *Bdellovibrio bacteriovorus* invades and proliferates inside other bacteria by non-binary division. Here we describe a fluorescence-based technique for the immediate evaluation of predator density independently of plaque formation, an optimized setup to monitor predation in microplates, and the CuRveR package to quantify both prey killing and predator proliferation dynamics. This protocol allows to assess the impact of mutations or chemicals on predation. CuRveR also constitutes a user-friendly tool to analyze growth or decay data unrelated to predation.

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Highlights

A protocol for routine culturing of *Bdellovibrio bacteriovorus* predatory bacteria

A quick fluorescence-based technique to obtain predator cells numbers

An automated assay of prey killing and predator proliferation in microplates

The CuRveR package to extract prey decay and predator growth parameters

Remy et al., STAR Protocols 3,
101104
March 18, 2022 © 2021 The
Author(s).
[https://doi.org/10.1016/
j.xpro.2021.101104](https://doi.org/10.1016/j.xpro.2021.101104)



Protocol

An optimized workflow to measure bacterial predation in microplates

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<https://doi.org/10.1016/j.xpro.2021.101104>

SUMMARY

The predatory bacterium *Bdellovibrio bacteriovorus* invades and proliferates inside other bacteria by non-binary division. Here we describe a fluorescence-based technique for the immediate evaluation of predator density independently of plaque formation, an optimized setup to monitor predation in microplates, and the CuRveR package to quantify both prey killing and predator proliferation dynamics. This protocol allows to assess the impact of mutations or chemicals on predation. CuRveR also constitutes a user-friendly tool to analyze growth or decay data unrelated to predation.

For complete details on the use and execution of this profile, please refer to Kaljević et al., 2021.

BEFORE YOU BEGIN

Bdellovibrio bacteriovorus is a model species for intra-periplasmic bacterial predation. Its cell cycle is composed of several stages: (1) attack phase, in which freely swimming *B. bacteriovorus* do not replicate (G1 phase) and attach to a prey bacterium (2) invasion of the prey envelope and modifications of the prey cell wall to shape a closed nest called bdelloplast (4) growth (S) phase: multiple DNA replication rounds and progressive chromosome segregation, predator filamentation and digestion of the prey content; (6) non-binary division; (7) release of newborn attack phase cells via lysis of the bdelloplast. This predatory life cycle has been reviewed previously (Sockett, 2009; Rotem et al., 2014; Laloux, 2020). As *B. bacteriovorus* preys upon most other Gram-negative species (Dashiff et al., 2011), a series of studies propose its potential use as a living antibiotic (Atterbury and Tyson, 2021). The sequenced wild-type HD100 strain of *B. bacteriovorus* is an obligate predator, hence its laboratory maintenance is achieved by co-culture with a suitable prey – usually *Escherichia coli*, either in the form of lysates (liquid medium) or as plaques (solid medium).

Assessing predation efficiency of different mutant predator/prey strains or under various settings ideally requires the control of predator cell numbers in each tested condition. Classical methods to evaluate cell density such as optical density (OD) or counting chambers are not applicable to *B. bacteriovorus*, probably owing to its small size in the attack phase ($1.26 \pm 0.14 \mu\text{m}$ long, Kaljević et al., 2021). Counting of plaque-forming units (PFU) is commonly used to determine predator numbers in lysates (Lambert and Sockett, 2008), but provides the information days after the experiment, preventing normalization across strains and conditions. Moreover, plaque formation depends on predation efficiency and thus may differ among mutant strains. Also, plaques are difficult to count with precision when confluent. Protein assays have been used to normalize *B. bacteriovorus*



samples, but subsequent PFU counting was still needed to evaluate cell numbers (Milner et al., 2020).

To assess the capacity of predators to kill prey cells, prey lysis and predator proliferation are recorded over time in killing assays. Here, prey lysis is typically evaluated via the decrease in absorbance, while predator numbers are estimated several days later by PFU counts from samples taken at selected time-points. Such experiments are therefore highly time- and material-consuming when several strains, time-points and replicates are considered (e.g., Lambert and Sockett, 2008; Rotem et al., 2015). Alternatively, the use of fluorescent *B. bacteriovorus* strains allows real-time detection of predator proliferation in a fluorimeter after prey and predator mixing (Mukherjee et al., 2015). However, PFU counting was needed to count predators over time. A method for extracting relevant quantitative parameters of prey killing and/or predator proliferation dynamics from absorbance and fluorescence reads was also lacking.

Here we describe (i) a standardized *B. bacteriovorus* culturing method, (ii) a fast protocol to count predator cells before any experiment without the need for PFU counts, (iii) a procedure for real-time monitoring of predation via absorbance and fluorescence kinetics reading in a microplate reader, and (iv) a user-friendly application called CuRveR for the automated quantitative analysis of prey lysis and predator proliferation. The protocol below describes the specific steps for monitoring both prey killing and predator proliferation using *E. coli* MG1655 as prey and *B. bacteriovorus* HD100 *bd0063-0064::pBioFab-mcherry* (constitutively producing mCherry from a chromosomal locus, abbreviated mCh *Bdellovibrio* below) as predator. However, this protocol can be adapted for different prey and *B. bacteriovorus* strains. Other chromosomal constructs allowing constitutive production of mCherry in *B. bacteriovorus* should work similarly as our strain. Although not thoroughly tested, our results indicate that other fluorescent proteins can also be used to monitor predator proliferation over time in a microplate reader. Non-fluorescent *B. bacteriovorus* strains can be used, but in this case only the prey killing can be monitored (via absorbance reading).

Preparation of prey suspension

⌚ Timing: 2 days

1. Day 1: Culture the prey bacteria
 - a. Option 1: inoculate 300 mL Luria Broth (LB) liquid medium with *Escherichia coli* MG1655 from a frozen stock of *E. coli*: scratch the frozen stock with a sterile loop and dip the loop in a glass flask containing 300 mL LB, as routinely done for *E. coli* cultures.
 - b. Option 2: inoculate 300 mL LB liquid medium with *E. coli* from a single colony:
 - i. Scratch the frozen stock with a sterile loop
 - ii. Streak on a LB agar plate
 - iii. Incubate the plate at 37°C overnight (i.e., 12–18 h).
 - iv. Pick a single colony with a sterile loop and dip it in a glass flask containing 300 mL LB

Note: If antibiotics are used in the experiment, the prey must carry the same resistance as the predator. Otherwise, no antibiotics are added in the plates or medium. Work under sterile conditions throughout.

Note: The nominal volume of the vessel should be at least 5 times larger than the volume of the culture to ensure proper oxygenation.

- c. Incubate overnight (12–18 h) at 37°C with 200 rpm shaking, to reach the stationary phase.
2. Day 2: Preparation of prey suspension
 - a. Transfer the culture in a centrifuge-compatible sterile bottle

Note: From this step, keep the cells on ice.

- b. Pellet the cells by centrifugation (4000×g, 10 min, 4°C)
- c. Wash twice as follows:
 - i. In the same vessel, resuspend the pellet with 10 mL 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid solution supplemented with 2 mM calcium chloride (CaHEPES).
 - ii. Bring the total volume to 100 mL with CaHEPES

Note: Other buffers or poor media could be used here instead of CaHEPES, although never tested systematically in our hands. The goal is to keep the prey in good condition (which can be checked by morphological observation under a benchtop microscope) and not affecting the growth of *B. bacteriovorus*.

- d. Final suspension: adjust to a final optical density measured at 600 nm (OD_{600}) of 10 using CaHEPES
- e. Store in 50 mL Falcon tubes at 4°C for up to 2 weeks

Revival of *Bdellovibrio bacteriovorus* from –80°C stocks

⌚ Timing: 3 days

3. Day 1: Inoculate *B. bacteriovorus* from a 15% glycerol stock stored at –80°C
 - a. Prepare the coculture mix in a sterile glass tube as follows:
 - i. Diluted Nutrient Broth (DNB) liquid medium
 - ii. Prey suspension (final OD_{600} = 1)

Note: A small volume is recommended to revive the predator from the –80°C frozen stock since the time required for clearance increases with the volume. Suggested volume: 3 mL.

