Fourth Edition of Mycobacteria Protocols

Measuring efflux and permeability in mycobacteria

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Abstract

Mycobacteria are intrinsically resistant to most antimicrobials, which is generally attributed to the impermeability of their cell wall that considerably limits drug uptake. Moreover, like in other pathogenic bacteria, active efflux systems have been widely characterized from diverse mycobacterial species in laboratory conditions, showing that they can promote resistance by extruding noxious compounds prior to their reaching their intended targets. Therefore, the intracellular concentration of a given compound is determined by the balance between permeability, influx and efflux.

Given the urgent need to discover and develop novel antimycobacterial compounds in order to design effective therapeutic strategies, the contributions to drug resistance made by the controlled permeability of the cell wall and the increased activity of efflux pumps, must be determined. In this chapter, we will describe a method that allows: i) the measuring of permeability and the quantification of general efflux activity of mycobacteria, by the study of the transport (influx and efflux) of fluorescent compounds, such as ethidium bromide; and (ii) the screening of compounds in search of agents that increase the permeability of the cell wall and efflux inhibitors that could restore the effectiveness of antimicrobials that are subject to efflux.

Keywords: *Mycobacterium*, permeability, efflux pumps, efflux inhibitors, ethidium bromide, fluorometry, accumulation assay, efflux assay.

1. Introduction

Bacterial efflux pumps are membrane proteins that can transport a broad range of substrates, including antimicrobials, from the cytoplasm to the exterior of the cell, fueled by the energy provided by ATP hydrolysis or the transmembrane proton gradient [1]. Consequently, increased expression of efflux pump genes confers a low-level resistance phenotype and it has been postulated that, under these conditions, bacteria have greater chances of acquiring chromosomal mutation(s) conferring stable and heritable higher levels of drug resistance [2, 3]. Mycobacteria, similarly to other bacterial genera, have a large number of active efflux pumps that have a role in low-level resistance to drugs such as tetracycline, aminoglycosides, fluoroquinolones [4, 5], macrophage induced drug tolerance to isoniazid and rifampicin [6], and other physiological processes such as redox potential and bacterial and colony morphology [7, 8]. Therefore, efflux pumps must be considered in drug discovery programs [5, 9-11] as their inhibition could contribute to new therapeutic strategies for tuberculosis and drug-resistant tuberculosis [12, 13].

Several methods have been used to detect the transport of radiolabeled, metal-labeled or fluorescent substrates through the bacterial cell wall, which can be correlated with altered permeability and/or efflux activity of the bacteria [7, 14-21]. We have developed a fluorometric method for the assessment of permeability and efflux activity in mycobacterial strains (reference strains, mutants impaired in efflux or permeability and clinical isolates) that uses ethidium bromide (EtBr), a common efflux substrate [18, 20-25]. EtBr has been shown to be a particularly suitable probe for these studies, since it emits weak fluorescence in aqueous solution (extracellular medium) and becomes strongly fluorescent as it accumulates in nonpolar and hydrophobic environments, such as the periplasmic space of Gram-negative bacteria or cytoplasm of Gram-positive bacteria [15]. This methodology allows easy and accurate detection and quantification of the transport of EtBr through the bacterial cell wall. Kinetics of EtBr accumulation and efflux can be assayed separately, hence examining the contribution of both processes to EtBr transport [18, 20-25].

Evaluation of accumulation or efflux of EtBr is performed on a real-time basis, with the possibility of testing many samples simultaneously and at different experimental conditions with the same set of bacterial suspensions, hence maximizing reproducibility and significance. Moreover, it also allows the screening of compounds as potential efflux inhibitors or cell wall permeabilizers that could be used in the future as adjuvants of antimycobacterial therapy [12].

The EtBr accumulation assay assesses the ability of the strain to handle increasing concentrations of EtBr. The concept behind this assay is that when the ability of the efflux systems to extrude EtBr is exceeded, EtBr will accumulate over a period of time, ultimately reaching a quasi steady-state. Figure 1 shows an example of this type of assay with *M. bovis* BCG. The accumulation of EtBr is relatively stable (no significant increase in fluorescence over time) at the concentration of 0.125 μg/mL, over a period of 60 min. In the presence of an efflux inhibitor (Fig. 2), such as chlorpromazine (CPZ), thioridazine (TZ), or verapamil (VP), accumulation of EtBr increases due to the inhibition of efflux in comparison with non-treated cells.

In an efflux assay (Fig. 3, adapted from ref. 25) bacterial cells are first de-energized with an efflux inhibitor and loaded with EtBr during a period of time (usually 1 h) and then placed in an EtBr-free solution. This assay allows the evaluation of efflux activity under different conditions: i) in the presence of a carbon source, such as glucose, efflux pumps are able to transport EtBr out and so fluorescence decreases quickly with time; and ii) in the presence of an efflux inhibitor (such as TZ or VP) efflux is abolished and there is no decrease in fluorescence.

In addition, accumulation and efflux assays can be used to quantify and compare EtBr transport activity between mycobacterial strains (*e.g.*, clinical isolates or mutants impaired in efflux or permeability). For example, Figure 4 (adapted from ref. 25) presents efflux of EtBr in two *M. bovis* BCG strains: *M. bovis* BCG (wild-type) and BCG-INH^R (an BCG derivative resistant to isoniazid by the activity of efflux pumps). The BCG-INH^R strain showed an increased efflux of

EtBr (evidenced by a faster decrease in fluorescence) relative to the wild-type strain, indicating that efflux pump activity is associated with the efflux of EtBr in *M. bovis* BCG [25].