- b. Scratch the frozen glycerol stock with a sterile loop and dip the loop in the coculture mix
- c. Incubate overnight at 30°C on a rotating wheel

Note: Our wheel rotates at about 60 rpm in the incubator. No humidity control is required. The same parameters remain true for all subsequent steps involving a rotating wheel and incubator.

Note: For larger volumes, it is possible to use an Erlenmeyer on a 200 rpm shaking plate.

4. Day 2: Subculture *B. bacteriovorus*
 - a. Prepare the coculture mix as described at step 3.a
 - b. Check the overnight culture from step 3.c on the benchtop microscope to confirm the presence of predator cells: using a micropipette, spot 2 µL of culture on a glass slide and cover with a coverslip. A 40X phase contrast objective is enough to visualize freely swimming predators.

Note: Clearance of the coculture mix is usually not complete at this stage.

- c. Based on the density of predators observed, inoculate the coculture mix with 100 µL (lots of predators, i.e., many predators per field of view) to 300 µL (few predators, e.g., only one or two predators per field of view) culture from step 3.c. Estimation of the most appropriate volume is empirical at this point; if in doubt, prepare several tubes with different inoculum volumes when performing this step for the first time, and adjust the inoculum volumes accordingly for future experiments.
- d. Incubate overnight at 30°C on a rotating wheel

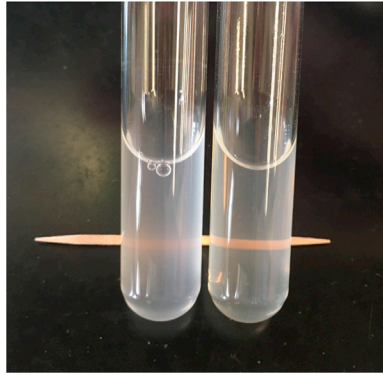


Figure 1. Revival of *B. bacteriovorus* from cryo-conservation

Aspect of the coculture mix inoculated with *B. bacteriovorus*, before (left) and after (right) overnight incubation.

5. Day 3: Cleared lysate

- a. Check the overnight culture tube from step 4.d: clearance should now be visible by eye (Figure 1). Observation on a benchtop microscope as described above should confirm the complete disappearance of prey cells and lots of predators swimming.

Optional: if the complete absence of remaining prey cells is critical, filter the lysate with 1.2- μm and 0.8- μm sterile syringe-filters. This step will let *B. bacteriovorus* cells go through and filter out *E. coli* cells.

Note: We routinely plug both filters at the tip of the syringe (so the flow will pass first through the 1,2- μm filter) to perform the filtration in a single step.

Note: All material for filtration needs to be pre-wet with sterile DNB prior to this step to minimize the inevitable loss of predators in the process. We usually observe up to 1-log less *B. bacteriovorus* cells after filtration compared to non-filtered lysates (which could result from a combination of cell death and cells sticking on the filtration membrane). However, this does not prevent the use of filtered lysates as starter *B. bacteriovorus* cultures (e.g. in steps 6.d.i., 8.b.). In our hands, the swimming of the predators is not affected by filtration.

- b. Store the cleared lysate (filtered or not) at 4°C for up to 2 weeks, or use directly in a subsequent step (e.g., in steps 6.d.i., 8.b.).

Solid culture of *Bdellovibrio bacteriovorus*

⌚ Timing: 2–5 days

6. Culture of *B. bacteriovorus* on a double-layer plate

Note: This step is required whenever the use of single *B. bacteriovorus* plaques is important for the experiment, e.g. to include biological replicates.

- a. Pre-heat the bottom agar plate(s) and the prey suspension at 30°C
- b. Melt or prepare fresh top agar, dispense 5–7 mL in a sterile tube and cool down to 55°C in a water bath until use
- c. Option 1: streak *B. bacteriovorus* on the bottom agar plate. This option allows to obtain isolated plaques by using a single plate per strain:
 - i. Pipet a 100 μL inoculum of cleared lysate from step 5.a. or 5.b. on a side of the plate.

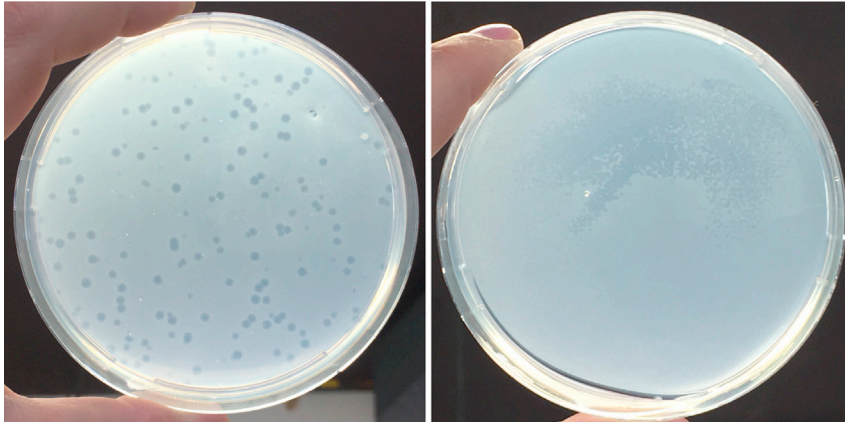


Figure 2. Solid culture of *B. bacteriovorus*

Left: aspect of plaques obtained with the dilution method. Right: aspect of plaques obtained with the streaking method.

- ii. Streak it with a sterile plastic or titanium loop.
- iii. Add the prey (final $OD_{600} = 1$) and the antibiotic if applicable, to the top agar
- d. Option 2: plate serial dilutions of *B. bacteriovorus*. This option requires more plates if used for PFU counting:
 - i. Prepare serial 10-fold dilutions of cleared lysate from step 5.a. or 5.b.
 - ii. Add 100 μL of each dilution, the prey (final $OD_{600} = 1$) and the antibiotic if applicable, to the top agar. We suggest the following range of dilutions for PFU counts: 10^{-3} to 10^{-7} . If the goal is only to obtain isolated plaques (as an alternative to the streaking method described in Option 1), the 10^{-4} dilution is usually giving optimal plaque isolation in our hands.
- e. Gently dispense the mix from c. or d. on top of the bottom agar plate without delay
- f. Let it solidify for 10 min at room temperature (RT, which is usually around 19°C – 23°C in our lab).
- g. Incubate upside down for 2–5 days at 30°C and check regularly for plaques (Figure 2).

Note: Contaminations are infrequent (especially when the lysate was filtered) but would appear as colonies developing on or within the top agar.

Note: When working with mutant *B. bacteriovorus* strains, we recommend including the wild-type HD100 strain as control to verify that the plate and mix permit plaque formation.

△ CRITICAL: At step e., avoid keeping the top agar mix out of the 55°C water bath for too long before pouring as it will quickly start solidifying, resulting in a lumpy top layer and difficulties to see plaques. Quickly tilt the plate after pouring the top agar mixture to ensure that it is fully covered before solidification. Step e. must be performed on a flat and leveled surface to ensure homogenous height of the top agar layer, which will impact the visibility of plaques. We recommend using 85-mm round plates and the top agar volume indicated in 6.b., to obtain a top agar depth that allows clear observation of plaques.

Liquid culture of *Bdellovibrio bacteriovorus*

⌚ Timing: 2–5 days

7. Option 1: from a single plaque on a double layer plate (step 6, Figure 2):
 - a. Prepare 200 μL (in a 96-well plate) or up to 500 μL (in a 1,5 mL tube) of the coculture mix as described at step 3.a.

- b. With a sterile pipette tip, pick and aspirate 10 μL from the top agar of a single plaque
- c. Resuspend in the coculture mix from step 7.a.
- d. Incubate at 30°C with 200 rpm shaking for 1–2 days and check regularly for clearance
8. Option 2: cultures for experiments from a cleared lysate:
 - a. Prepare a sterile glass tube with the coculture mix as described at step 3.a
 - b. Add 100–300 μL of cleared lysate from step 5 or 7 (when cultures from isolated plaques is required)
 - c. Incubate overnight at 30°C on a rotating wheel
 - d. Check the overnight culture regularly until cleared. Observation at the microscope should show complete disappearance of the prey and lots of predators swimming. If not, see [troubleshooting 1](#) and [2](#).

Note: When needed, larger cultures can be prepared in sterile glass flasks by scaling up the mix and lysate volumes, as long as the vessel nominal volume is at least 5-times bigger than the coculture volume.