The protocols described in the previous edition of this publication have been used in several other reports, in which increased accumulation of EtBr was found indicative of increased cell wall or membrane permeability. In *M. tuberculosis* mutants defective in enzymes responsible for ligating arabinogalactan to peptidoglycan, an increase in EtBr accumulation rate was detected. This was attributed to a general increase in cell wall permeability, rather than to changes in efflux pump activity [26]. Similarly, an *M. tuberculosis* mutant defective in a putative iron dicitrate-binding protein accumulated significantly more EtBr than wild-type cells, independently of the activity of efflux pumps [27]. In *Mycobacterium marinum*, a series of suppressor strains carrying defects in diverse genes related with the function of Esx-1 secretion system, were shown to have higher levels of EtBr accumulation, hence demonstrating that such alterations had resulted from increased cell wall permeability [28]. Bacteriocin AS-48 kills *M. tuberculosis* and other mycobacteria by interfering with cell membrane functions, and this was demonstrated since, in the presence of AS-48, EtBr accumulation increased notably in comparison with non-treated cells [29].

Also, these protocols were used to test whether diverse compounds could have any efflux inhibitory activity. It was hypothesized that pyronaridine, an antimalarial drug for which antituberculosis activity was recently reported [30], could function as efflux inhibitor since it modulates the activity of P-glycoprotein. However, EtBr accumulation experiments could not demonstrate any significant activity of pyronaridine as efflux inhibitor in *M. tuberculosis* [30]. In another report, a series of inhibitors of the sole signal peptidase in *M. tuberculosis* did not significantly alter accumulation of EtBr, demonstrating their specificity against this target [31]. Natural product carvacrol has both antimicrobial and efflux inhibitory activities against several bacterial species. In *M. tuberculosis*, EtBr accumulation was greatly increased in the presence of carvacrol and derivatives, being this effect notably higher than that produced by VP, hence demonstrating efflux inhibitory activity of this series of compounds [32].

Finally, other reports have used these protocols with the aim of assessing general efflux activity of mycobacterial strains. VP, TZ, CPZ, flupentixol and haloperidol increased accumulation and reduced efflux of EtBr in a set of multidrug and extensively drug resistant strains of *M. tuberculosis*, hence demonstrating its activity as efflux inhibitors in addition of being ion channel blockers [33]. Similarly, VP, TZ and CPZ were used to demonstrate innate efflux activity in a series of clinical strains of *M. tuberculosis* having different resistance-conferring mutations [34].

In summary, these methods can be used to evaluate influx and efflux of fluorescent substrates by mycobacteria, providing valuable information on cell wall and membrane permeability, efflux inhibitory activity of compounds and, thus, are useful to predict the contribution of permeability and efflux to the drug resistance phenotype.

2. Materials

2.1 Determination of Minimum Inhibitory Concentration (MIC)

1. Mycobacterial strains. Commonly, laboratory reference strains such as *M. tuberculosis* H37Rv can be used for these protocols. Other strains such as mutants deleted in genes encoding efflux pumps, clinical isolates or other mycobacterial species can be used as well (*see* **Note 1**).

2. 7H9 medium plus Tween: dissolve 4.7 g Middlebrook 7H9 broth medium in 900 mL water, add Tween 80 to 0.05% w/v. Autoclave for 15 min at 121°C and add 100 mL of oleic acid-albumin–dextrose–catalase supplement (OADC) (Becton Dickinson) (*see* **Note 2**).

3.7H9 medium plus glycerol: dissolve 4.7 g Middlebrook 7H9 broth medium in 900 mL water, add glycerol to 0.5% w/v. Autoclave for 15 min at 121°C and add 100 mL of oleic acid-albumin–dextrose–catalase supplement (OADC) (Becton Dickinson) (*see* **Note 2**).

4. 96-well microtiter plates.

5. EtBr (3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide): stock solution of 1 mg/mL in deionized, sterile water. Store at 4°C and protect from light (*see* **Note 3**).

6. CPZ: stock solution of 1 mg/mL in deionized, sterile water. Store at -20°C and protect from light.

7. TZ: stock solution of 1 mg/mL in deionized, sterile water. Store at -20°C and protect from light.

8. VP: stock solution of 10 mg/mL in deionized, sterile water. Store at -20°C and protect from light.

9. Resazurin: stock solution of 0.1 mg/mL in deionized, sterile water. Store at 4°C and protect from light (*see* **Note 4**).

2.2 Preparation of Cultures

1. 7H9 medium: *see* Subheading 2.1, items 2 and 3.

2. Phosphate buffered saline (PBS): dissolve 8 g NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of deionized water. Adjust the pH to 7.4 with HCl and the volume to 1 L with additional deionized water. Sterilize by autoclaving for 15 min at 121°C (*see* **Note 5**).

3. PBS-Tw: PBS (*see* Subheading 2.2 item 2) plus Tween 80 0.05% (w/v).

2.3 Determination of the Steady-State EtBr Concentration

1. Glucose: 20% (w/v) stock solution in deionized, sterile water. Store in aliquots at 4°C (*see* **Note 6**).

2. 0.2 mL microtubes.

3. PBS: *see* Subheading 2.2, item 2.

4. PBS (*see* Subheading 2.2 item 2) plus 0.4% (w/v) glucose.

5. EtBr: *see* Subheading 2.1, item 5.

6. Fluorometer (*e.g.*, Rotor Gene™ 3000/6000) (*see* **Note 7**).

2.4 Demonstration of the Effect of Efflux Inhibitors and Cell Wall Permeabilizers on EtBr Accumulation

1. PBS: *see* Subheading 2.2, item 2.

2. Glucose: *see* Subheading 2.3, item 1.

3. PBS (*see* Subheading 2.2 item 2) plus 0.4% (w/v) glucose.

4. 0.2 ml microtubes.

5. EtBr: *see* Subheading 2.1, item 5.

6. Fluorometer (*e.g.*, Rotor Gene™ 3000/6000) (*see* **Note 7**).

7. CPZ: *see* Subheading 2.1, item 6.

8. TZ: *see* Subheading 2.1, item 7.

9. VP: *see* Subheading 2.1, item 8.

2.5 EtBr Efflux Assay

See Subheading 2.4, items 1–9.

3. Methods

3.1 Determination of Minimum Inhibitory Concentration (MIC)

Before starting any EtBr accumulation or efflux assay, the MIC of EtBr and compounds to be tested must be determined:

1. Grow mycobacterial strains at 37°C in 10 mL of 7H9 medium plus Tween until an OD_{600} of 0.6–0.8.

2. Dilute the mycobacterial cultures in 7H9 medium plus glycerol in order to obtain a final concentration of 10^5 CFU/mL (*see* **Note 8**).

3. Transfer 100 μL to the wells of a 96-well plate that contain 100 μL of each compound (*e.g.* EtBr and efflux inhibitors CPZ, TZ and VP) at concentrations prepared from two-fold serial dilutions in 7H9 medium plus glycerol.

4. Incubate the plates at 37°C for the appropriate period of time (*see* **Note 9**).

5. Add 30 μL of resazurin and incubate at 37°C for the appropriate period of time (*see* **Note 10**).

6. A change from blue to pink indicates reduction of resazurin and, therefore, bacterial growth. The MIC is defined as the lowest concentration of compound that prevents this color change [7, 35].

3.2 Preparation of Cultures

1. Grow mycobacterial strains (*see* **Note 1**) at 37°C in 50 mL (*see* **Note 11**) of 7H9 medium plus Tween until they reach OD_{600} of 0.6–0.8.

2. Centrifuge cultures at 3,000–4,000 × g for 10 min and discard the supernatant.

3. Wash the pellet in 25 mL of PBS-Tw (*see* **Note 12**) and adjust the OD_{600} to 0.8 with PBS (*see* **Note 13**).

3.3 Determination of the Steady-State EtBr Concentration

1. Add glucose to yield a final concentration of 0.4% (w/v) to the bacterial suspension prepared in Subheading 3.2 (*see* **Note 6**).

2. In 0.2 ml microtubes (*see* **Note 14**), prepare serial dilutions of EtBr from 0.125 to 4 μg/mL (do not exceed ¼ MIC) in PBS plus 0.4% (w/v) glucose in a volume of 50 μL per tube.

3. Aliquot 50 μL of bacterial suspension into 0.2 ml microtubes containing the serial dilutions of EtBr (*see* **Note 15**). A final volume of 100 μL should be obtained in each tube.

4. Include the following controls:

Control 1: add 50 μL of PBS to 50 μL of EtBr at the selected concentrations; no bacterial cells must be added.

Control 2: add 50 μL of PBS and 50 μL of the bacterial suspension with no EtBr.

5. Place the 0.2 mL microtubes in the Rotor-Gene fluorometer and program the instrument with the following settings: a temperature of 37°C; a unit of time for each measurement (cycle) of 60 s; the number of cycles necessary to obtain the total period of time (*i.e.*, 60 cycles to obtain a 60 min assay); and excitation and detection wavelengths of 530 nm and the 590 nm, respectively (*see* **Notes 7** and **16**).

6. Select the highest EtBr concentration that allows readable steady-state equilibrium without compromising bacterial viability, for use in demonstration of the effect of efflux inhibitors or permeabilizing agents on EtBr accumulation (*see* **Note 7**).

3.4 Determination of the Effect of Potential Efflux Inhibitors / Permeabilizing Agents on EtBr Accumulation

1. Prepare a dilution of EtBr in PBS plus 0.4% (w/v) glucose to reach two times the highest EtBr concentration that allows readable steady-state equilibrium without compromising bacterial viability, selected as described in Subheading 3.3. Transfer 47.5 μL of this EtBr dilution to 0.2 mL microtubes.

2. Add 5 μL of one compound (CPZ, TZ, VP or any other, *see* **Note 17**) at ¼ MIC.

3. Add 47.5 μL of bacterial suspension prepared as described in Subheading 3.2.

4. Include the following controls:

Control 1: add 50 μL of PBS to 50 μL of EtBr at the selected concentration; no bacterial cells must be added.

Control 2: add 50 μL of PBS and 50 μL of the bacterial suspension with no EtBr.

Control 3: mix 95 μL of PBS and 5 μL of efflux inhibitor.

Control 4: mix 47.5 μL of PBS, 47.5 μL of EtBr dilution and 5 μL of efflux inhibitor.

5. Place the 0.2 mL microtubes in the Rotor-Gene fluorometer and program the instrument with the following settings: a temperature of 37°C; a unit of time for each measurement (cycle) of 60 s; the number of cycles necessary to obtain the total period of time (*i.e.*, 60 cycles to obtain a 60 min assay); and excitation and detection wavelengths of 530 nm and the 590 nm, respectively (*see* **Notes 7** and **16**).