⏸ Pause Point: The cleared lysate can be left on the bench for several hours.

Preparation of the SYBR green standard curves

⌚ Timing: 1–2 weeks

Note: In order to circumvent PFU counting to estimate the number of predator cells engaged in a given experiment, we developed an assay based on the fluorescence labeling of *B. bacteriovorus* DNA by the SYBR Green dye (see “[step-by-step method details, normalization by SYBR green labeling](#)” for more details). Hence, the goal of the following steps is to set the relationship between the fluorescence values obtained upon labeling of a predatory lysate (and subtraction of the fluorescence contribution from residual *E. coli*) to a number of *B. bacteriovorus* cells.

9. Preparation of SYBR Green aliquots, dilution 1:30
 - a. In black or foil-wrapped 1.5 mL Eppendorf tubes, add 1160 μL of DMSO.
 - b. Add 40 μL from the 10,000X SYBR Green stock.
 - c. Vortex well and store at -20°C .

⚠ CRITICAL: The SYBR Green dye should be handled with extra care. This nucleic acid stain is sensitive to light. Therefore, work with a minimum of light in the room and with dark containers only.

Note: Wear gloves since SYBR Green is considered as a potential carcinogen.

10. Standard DNA curve

Note: The main goal of this curve is to define the best gain used by the plate reader to detect the largest dynamic range of SYBR Green values and to keep the same settings across experiments. It also gives the relationship between the SYBR Green value detected and the amount of DNA in the sample.

- a. Turn on the plate reader and prepare the protocol on the plate reader controlling software as follows:
 - i. Pre-set temperature: 25°C.
 - ii. Continuous shaking (double orbital shaking, frequency 282 cpm, 15 min).
 - iii. SYBR Green fluorescence read: 490 nm excitation, 520 nm emission, autogain.
- b. Prepare dilutions in sterile milliQ water from a sample with a known concentration of DNA.

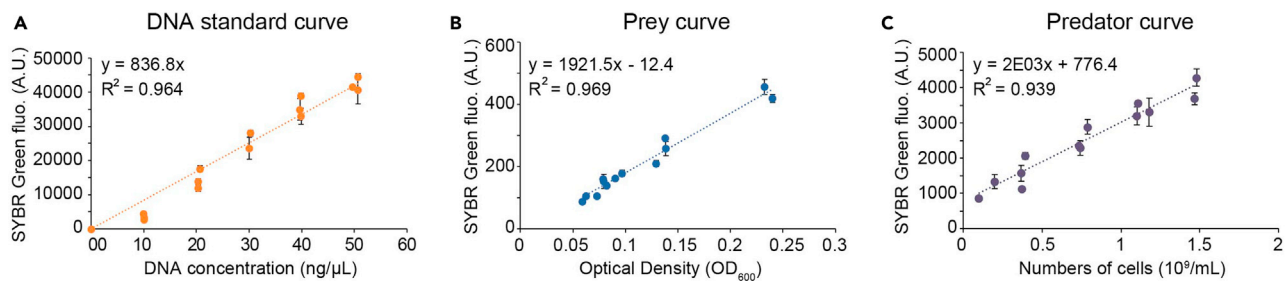


Figure 3. SYBR Green standard curves

For each curve, the mean and standard deviation of 3 wells from the same dilution of a representative experiment is plotted.

(A) DNA standard curve.

(B) Prey curve. The prey is an *E. coli* prey suspension prepared as described in step 2.

(C) Predator curve with the *B. bacteriovorus* HD100 strain.

Note: We used salmon sperm DNA for our DNA standard. Although we did not prepare standard curves with DNA from other sources, it is very likely that the same results would be obtained (e.g. with other in-house DNA preparations with defined concentrations or commercially available cloning vectors).

- c. Confirm the DNA concentration using a NanoDrop (or a similar microvolume spectrophotometer – see [materials and equipment](#)). We recommend using a blank, such as sterile milliQ water used in 10.b., before and between each dilution. For the curve in [Figure 3A](#) (see step 10.h. below), the DNA concentration of each dilution was measured 3 times and the mean was used for the X values.
- d. For each dilution, do triplicates by transferring 198 μL into 3 wells of a 96-well black plate with transparent flat bottom. Include 3 wells with only the medium used for the dilutions. The mean of these 3 wells will serve as blank.
- e. With gloves and protected from light, add 2 μL of diluted SYBR Green (see 9.) in each well.
- f. Quickly load the plate without lid inside the plate reader and run the protocol saved in a.

Note: The 15-min shaking step can be done at room temperature outside the plate reader. If chosen, this condition should be kept for all following experiments.

- g. Export the values and note the gain used by the plate reader. Repeat twice more with this gain.
- h. To construct the standard DNA curve, the SYBR Green fluorescence value of the blank is removed from the SYBR Green fluorescence value for each well. The means of each dilution per experiment are plotted with standard deviation, except if the standard deviation represents more than 15% of the mean. The curve follows a linear fit with the axes intercept set at (0,0). An example is found in [Figure 3A](#). [Troubleshooting 3](#).

11. Standard curve for the prey (*E. coli* MG1655)

Note: The main goal of the prey standard curve is to calculate the contribution of the remaining *E. coli* in the cleared culture to exclude it from the total SYBR Green fluorescence value obtained from a sample. Since the *B. bacteriovorus* culture is made with prey at $\text{OD}_{600} = 1$ and the preys are expected to be lysed overnight, higher values of OD_{600} are not necessary to draw this standard curve.

- a. Turn on the plate reader and prepare the protocol on the plate reader controlling software as in 10.a. using the gain set with the standard DNA curve in step 10.g. and by adding an absorbance read at 600 nm immediately after the SYBR Green fluorescence acquisition.
- b. Prepare 2-fold serial dilutions from the prey suspension from step 2.

- c. Do the SYBR Green assay by following the steps from 10.d to 10.f with the protocol set in a.
- d. To construct the standard curve for the prey, plot the mean of the SYBR Green fluorescence values for each dilution and per experiment and the corresponding OD₆₀₀ mean. Draw a linear fit for which the interception does not have to be set at (0,0) (see [Figure 3B](#)). [Troubleshooting 3](#).

Note: The blank values are not removed from the sample values at this point.

Note: Dilutions of *E. coli* with an OD₆₀₀ value below or equal to the blank should not be taken in account. Triplicates with a standard deviation that represents more than 15% of the mean are also excluded.

Note: The absolute OD₆₀₀ values acquired in 1-cm cuvettes and in microwells are different. Please refer to your plate reader user information if a conversion is needed. Data presented here use unmodified absorbance values from the plate reader.

12. Standard curve for the predator (*B. bacteriovorus* HD100)

Note: The main goal of the predator standard curve is to correlate the number of *B. bacteriovorus* cells with the corresponding SYBR Green values measured by the plate reader. Since *B. bacteriovorus* cells are too small to be counted in standard counting chambers or by optical density measurements, this preparatory step requires that the density of the predator is determined by plaque-forming unit (PFU) counting. After establishment of the predator standard curve, the SYBR Green measure will be sufficient to estimate the density of predators in a sample, hence circumventing the need for the formation and counting of plaques.

- a. Prepare an overnight culture with one clone isolated by plating (step 7).
- b. For the SYBR Green assay:
 - i. From the cleared culture from step a, prepare 2-fold serial dilutions.
 - ii. Follow the protocol from 10.d to 10.f
 - iii. Use the same plate reader settings as in step 10.a.
- c. For PFU counting:
 - i. From the cleared culture from step a, prepare 10-fold serial dilutions to 10⁻⁷.
 - ii. From each of the 10⁻⁵ to 10⁻⁷ dilutions, plate 100 μL on 3 plates (triplicates) with the method described at step 6.d.
 - iii. Incubate at 30°C for 2 days.

⏸ Pause Point: Store plates from iii at 4°C until plaques counting.