6. Select the compound resulting in the highest accumulation of EtBr by bacterial cells for being used in EtBr efflux assays.

3.5 EtBr Efflux Assay

1. Prepare 10 mL of bacterial suspension prepared as described in Subheading 3.2. Add EtBr (at the concentration selected in Subheading 3.3) and the compound selected in subheading 3.4 step 6. (*see* **Note 17**).

2. Mix and incubate at 25°C for 60 min (*see* **Note 18**).

3. Harvest the EtBr-loaded cells at 3,000–4,000 × g for 5 min at 4°C and discard the supernatant.

4. Resuspend to an OD_{600} of 0.8 in EtBr-free cold PBS (*see* **Note 13**).

5. Keep cells on ice until use in order to minimize EtBr efflux; otherwise, EtBr efflux would promptly start (*see* **Note 19**). Proceed to the next step immediately.

6. Aliquot 95 μL of cells into 0.2 mL microtubes (*see* **Note 20**).

7. Add glucose to yield a final concentration of 0.4% and add the efflux inhibitors at ¼ the MIC in order to obtain the following conditions: (a) Minimal efflux = bacteria without glucose + efflux inhibitor; (b) Baseline efflux = bacteria without glucose; (c) Maximal efflux = bacteria with glucose; and (d) Efflux inhibitor = bacteria with glucose + efflux inhibitor.

8. Place the 0.2 mL microtubes in the Rotor-Gene fluorometer and program the instrument with the following settings: a temperature of 37°C; a unit of time for each measurement (cycle) of 60 s; the number of cycles necessary to obtain the total period of time (*i.e.*, 60 cycles to obtain a 60 min assay); and excitation and detection wave- lengths of 530 nm and the 590 nm, respectively (*see* **Note 21**).

3.6 Normalization of Data and Presentation of Results

The final results of the accumulation assay (determination of EtBr steady-state) are calculated as follows:

1. Subtract the EtBr baseline fluorescence obtained in Control 1 (PBS + EtBr, with no bacterial cells) from the one(s) corresponding to the bacteria + EtBr.

2. Make sure that fluorescence measurements in Control 2 should be very low, below those in Control 1, with no increases over time.

3. If the latter is not the case, conditions should be checked.

The results of the accumulation assay in the presence of efflux inhibitors are calculated as follows:

1. Subtract the EtBr baseline fluorescence obtained in Control 4 (PBS + EtBr + efflux inhibitor, with no bacterial cells) from the one(s) corresponding to the bacteria + EtBr + efflux inhibitor.

2. Make sure that fluorescence measurements in Control 3 should be very low, below those in Control 4, with no increases over time.

3. If the latter is not the case, conditions should be checked.

In any of these experiments, fluorescence can be expressed in terms of relative final fluorescence (RFF) at the last time point (minute 60) of the assay in comparison with reference conditions by using the formula $(RF_{assay} - RF_{ref})/RF_{ref}$, where RF_{assay} is the relative fluorescence at the last time point of the EtBr accumulation assay and RF_{ref} is the relative fluorescence at the last time point of the EtBr accumulation assay under the reference conditions [23, 36] (*see* **Note 22**).

The results of efflux experiments are presented in terms of relative fluorescence, calculated by comparing the data obtained for the bacterial population under conditions that allow maximum efflux (bacteria in the presence of glucose and absence of efflux inhibitor) against the data obtained from the control that contains the EtBr-loaded cells under conditions that restrict efflux

(presence of efflux inhibitor and no glucose). The relative fluorescence thus corresponds to the ratio of fluorescence that remains per unit of time, relative to the EtBr-loaded cells.

4. Notes

1. Some mycobacterial strains are pathogenic, so cultures and further manipulations must be done in biosafety level 2 or 3 laboratories according to the requirements for each specific strain.

2. According to the manufacturer, OADC must be added to Middlebrook 7H9 broth medium. However, in many laboratories, ADC (albumin-dextrose-catalase) is used instead of OADC. The choice between OADC and ADC may depend on many factors, such as availability, price, and the particular growth requirements of mycobacterial species or strains to be assayed.

3. EtBr is a powerful teratogen. Safety precautions must be followed when working with EtBr. Non-absorbent gloves (*e.g.*, nitrile) should be worn when handling EtBr and stock solutions should be prepared under a fume hood. Care should be taken to avoid contaminating the working area. All disposable materials used for EtBr preparation or manipulation should be identified as contaminated and disposed according to local laws and regulations, such as in appropriate containers for subsequent incineration.

4. Resazurin stock solutions stored at 4°C may be used for no longer than 2 weeks.

5. Alternatively, some suppliers provide PBS tablets (Sigma Aldrich). In this case, dissolve one tablet in 200 mL of deionized sterile water, according to the manufacturer's instructions.

6. Glucose solutions do not need to be sterilized if used immediately. If they are kept at 4°C they should be used within 3 days of preparation. Glucose is added to provide an energy source for the activity of the efflux systems.

7. This method can be performed using several types of equipment, for example real-time PCR instruments (*e.g.* Rotor-Gene™ 3000/6000, Corbett Life Science) and 96-well plate

fluorometers (*e.g.* Safire$^{2\ TM}$, Tecan). Both platforms allow accurate real-time measurements of accumulation and efflux of EtBr (18, 20-25). Other fluorometers may also be used. In our experience, the Rotor Gene™ 3000/6000 (0.2 mL microtube system) and the Safire$^{2\ TM}$ (microplate system) are suitable instruments to be used in these assays. Any equipment should be set up for excitation at 530 nm and detection of fluorescence of EtBr at 590 nm. Most fluorometers provide measurements of fluorescence at every time point during the course of the experiment, along with a graphical plot of the increase of fluorescence over time. This allows the real-time visualization of when the steady-state equilibrium is reached at a given EtBr concentration (such as that shown in Fig. 1 for a concentration of 0.125 μg/mL). This should be reached at no more than 10% of the relative fluorescence that the instrument can record [21].

8. The correlation between CFU/mL and OD_{600} measurement must be determined prior to carrying out the protocol, as different results may be observed between laboratories depending on the equipment used to measure the OD_{600} [37]. 9. Incubation time varies according to the mycobacterial species: 6 days in the case of *M. tuberculosis* and other slow growing mycobacteria, 3 days for fast growers like *M. smegmatis*.

10. Incubation time with resazurin varies according to the mycobacterial species, for example, 2 days in the case of *M. tuberculosis* and up to 24 h for *M. smegmatis* [38]. A word of caution with the readout of the resazurin signal:background, especially to assess the antimycobacterial activity of compounds that affect energy/ATP production (*e.g.* bedaquiline or delamanid), due to the spectral overlap of the oxidized resazurin with the resorfin product. Direct measurement of intracellular ATP in mycobacterial cells is a confirmatory and more sensitive and reliable viability readout in these situations [39].

11. A culture of 50 mL will give approximately 35 mL of bacterial suspension at an OD_{600} of 0.8, which is a volume large enough to test several experimental conditions and to carry out the control experiments. The volume of the culture can be scaled up or down depending on the number of experiments to be done.

12. PBS is supplemented with Tween 80 at a final concentration of 0.05% to get disperse and homogenous suspensions of mycobacterial cells and avoid the formation of bacterial clumps.

13. If using bacterial suspensions adjusted at an OD_{600} of 0.8 does not result in detection of accumulation or efflux, test other bacterial suspensions adjusted at an OD_{600} of 0.4, 0.6, or 1.0. This optimization may be necessary according to the instrument used to record the fluorescence emitted by the fluorescent substrate accumulated inside the mycobacterial cells.

14. The assay can be done in 0.2 mL microtubes or in 96-well plates depending on the requirements of the fluorometer to be used.

15. Other fluorescent compounds such as Hoechst 33342, ethidium monoazide (EMA), rhodamine 123 or orange acridine can be used instead of EtBr. If so, excitation and emission wavelengths should be modified, and the levels of relative fluorescence sensitivity of the assays will also vary according to the excitability threshold of the compound used for efflux substrate. Optical density of the bacterial suspension may have to be adjusted as well.

16. When setting up a new protocol in the Rotor-Gene, it may be necessary to perform the "Gain Optimization" to determine the best gain setting. This will ensure that all data is collected within the dynamic range of the detector. If the gain is too low, the signal will be lost in background noise. If it is too high, all signal will be lost off scale (saturated). In the case of the Rotor-Gene, the gain range for each channel is –10 to 10, where –10 is the least sensitive and 10 is the most sensitive. By our experience a gain of 8 is appropriate for most experiments.

17. Other compounds such as Phe-Arg-β-naphthylamide (PAβN) may be used. MICs of the compounds to be tested on each bacterial strain must be conducted prior to their use, to test for antibacterial activity. Each inhibitor is used at a final concentration that should not exceed ¼ MIC in order to not compromise the cellular viability.

18. In order to measure EtBr efflux, it is necessary to promote the maximum accumulation of EtBr. This is accomplished by exposing the bacteria to EtBr under conditions that limit efflux to

its minimal activity, namely: a temperature of 25°C, absence of glucose, and the presence of the most effective compound selected from accumulation experiments. All these conditions that decrease efflux must be optimized experimentally according to the instrument used to record the fluorescence emitted by the fluorescent substrate accumulated inside the mycobacterial cells [18, 20, 21].

19. We have found that for most strains, efflux of EtBr takes place within the first 10–15 min of the assay at 37°C, and this period of time may be shorter in the case of drug resistant strains or strains overexpressing efflux pumps.

20. It is advisable to keep the microtubes or the 96-well plate on ice until all reagents are added.

21. In the case of strains with overexpression of efflux pumps it might be necessary to adjust the measurement time, performing shorter acquisition cycles (*e.g*. every 30 s) due to the increased efflux activity.

22. When assaying different strains and mutants of *M. tuberculosis*, the reference condition could be the assay of reference strain H37Rv, whereas when assaying the effect of a compound in the accumulation of efflux of EtBr, the reference condition is the assay in the absence of any such compound. In both cases, high RFF values indicate that cells accumulate more EtBr under the tested conditions than under the reference conditions and *vice versa* for negative RFF values.

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Figure Captions

Fig. 1 Accumulation of EtBr by *M. bovis* BCG.

Fig. 2 Effect of efflux inhibitors on the accumulation of EtBr by *M. bovis* BCG. EtBr was used at a concentration of 0.125 μg/mL and efflux inhibitors CPZ, TZ and VP at 5, 2.5 and 80 μg/mL, respectively (¼ MIC – ref. 25) to not compromise cellular viability. CPZ, chlorpromazine; EI, efflux inhibitor; TZ, thioridazine; VP, verapamil.

Fig. 3 Efflux of EtBr by *M. bovis* BCG. Efflux takes place at 37°C in the presence of glucose and is inhibited by TZ (adapted from ref. 25). EI, efflux inhibitor; TZ, thioridazine.