- iv. Count plaques and calculate PFU (see 12.e.i.).
- d. Repeat twice more from 12.a. to 12.c.iv. with new clones. For an overview of the procedure, see [Figure 4](#).
- e. For the curve:
 - i. Count the number of plaques on each plate where all plaques are sufficiently isolated. Typically for the wild-type strain, ± 15 plaques were counted with 10⁻⁷ dilution and ± 150 with 10⁻⁶. For each dilution, calculate first the mean PFU of the 3 plates. Multiply this number by the dilution factor × 10 (to account for the 100 μL initial inoculum). The average value corresponds to the number of predators per milliliter of culture.
 - ii. Using the prey standard curve ([Figure 3B](#)), subtract for each well the SYBR Green fluorescence values due to *E. coli* from the total SYBR Green fluorescence values to obtain the values generated by *B. bacteriovorus*. Then, subtract from these values the mean SYBR Green fluorescence value from the blank.
 - iii. For each dilution and per experiment, plot the mean of the SYBR Green fluorescence values obtained in 12.e.ii. and the corresponding *B. bacteriovorus* numbers.

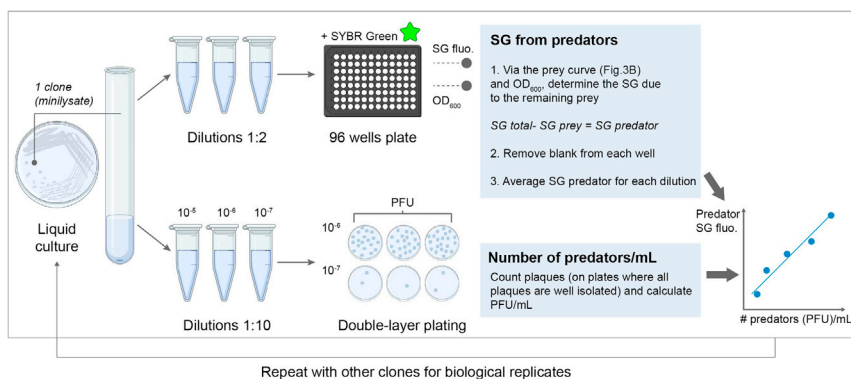


Figure 4. Overview of the steps to plot the predator SYBR Green predator standard curve
SG: SYBR Green. Figure created with [BioRender.com](https://www.biorender.com).

iv. Draw a linear fit (see [Figure 3C](#)). [Troubleshooting 3](#).

△ **CRITICAL:** Before the dilutions, the overnight culture must be checked by microscopy. There should be almost no prey or bdelloplast left. If this is not the case, let the culture shake at 30°C for a few more hours. The bacterial predators should be actively swimming. If they are swimming slowly, repeat step 12.a.

Note: When the standard deviation of SYBR Green fluorescence represents more than 15% of the mean of the wells from the dilution, exclude this mean in the final graph.

Installing CuRveR

13. Run Rstudio
14. Install this paper version of CuRveR from GitHub by running “devtools::install_github(“geraldin-laloux /curver”)” in the R console. If installation is unsuccessful, see [troubleshooting 7](#).

Optional: Install the latest version of CuRveR by running “devtools::install_github(“Giatomo/curver”)” in the R console.

Note: Differences between the latest version and the one presented in this paper can occur (see [materials and equipment](#)).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>B. bacteriovorus</i> HD100	Kind gift from R.E. Sockett (Rendulic et al., 2004)	GL499
<i>B. bacteriovorus</i> HD100 <i>bd0063-0064::pBioFab-mcherry</i> (named here mCh <i>Bdellovibrio</i> for simplicity)	Kaljević et al., 2021	GL1025
<i>E.coli</i> MG1655	Lab collection	GL655
<i>E.coli</i> MG1655 / pSEVA281- Δ oriT	Lab collection: Kan ^R prey strain from (Kaljević et al., 2021), obtained upon removal of <i>oriT</i> from pSEVA281 (Silva-Rocha et al., 2012) by DNA assembly	GL818

(Continued on next page)

<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Biological samples</i>		
Salmon sperm double-strand DNA	Lab stock at 2 µg/µL	n/a
<i>Chemicals, peptides, and recombinant proteins</i>		
LB Miller	Sigma-Aldrich	CAT# L3152
LB agar	MP Biomedicals	CAT# 3002232
DNB (Difco Nutrient Broth)	BD Bioscience	CAT# 234000
HEPES	Sigma-Aldrich	CAT# H3375
CaCl ₂	Sigma-Aldrich	CAT# C3306
MgCl ₂	Sigma-Aldrich	CAT# 1058330250
Kanamycin sulfate	Sigma-Aldrich	CAT# K4000
Gentamicin sulfate	Sigma-Aldrich	CAT# G1914
Bacto Agar	Fisher Scientific	CAT# 214010
SYBR Green	Thermo Fisher Scientific	CAT# S7563
<i>Deposited data</i>		
Dataset used for Figures 6 and 7	This study	https://github.com/geraldinelaloux/CuRveR/tree/main/Sample%20data ; https://doi.org/10.5281/zenodo.5767031
<i>Software and algorithms</i>		
CuRveR	This study and Kaljević et al., 2021	https://github.com/geraldinelaloux/CuRveR ; https://doi.org/10.5281/zenodo.5767031
Gen5™ Data Analysis Software	BioTek Instruments	v2.06.10
Rstudio	RStudio Team (2020)	https://rstudio.com v4.1.0 or higher
<i>Other</i>		
Multimode microplate reader	BioTek	Synergy™ H1M
NanoDrop One ^c microvolume spectrophotometer	Thermo Fisher Scientific	ND-ONEC-W
Primo Star benchtop microscope	Carl Zeiss	490701-9850-000
Plan-ACHROMAT 40x/0.65 Ph2 phase contrast objective	Carl Zeiss	415500-1603-001
BRAND® microplate BRANDplates®, pureGrade size 96 wells, size 330 µL, flat bottom, black, with transparent bottom	Sigma-Aldrich	BR781671
Stericup Quick Release-GP Sterile Vacuum Filtration System 0,22 µm	Merck Millipore	S2GPU05RE
Millex® filter unit with MF-Millipore™ Membrane 0.8 µm (or equivalent)	Merck Millipore	SLAA033SS
Minisart Syringe filter, SFCA, pore size 1.2 µm (or equivalent)	Sartorius	17593-K
Sterile inoculating loops	VWR	612-9360

MATERIALS AND EQUIPMENT

Synergy H1M plate reader setup: the protocol used for each step performed in the plate reader is described in the corresponding section. The user should refer to the manufacturer's manual for details on the use of the plate reader and the controlling software Gen5.

Alternatives: Other microplate readers than the one listed in the Key Resources Table can be used, as long as they allow continuous orbital shaking and temperature control during kinetics acquisition of fluorescence (with compatible excitation and emission wavelengths, see also [limitations](#)) and 600 nm absorbance measurements.

NanoDrop One setup: DNA concentration is measured in the dsDNA mode, after blanking with milliQ water.

Alternatives: Other spectrophotometers that measure DNA concentration from microvolume drops can also be used. Note that measurements on this equipment are mostly needed to confirm the expected concentrations of the serial dilutions in step 10 in order to plot a standard curve with more precision (for the values within the dynamic range of sensitivity of the spectrophotometer). Hence, if the initial DNA concentration is known, this step can also be omitted if no similar device is available.

CuRveR: CuRveR is an application written in R and used to analyze and plot proliferation and killing of cells.

Alternatives: The version presented in this paper (<https://github.com/geraldinelaloux/CuRveR>) is subjected to future modifications that will be documented. The latest version of CuRveR will be available from Github (<https://github.com/Giatomo/CuRveR>).

LB Miller medium

Reagent	Final concentration	Amount
LB powder	25 g/L	25 g
ddH ₂ O	n/a	Up to 1 L
Total	n/a	1 L

Autoclave. Store at room temperature for up to 1 month.

LB agar plates

Reagent	Final concentration	Amount
LB agar powder	40 g/L	40 g
ddH ₂ O	n/a	Up to 1 L
Total	n/a	1 L

Autoclave. Pour in Petri dishes. When solidified, store the plates at 4°C for up to 1 month.

DNB (Diluted Nutrient Broth) liquid medium

Reagent	Final concentration	Amount
DNB (Difco Nutrient Broth) powder	0.8 g/L	0.8 g
ddH ₂ O	n/a	Up to 1 L
Total	n/a	1 L

Adjust to pH 7.4 with NaOH 2M. Autoclave, then add 2 mM CaCl₂ and 3 mM MgCl₂. Store at room temperature for up to 1 month.