Fig. 4 Efflux of EtBr by wild-type *M. bovis* BCG and BCG-INH^R(isoniazid resistance is induced by overexpression of efflux pump genes) (adapted from ref. 25).

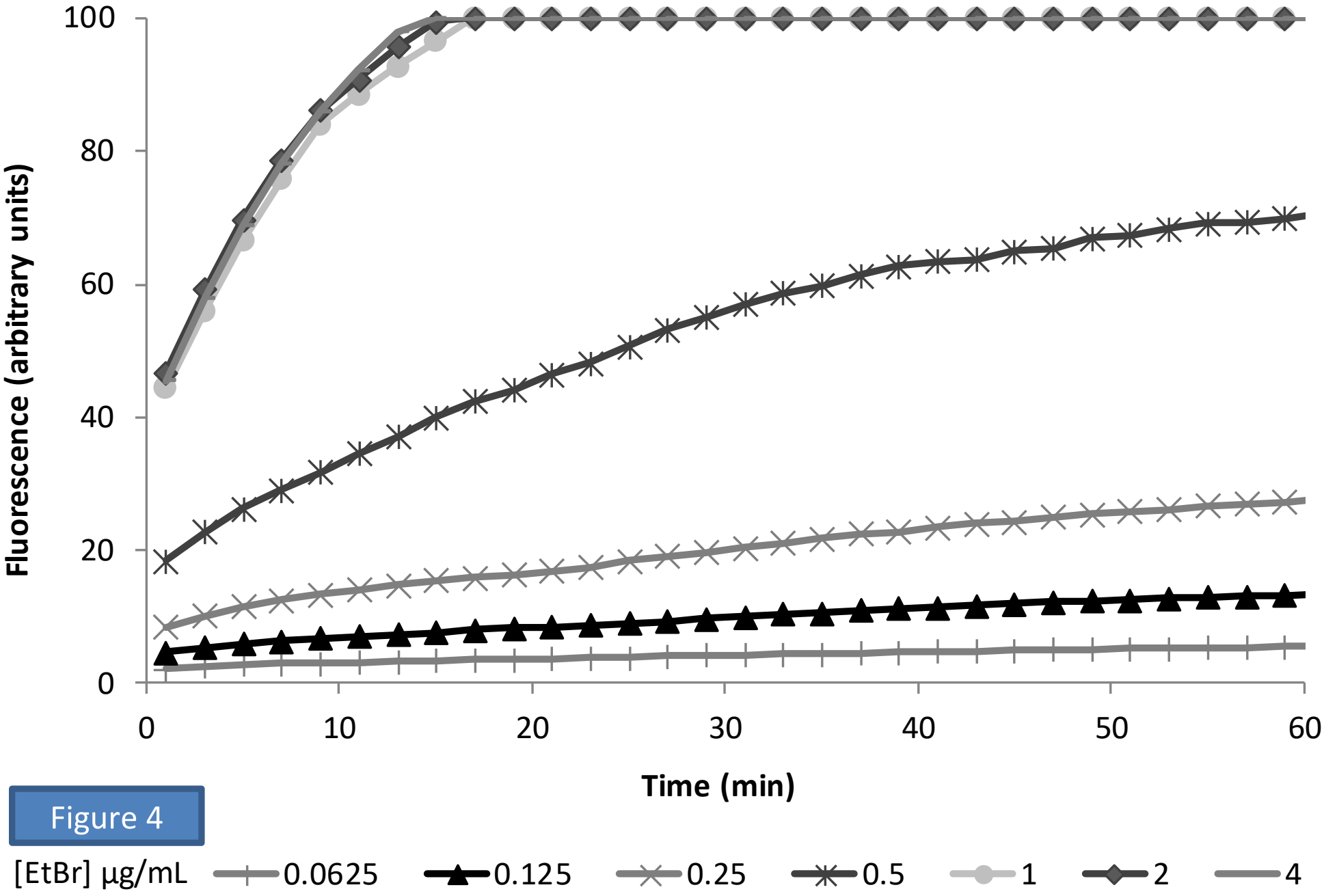
100
80
60
40
20
0
Fluorescence (arbitrary units)
0
10
20
30
40
50
60
Time (min)
[EtBr] µg/mL
0.0625
0.125
0.25
0.5
1
2
4