Bottom DNB agar plates

Reagent	Final concentration	Amount
DNB (Difco Nutrient Broth) powder	0.8 g/L	0.8 g
Bacto Agar	15 g/L	15 g
ddH ₂ O	n/a	Up to 1 L
Total	n/a	1 L

Adjust to pH 7.4 with NaOH 2M. Autoclave. Add 2 mM CaCl₂ and 3 mM MgCl₂ and pour directly in Petri dishes on a flat and level surface. When solidified, store the plates at 4°C for up to 1 month.

Top DNB agar

Reagent	Final concentration	Amount
DNB powder	0.8 g/L	0.8 g
Bacto Agar	7 g/L	7 g
ddH ₂ O	n/a	Up to 1 L
Total	n/a	1 L

Adjust to pH 7.4 with NaOH 2M. Autoclave. Add 2 mM CaCl₂ and 3 mM MgCl₂. Use immediately.

Optional: store at room temperature before adding the salts and melt when needed, either in a microwave with 10–20 s pulses at 700 W until liquid (making sure that the medium does not boil over), or by a second autoclaving cycle. Add 2 mM CaCl₂ and 3 mM MgCl₂.

CaHepes buffer

Reagent	Final concentration	Amount
HEPES powder	25 mM	5.96 g
ddH ₂ O	n/a	Up to 1 L
Total	n/a	1 L

Adjust to pH 7.6–7.8 with NaOH 2M. Autoclave then add 2 mM CaCl₂. Store at room temperature for up to 1 month.

CaCl₂ stock solution

Reagent	Final concentration	Amount
CaCl ₂ ·2H ₂ O	2 M	88.21 g
ddH ₂ O	n/a	Up to 300 mL
Total	n/a	300 mL

Stir until completely dissolved and filter with 0.22 μm pore size. Store at room temperature for up to 12 months.

MgCl₂ stock solution

Reagent	Final concentration	Amount
MgCl ₂ ·6H ₂ O	3 M	182.97 g
ddH ₂ O	n/a	Up to 300 mL
Total	n/a	300 mL

Stir until completely dissolve and filter with 0.22 μm pore size. Store at room temperature for up to 12 months.

NaOH stock solution

Reagent	Final concentration	Amount
NaOH	2 M	16.0 g
ddH ₂ O	n/a	Up to 200 mL
Total	n/a	200 mL

Stir until completely dissolved. Store at room temperature for up to 12 months. Prepare and handle the solution under a chemical hood and wear lab coat, gloves and goggles.

NaCl stock solution

Reagent	Final concentration	Amount
NaCl 99.5%	100 g/L	5 g
ddH ₂ O	n/a	Up to 50 mL
Total	n/a	50 mL

Stir until completely dissolved and filter with 0.22 μm pore size. Store at room temperature for up to 12 months.

Kanamycin stock solution

Reagent	Final concentration	Amount
Kanamycin sulfate	50 mg/mL	2.5 g
ddH ₂ O	n/a	50 mL
Total	n/a	50 mL

Stir until completely dissolved and filter with 0.22 μm pore size. Store at –20°C for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Normalization by SYBR green labeling

⌚ Timing: 20 min

The SYBR Green assay that we describe here allows to quickly determine, before the experiment and regardless of plaque formation capacities, the number of *B. bacteriovorus* present in different cultures. This allows sample normalization for the comparison of predation dynamics (predator proliferation and/or prey killing).

⚠ **CRITICAL:** The cleared culture must be checked by microscopy prior to the SYBR Green assay to verify the status of the predators. They should be actively swimming and there should be almost no prey or bdelloplast left ([troubleshooting 1](#) and [2](#)).

1. Start an overnight culture from a double layer plate ([before you begin](#), step 7) or from a revived clone ([before you begin](#), step 8).
2. Turn on the plate reader and prepare the protocol on the plate reader controlling software as follows:
 - a. Pre-set temperature: 25°C.
 - b. Continuous shaking (double orbital shaking, frequency 282 cpm, 15 min).
 - c. SYBR Green fluorescence read: 490 nm excitation, 520 nm emission, gain set with the Standard DNA curve (see [before you begin](#), step 10).
 - d. Absorbance read at 600 nm (OD₆₀₀).
3. For each cleared culture, prepare replicates by transferring 198 μL into 3 different wells of a black 96-well plate with transparent flat bottom.
4. With gloves and protected from light, add 2 μL of SYBR Green from the pre-diluted stock (1:30, see [before you begin](#), step 9).
5. Quickly load the plate without lid inside the plate reader.
6. For each culture, calculate the average SYBR Green fluorescence value due to *Bdellovibrio bacteriovorus* by subtracting from the average total SYBR Green fluorescence value the average SYBR Green fluorescence value due to *E. coli* (determined from the OD measures and the prey standard curve, see [before you begin](#), step 11). Using the predator standard curve (see [before you begin](#), step 12), the number of *B. bacteriovorus* cells can be extrapolated for each condition.

Note: When the standard deviation of SYBR Green fluorescence represents more than 15% of the mean from the triplicate wells, exclude the outlier to calculate the mean.

Prey killing and predator proliferation assay

⌚ Timing: 18 h

Quantification of predatory parameters is achieved upon an automated killing assay procedure, which allows live monitoring of both prey decay and predator proliferation, using absorbance and

fluorescence measurements, respectively. Drugs or other chemicals, as well as mutations in either the prey or the predator, can be included to test their effect on these two aspects of predation.

7. Following the SYBR Green assay, the killing kinetics of the *Bdellovibrio* predator is tested using a killing assay in a 96-well plate. The use of the mCh *Bdellovibrio* strain in which the mCherry fluorescent protein is produced constitutively, allows for monitoring of predator growth over time. [Before you begin](#), set the plate reader protocol for the killing assay as follows:
 - a. Temperature: 30°C
 - b. Continuous shaking (double orbital shaking, frequency 282 cpm).
 - c. Absorbance read at 600 nm (OD₆₀₀)
 - d. mCherry fluorescence read: 587 nm excitation, 610 nm emission, gain 70.

Note: Since the fluorescence values might differ between plate readers, the gain should be optimized in your setup as explained in the “[before you begin](#)” section, step 10.

- e. Intervals between acquisitions: 20 min
 - f. Total kinetics duration: 18 h
8. Once the plate reader is set, prepare a black 96-well plate with transparent flat bottom by adding first the medium, i.e., DNB supplemented with 2 mM CaCl₂ and 3 mM MgCl₂, then the prey suspension (final OD₆₀₀ = 1), and finally the *B. bacteriovorus* predator. We recommend a concentration of 5–10 × 10⁷ cells/mL, which corresponds to approximately 20–40 μL of a clear lysate at 6000 units obtained in the SYBR Green assay (“[before you begin](#)” section, step 12). The recommended final volume is 300 μL per well.

△ CRITICAL: When killing kinetics of several *B. bacteriovorus* strains are compared, the volume of *B. bacteriovorus* inoculum must be adjusted according to the results in the SYBR Green assay in order to engage the same initial amount of predators.

Note: For this step, we recommend using repetitive pipettes to ensure accurate liquid transfer and reproducibility over technical and biological replicates.

Note: If antibiotics are used in the experiment, the prey must carry the same resistance as the predator. From our experience, *B. bacteriovorus* strains carrying replicative plasmids do not lose their plasmid when selective antibiotics have been removed for the last overnight culture only. In order to avoid any possible impact of the selective pressure on the comparison of predation efficiencies between strains carrying plasmids with different antibiotics resistance cassettes and/or strains without replicative plasmid, we usually subculture the antibiotic-resistant *B. bacteriovorus*, from a starter culture containing the antibiotics, in medium without antibiotics before the killing assay. In this case, a wild-type *E. coli* strain can be used as prey for both the subculture and the assay.

9. In addition to the predatory mix in 7., a couple of controls must be added in the same 96-well plate:
 - a. the prey alone in DNB supplemented with salts to both monitor the predation-independent lysis occurring during the experiment, and as a blank for data normalization.
 - b. the *Bdellovibrio* predator alone to monitor the fluorescence signal in absence of prey.

△ CRITICAL: In these control wells, keep the same density of predator or prey cells as used in the predatory mix in a 300 μL final volume.

10. Quickly load the 96-well plate without lid in the plate reader and start the killing kinetics for 18 h using the settings described in 7. [Troubleshooting 6](#) and [7](#).

Table 1. Example of raw data measured on a plate reader

Time	Temperature	A1	A2	A3	A4	A5	A6	...
0:00:00	30.1	696	728	727	674	710	729	780
0:10:00	30.0	679	714	705	641	677	716	758
00:20:00	30.0	633	706	691	623	675	691	761
00:30:00	29.9	626	662	651	613	659	670	707

Quantitative analysis of prey killing and predator proliferation

⌚ Timing: 30 min

11. Formatting the input file. The provided pipeline is designed to work with Excel files containing in one sheet the OD_{600} data recorded in the plate reader and in another sheet the fluorescence data. All data should be formatted following the provided template (Table 1 and <https://github.com/geraldinelaloux/CuRveR/tree/main/Sample%20data>).

Note: The recorded temperature data are not useful in the analysis.

12. Launch Rstudio
13. Execute CuRveR by running "CuRveR::run_curver()" in the R console. This will open the GUI used for the following steps (Figure 5A).
14. Load the data into the software:
 - a. Select the data filetype
 - b. If the data are in XLSX format, select whether data are split across several files or several sheets within the same file. In the latter case, select your XLSX file.
 - c. Select the number of signals (e.g., mCherry, OD_{660}) to analyze.
 - d. Select the file/sheet where the data are located and name the corresponding signal. Make sure the data are formatted as shown in Table 1.
 - e. Press load to import your data and get an overview of your experiment.
15. Classify replicates into conditions and blanks. If the experiment includes different conditions and blanks, simply drag and drop each replicate and the corresponding blanks using the provided interface.
16. Select data range. In some cases, not all time points should be fitted to a sigmoid curve, e.g., when bacteria start dying after the exponential phase. Only the portion of the data that are following the shape of a sigmoid curve should be selected.
17. Select the model (currently only "Richard" is available), the loss function (how close the model parameters are from the data: Least Absolute Deviation (LAD) or Ordinary Least Square (OLS)) and the optimization method (how to optimize the parameters to minimize the loss function: currently only Genetic Algorithm (GA)), then press fit.

Parameters obtained with the "Richard" model are extracted from this modification of the generalized logistic equation:

$$P(t) = P_{min} + \frac{P_{max} - P_{min}}{1 + e^{\frac{4 \times r_{max}}{t-s}}}$$

where $P(t)$: Population at time t (number of cells, OD_{600} or fluorescence unit), P_{min} : Minimal population, lower asymptote (number of cells, OD_{600} or fluorescence unit), P_{max} : Maximal population, upper asymptote (number of cells, OD_{600} or fluorescence unit), r_{max} : Maximum rate, when $t = s$, positive if the population grows (i.e., growth rate), negative if the population declines (i.e., killing rate) (number of cells, OD_{600} or fluorescence unit), s : Inflection point, time when the rate (i.e., the value of the derivative of the curve) is an extremum (hours, h), e.g., if s is equal to 4, r_{max} is reached at 4 h.

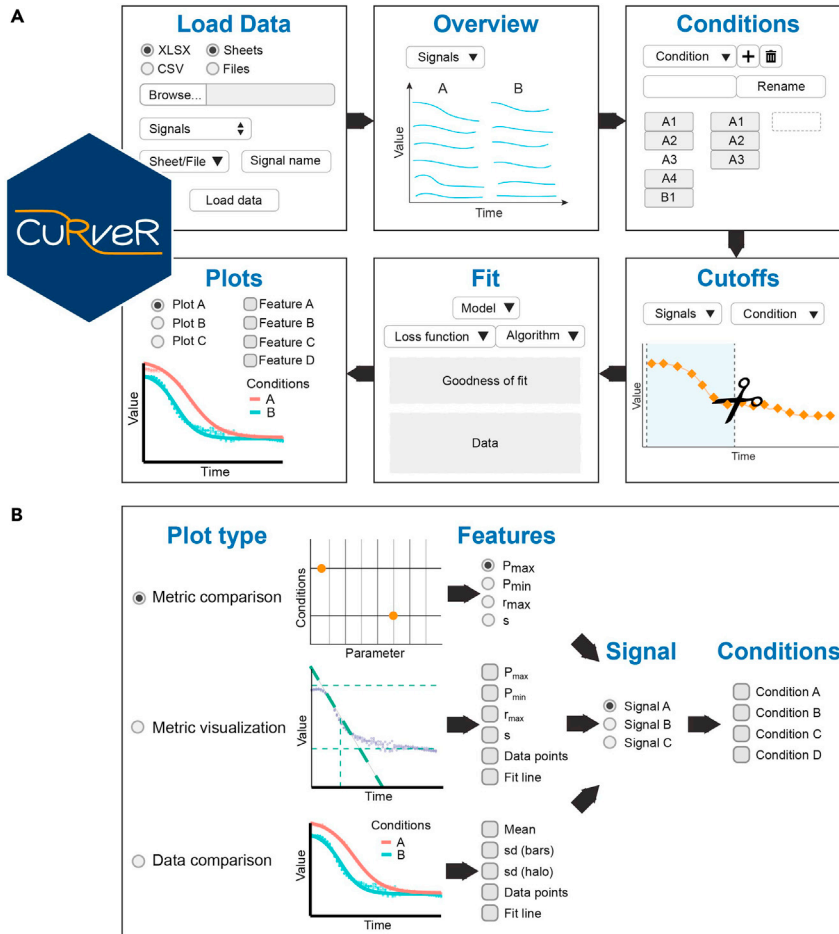


Figure 5. Overview of the CuRveR application

(A) A pipeline to analyze plate reader data.

(B) Several plotting options and features in CuRveR.

Multiple metrics regarding the goodness of fit are provided as well as a dataframe containing the raw, the fitted data and the model parameters. The two easiest metrics to check are the variance explained (VE_{cv}) and Willmott et al.'s refined index of agreement (dr) (formulas for the metrics are available in Li (2017) and implementation of these are available in the CuRveR source code). These two metrics range from 0 (bad fit) to 100 (perfect fit). [Troubleshooting 8](#).

Note: Parameters are extracted on a condition-basis (where technical replicates are pooled for each condition) rather than on a well-basis. This results in a higher precision, especially when there are substantial differences between replicates or experiments. Precision is indeed proportional to the square root of the number of degrees of freedom, which is higher when replicates are pooled than when taken individually

18. Plot your data. Several options are available ([Figure 5B](#)).

EXPECTED OUTCOMES

The expected outcomes after step 10 are the following. In wells containing prey only, the OD₆₀₀ values should plateau over time with no significant decrease or increase (if not, see [troubleshooting 5](#) and [6](#)). In contrast, wells containing both predator and prey cells will display a decrease in OD₆₀₀

values starting from 3 to 4 h after the start of the reading (when using a predator strain with wild-type predation dynamics), indicative of *B. bacteriovorus*-induced lysis of *E. coli* and corresponding to the average duration of one infection cycle (i.e., prey invasion, intra-periplasmic growth, release of newborn predator cells and lysis of the bdelloplast). In parallel, fluorescence associated with the *B. bacteriovorus* cells will increase, showing growth and proliferation of predator cells.

Assessing predator efficiency relies on several specific parameters that can be extracted by our CuRveR package (see steps 11–18). In this section, we provide simple examples of the use of CuRveR to compare several predator strains or to assess predation in the presence of specific compounds. CuRveR is used both to plot absorbance and fluorescence data over time, and to extract prey killing and/or predator proliferation parameters in a user-friendly and automated manner.

As a first example, we compared the killing efficiency of two predator strains: the mCh *Bdellovibrio* strain, which produces mCherry constitutively and allows us to monitor predator growth using fluorescence increase as a proxy (see below), and the WT *B. bacteriovorus* strain. In Figure 6A, we show that the mCh *Bdellovibrio* killing parameters (right panel) are similar to those of the WT strain (left panel). For instance, the time to reach the maximum killing rate (given by the s parameter) is 4 h 21 min and 4 h 34 min for the WT and the mCh *Bdellovibrio* strains, respectively. Based on these results, we concluded that the mCh *Bdellovibrio* strain can be used as a WT strain regarding predation dynamics in subsequent experiments.