Figure 4

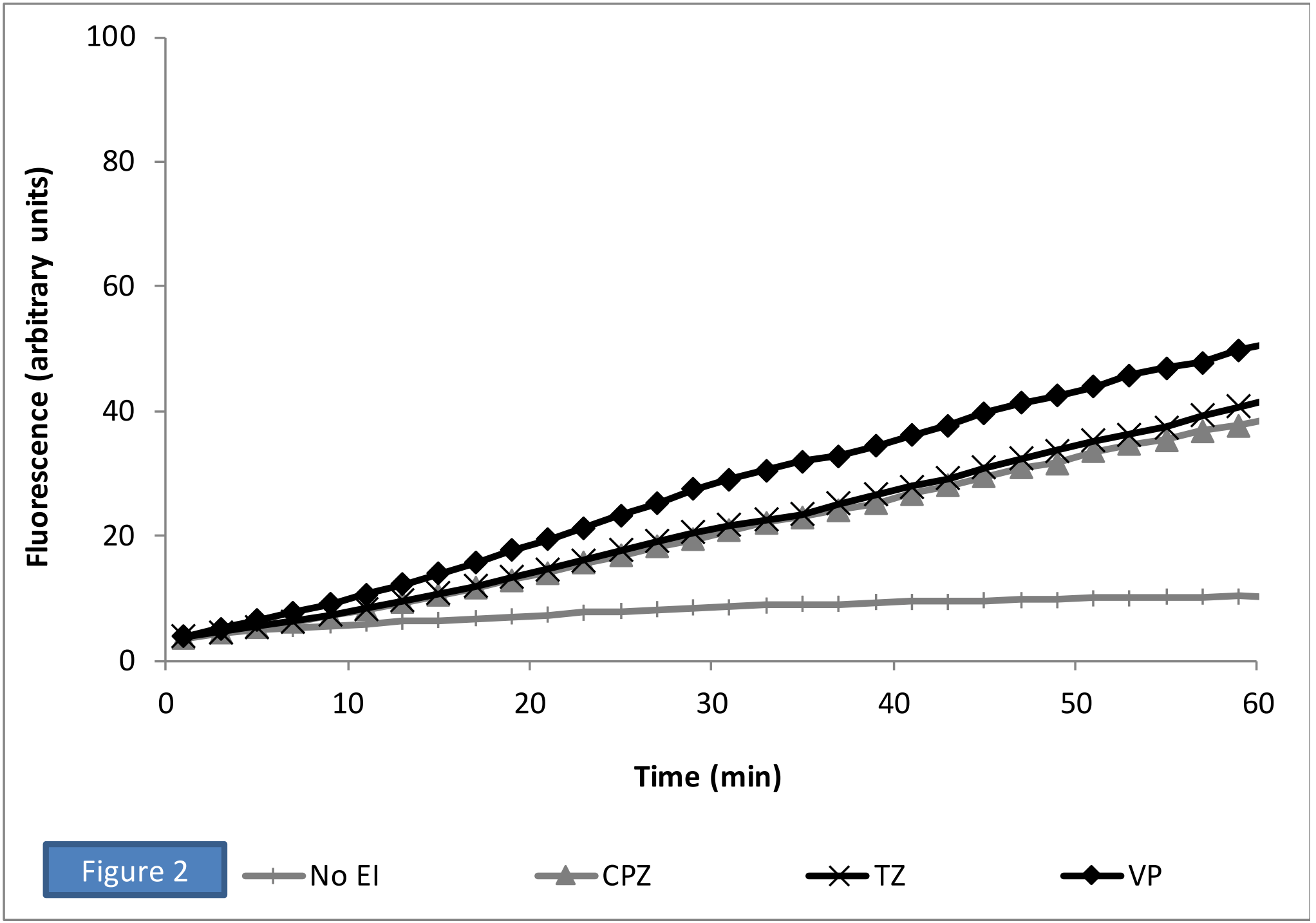
100
80
60
40
20
0
Fluorescence (arbitrary units)
0
10
20
30
40
50
60
Time (min)
No EI
CPZ
TZ
VP

Figure 2

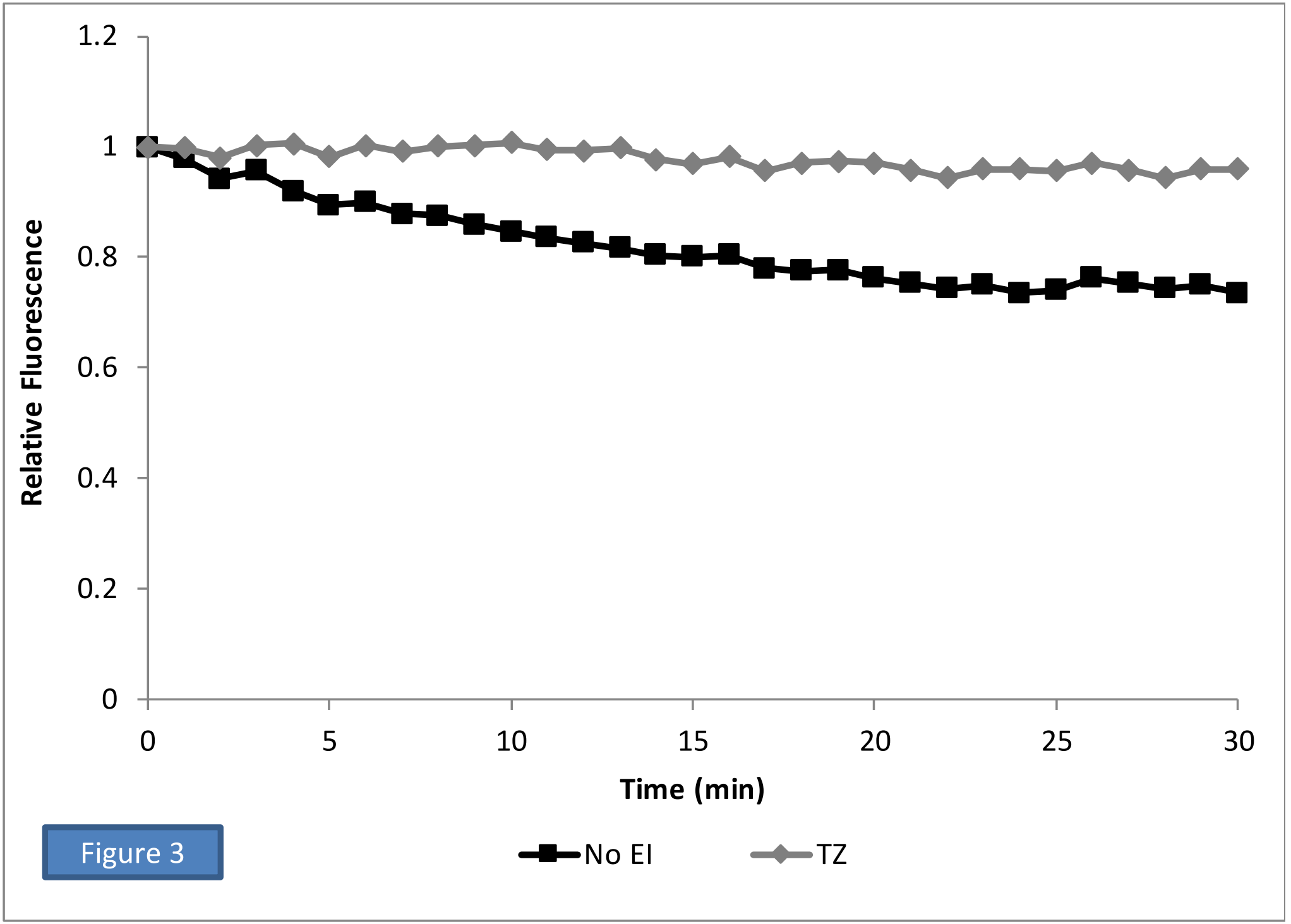
1.2
1
0.8
0.6
0.4
0.2
0
Relative Fluorescence
0
5
10
15
20
25
30
Time (min)
No El
TZ

Figure 3

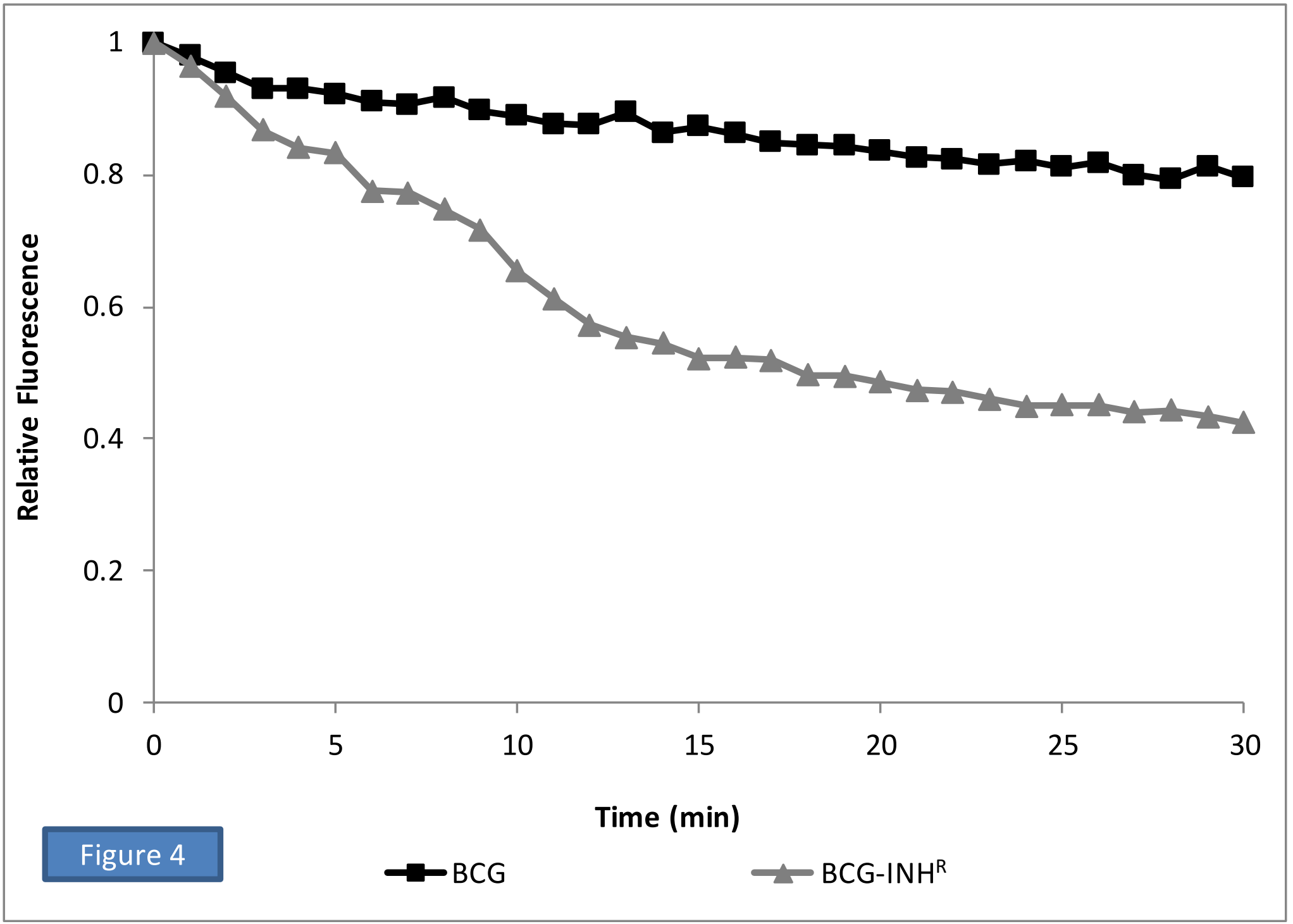
1
0.8
0.6
0.4
0.2
0
Relative Fluorescence
0
5
10
15
20
25
30
Time (min)
BCG
BCG-INH^R

Figure 4