Since the initial concentration of predators is important for predation efficiency (Varon and Shilo, 1968), we decided to determine predation parameters using gradual concentrations of mCh *Bdellovibrio* cells (Figures 6B and 6C). Results showed that while the r_{max} is similar across conditions, ranging from -0.041 to -0.049 $OD_{600}\cdot h^{-1}$ (killing rate), and from 257 to 295 $mCh_{fluO}\cdot h^{-1}$ (growth rate), the s parameter is negatively correlated with the initial concentration of *B. bacteriovorus* cells. In other words, the r_{max} is reached sooner when higher initial concentrations of predators are used. Interestingly, we also found that regardless of the initial density of *B. bacteriovorus* cells, the maximal killing rate ($OD_{600} r_{max}$) is reached on average 2.47 h prior the maximal growth rate ($mCh_{fluO} r_{max}$). This means that the *B. bacteriovorus*-mediated prey killing is not correlated in time with the growth of the predator, consistent with the non-binary proliferation mode of *B. bacteriovorus*, i.e., the production of more than two daughter cells per generation (Laloux, 2020).

To go further, we decided to explore the effects of different compounds on the predation parameters. Sodium chloride (NaCl) and kanamycin, which have been shown to impair *Bdellovibrio* predation (Varon and Shilo, 1968), were used in this assay (Figure 7). As expected, both NaCl (Figures 7A and 7B) and kanamycin (Figures 7C and 7D) inhibit *Bdellovibrio* predation in a dose-dependent manner. In both cases, the addition of gradual concentrations of NaCl or kanamycin decreases the r_{max} , and increases s values. For instance, the addition of 2% of NaCl or 25 $\mu g\cdot\mu L^{-1}$ of kanamycin decreases by 7-fold the r_{max} values and increases s values by about 5–6 h. Furthermore, while the *E. coli* prey survival (i.e., $OD_{600} P_{min}$) is positively correlated with NaCl and kanamycin concentrations (Figures 7A and 7C), predator proliferation ($mCh_{fluO} P_{max}$) is negatively correlated with the dose of these compounds (Figures 7B and 7D).

Thus, across these different examples, we demonstrate that our killing assay protocol and the use of our CuRveR package allow for the extraction of key parameters of prey killing and/or predator proliferation in a sensitive, robust and automated manner.

LIMITATIONS

Viability. SYBR Green is a membrane-permeant dye. Therefore, dead *B. bacteriovorus* also contribute to the total SYBR Green fluorescence value. It is thus critical to observe the status of the predators before performing the SYBR Green assay and cancel it if the viability of the population

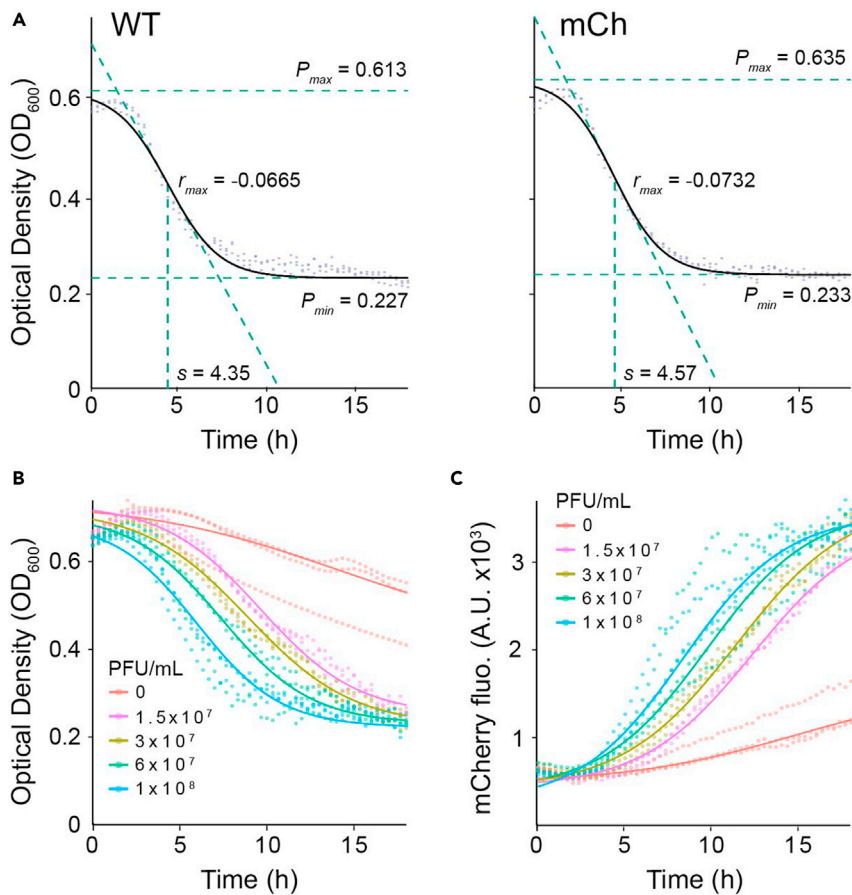


Figure 6. Expected outcomes: killing assay using the mCh *Bdellovibrio* strain

(A) Killing efficiency of the mCh *Bdellovibrio* strain (right) is similar to the WT strain (left). MG1655 *E. coli* preys were co-incubated with the indicated predator for 18 h at 30°C. The evolution of OD₆₀₀ as well as the metrics provided by CuVrR i.e., P_{max} and P_{min} (maximal or minimal population predator population, respectively); r_{max} (killing rate); s (time point when r_{max} is maximum) are shown. The black line corresponds to the fitting model.

(B and C) Killing efficiency depends of the initial predator cells concentration. MG1655 *E. coli* preys were co-incubated with the indicated amount of mCh *Bdellovibrio* predators (in PFU/mL, calculated via the Sybr Green assay) for 18 h at 30°C. The evolution of OD₆₀₀ (B) and mCherry fluorescence (C) are shown. Colored lines correspond to the fit and dots are values obtained from three replicates.

is uncertain. A potential amelioration of this assay would be to couple the SYBR Green assay with a membrane-impermeant dye (e.g., propidium iodide) to evaluate the cellular viability (e.g., (Duedu and French, 2017)).

Fluorescence spectrum compatibility with SYBR Green. The excitation-emission spectrum of SYBR Green overlaps the spectrum of the Green Fluorescent Protein. Hence, the number of bacteria carrying GFP fusions (or other fluorescent proteins with overlapping spectra) cannot be assessed with the SYBR Green assay. A possible alternative is the use of another cell-permeant nucleic acid dye with different excitation-emission peaks (e.g., Hoechst 33342 or SYTO dyes). In this case, a new standard curve has to be prepared for each dye. The compatibility of fluorescence spectra also depends on the plate reader fluorescence excitation and emission mode (monochromatic or filter-based), and this should be verified for each combination of dye, fluorescent protein, and plate reader.

Strain construction and fluorescence spectrum compatibility with mCherry. When the proliferation of different *B. bacteriovorus* strains is compared using the fluorescence-based kinetics assay

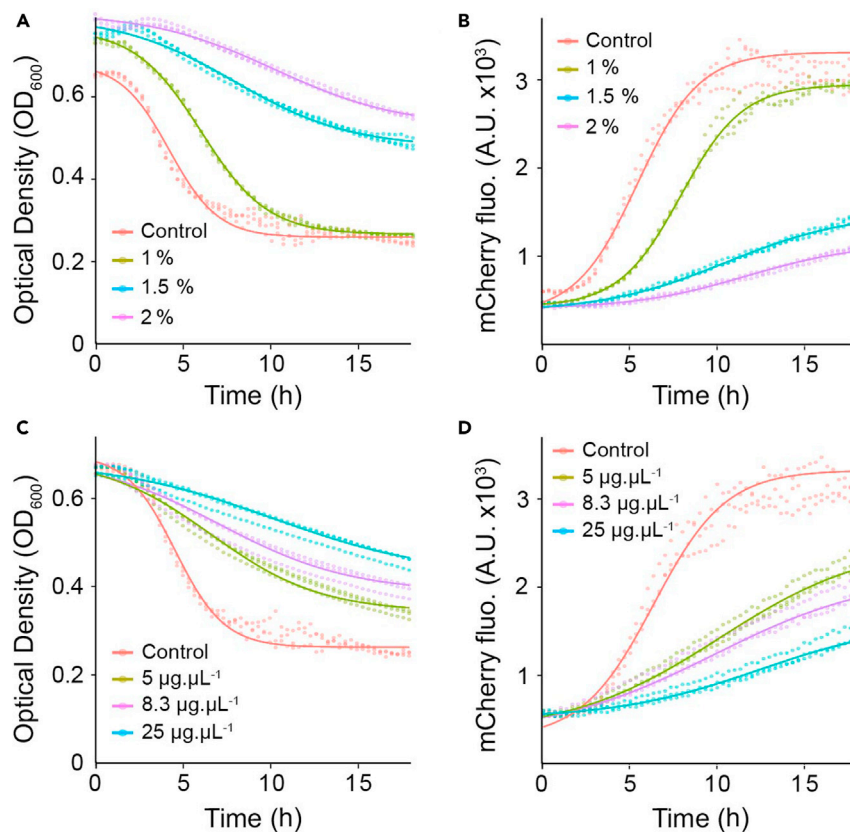


Figure 7. Expected outcomes: impact of compounds on predation

(A–D) The mCh *Bdellovibrio* strain was co-incubated with MG1655 *E. coli* preys in presence of different concentrations of NaCl (A and B) or kanamycin (C and D). The evolution of OD₆₀₀ (left) and mCherry fluorescence (right) are shown. Colored lines correspond to the fit and dots are values obtained from three replicates.

presented here, they would need to be constructed with the same background allowing constitutive fluorescence (for instance *bd0063-0064::pBioFab-mcherry*). Fluorescence compatibility must be taken into account, especially when using strains that produce fluorescently-tagged proteins for other purposes (e.g., imaging). In such a case, it is important to consider changing either the fluorescent proteins used for tagging the protein of interest or the one used for monitoring proliferation. The excitation and emission settings for fluorescence acquisition on the plate reader must also be adapted accordingly.

TROUBLESHOOTING

Problem 1

Preys are not completely lysed in the overnight culture, i.e., remaining uninfected prey cells or bdelloplasts are observed (refers to the “*liquid culture of B. bacteriovorus*” section).

Potential solution

Incubate the tube at 30°C for a few more hours. Filtration is not recommended because this process causes a loss of *B. bacteriovorus*.

Problem 2

All prey cells are lysed overnight but the predators are not swimming or swimming slowly in the overnight culture (refers to the “*liquid culture of B. bacteriovorus*” section).

Potential solution

The predators have probably been starving for some hours. It is recommended to start a fresh culture and check predators at the microscope more frequently.

Problem 3

The data for plotting standard curves do not align well with linear fits (refers to the “[preparation of the SYBR green standard curves](#)” section).

Potential solution

For all three standard curves, we recommend to calculate the R^2 , which indicates the goodness of the linear fit and therefore constitutes a very useful cue to assess the quality of the corresponding standard curve. In our hands, R^2 values were always > 0.9 , but values > 0.85 should be considered acceptable. The quality of the final predator curve can also be evaluated by testing the reproducibility of the correspondence between SYBR Green values and the number of PFU.

Considering that SYBR Green is widely used for the linearity between the resulting fluorescence signal and the amount of DNA stained (e.g., in RT-qPCR), we expect no major issue when plotting the DNA standard curve with the present protocol. However, it is important to verify the sensitivity range of the NanoDrop or the other device used to measure DNA concentration to avoid out-of-range values that would introduce a bias in the linear fit. Discrepancies between the estimated DNA concentrations (based on the known concentration of the starting DNA sample) and the measured concentrations should raise the attention of the user on that aspect. We explained in the corresponding sections how to deal with outliers for each curve.

If reproducibility or linearity issues occur with the preparation of the prey or predator curve, we recommend repeating the experiment using fresh material and making sure that colonies or plaques are counted carefully. In line with the membrane-permeability of SYBR Green and the linearity of DNA staining mentioned above, we repeatedly found that a linear fit was the most appropriate model for our prey and predator curves. However, we do not exclude that other types of fit may better represent the relationship between SYBR Green values and the number of bacteria. The ultimate goal here is to verify the reproducibility of these correlations, so they can be used to normalize samples before killing assays or other experiments that benefit from controlled predator input.

Problem 4

Little or no prey killing occurs during the experiment (refers to the “[prey killing and predator proliferation assay](#)” section).

Potential solution

In those cases where killing kinetics seems abnormally low, two main reasons can be envisioned. First, the initial viability and/or the density of the *B. bacteriovorus* strain was not good for the experiment. Since viability cannot be assessed using the SYBR Green assay (see “[limitations](#)” section), we recommend repeating the experiment using a fresh initial co-culture (see “[before you begin](#)” section, step 7). If the density of the *B. bacteriovorus* strain seems too low, perform the killing experiment using different concentrations of *B. bacteriovorus* cells. Another reason for low killing kinetics could be the degradation of the initial prey suspension upon storage. We therefore recommend using fresh prey suspension whenever possible to ensure proper killing. Alternatively, the user may want to verify the absence of contaminants by plating the non-killed sample on LB plate and observing the colony aspect and/or cell morphology at the microscope. Here also, starting with a fresh prey suspension should solve the issue.

Problem 5

Prey cells lyse during the killing experiment (i.e., OD_{600} decreases substantially over time) in absence of the predator (refers to the “[prey killing and predator proliferation assay](#)” section).

Potential solution

During a killing assay, cell death can occur for multiple reasons. This could be due to the initial degradation of the prey suspension (see “[problem 3](#)”), or the use of additional compounds in the medium. A simple solution to this issue is to adjust the composition of the medium according to the current research. Another reason for cell death is phototoxicity due to the acquisition set up. If cells are too sensitive to illumination, try increasing the interval time between acquisitions. We also noticed that the total volume per well is critical to avoid predation-independent lysis of the prey suspension. While 300 μ L is optimal in our hands, testing a small range of volumes around this value may help decreasing prey death.

Problem 6

Prey cells grow during the killing experiment (i.e., OD_{600} increases substantially over time) in absence of predator (refers to the “[prey killing and predator proliferation assay](#)” section).

Potential solution

While DNB medium and 300 μ L work optimally in our hands and do not favor prey growth ([Figure 6B](#)), we recommend testing a small range of total volumes per well around this value, and/or using another medium with lower or no nutrient content, such as CaHEPES, if such problem arises.

Problem 7

Unable to install CuRveR (refers to the “[installing CuRveR](#)” section).

Potential solution

Try running again “`devtools::install_github("geraldinelaloux/curver")`”. In case it does not work, try a manual installation with “`install.packages("name_of_missing_package")`” of missing packages if any. If unsuccessful again, search for similar issues at <https://github.com/Giatomo/CuRveR/issues> or post a new one there with the description of the problem and the error message.

Problem 8

Goodness of fit is bad, e.g., variance explained (VEcv) or Willmott et al.’s refined index of agreement (dr) are < 80 (refers to “[quantitative analysis of prey killing and predator proliferation](#)” section).

Potential solution

Make sure that data are correctly formatted and that the selected range of data points has a sigmoid shape. In other cases, search for similar issues at <https://github.com/Giatomo/CuRveR/issues> or post a new one there.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Géraldine Laloux (geraldine.laloux@uclouvain.be).

Materials availability

This study did not generate new unique strains or reagents.

Data and code availability

The datasets and the code generated during this study are available on Github: <https://github.com/geraldinelaloux/CuRveR> and have been deposited on Zenodo: <https://doi.org/10.5281/zenodo.5767031>. Original data for [Figures 6](#) and [7](#) are available on Github: and have been deposited on Zenodo: <https://doi.org/10.5281/zenodo.5767031>.

ACKNOWLEDGMENTS

We are grateful to all members of the Laloux lab for discussions and thorough testing of the protocol. This work was supported by the European Commission (ERC starting grant PREDATOR 802331). T.L. is a FRIA grantee of the F.R.S.-FNRS. J.K. is a Research Fellow of the F.R.S.-FNRS. G.L. is a Research Associate of the F.R.S.-FNRS.

AUTHOR CONTRIBUTIONS

Conceptualization, O.R., T.L., Y.S., and G.L.; Methodology, all authors; Resources, G.L.; writing – original; Draft preparation, all authors; Writing – review and editing, all authors; Supervision and funding acquisition, G.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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