TITLE:

In Vitro Drug Screening Against All Life Cycle Stages of *Trypanosoma cruzi* Using Parasites Expressing β-galactosidase

AUTHORS AND AFFILIATIONS:

Victoria Lucia Alonso[1,2], Romina Manarin[1], Virginia Perdomo[1], Ernesto Gulin[3], Esteban Serra[1,2], Pamela Cribb[1,2]

[1]Área Parasitología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario (UNR), Rosario, Argentina
[2]Instituto de Biología Molecular y Celular de Rosario (IBR), CONICET, Rosario, Argentina
[3]Instituto de Investigaciones Biomédicas (INBIOMED), Universidad de Buenos Aires (UBA) Facultad de Medicina, CONICET, Buenos Aires, Argentina

Email addresses of co-authors:

Romina Manarin (rmanarin@fbioyf.unr.edu.ar)
Virginia Perdomo (vperdomo@fbioyf.unr.edu.ar)
Ernesto Gulin (jgulin@fvet.uba.ar)
Esteban Serra (serra@ibr-conicet.gov.ar)

Corresponding authors:

Victoria Lucia Alonso (alonso@ibr-conicet.gov.ar)
Pamela Cribb (cribb@ibr-conicet.gov.ar)

SUMMARY:

We describe a high-throughput colorimetric assay measuring β-galactosidase activity in three life cycle stages of *Trypanosoma cruzi*, the causative agent of Chagas disease. This assay can be used to identify trypanocidal compounds in an easy, fast, and reproducible manner.

ABSTRACT:

Trypanosoma cruzi is the causative agent of Chagas disease (ChD), an endemic disease of public health importance in Latin America that also affects many non-endemic countries due to the increase in migration. This disease affects nearly 8 million people, with new cases estimated at 50,000 per year. In the 1960s and 70s, two drugs for ChD treatment were introduced: nifurtimox and benznidazole (BZN). Both are effective in newborns and during the acute phase of the disease but not in the chronic phase, and their use is associated with important side effects. These facts underscore the urgent need to intensify the search for new drugs against *T. cruzi*.

T. cruzi is transmitted through hematophagous insect vectors of the Reduviidae and Hemiptera families. Once in the mammalian host, it multiplies intracellularly as the non-flagellated amastigote form and differentiates into the trypomastigote, the bloodstream non-replicative infective form. Inside the insect vector, trypomastigotes transform into the epimastigote stage and multiply through binary fission.

45 This paper describes an assay based on measuring the activity of the cytoplasmic β-galactosidase
46 released into the culture due to parasites lysis by using the substrate, chlorophenol red β-D-
47 galactopyranoside (CPRG). For this, the *T. cruzi* Dm28c strain was transfected with a β-
48 galactosidase-overexpressing plasmid and used for *in vitro* pharmacological screening in
49 epimastigote, trypomastigote, and amastigote stages. This paper also describes how to measure
50 the enzymatic activity in cultured epimastigotes, infected Vero cells with amastigotes, and
51 trypomastigotes released from the cultured cells using the reference drug, benznidazole, as an
52 example. This colorimetric assay is easily performed and can be scaled to a high-throughput
53 format and applied to other *T. cruzi* strains.
54
55 **INTRODUCTION:**
56 Chagas disease (ChD), or American trypanosomiasis, is a parasitic disease caused by the
57 flagellated protozoan, *Trypanosoma cruzi* (*T. cruzi*). ChD begins with an asymptomatic or
58 oligosymptomatic acute phase that is usually undiagnosed, followed by a lifelong chronic phase.
59 In the chronicity, ~30% of patients manifest—decades after the infection—a variety of
60 debilitating conditions, including myocardiopathy, mega-digestive syndromes, or both, with a
61 mortality rate ranging from 0.2% to 20%[1–3]. Asymptomatic chronic patients may have no clinical
62 signs but remain seropositive throughout their life.
63
64 Estimations suggest that ~7 million people are infected worldwide, mostly from Latin America,
65 where ChD is endemic. In these countries, *T. cruzi* is mainly transmitted through infected blood-
66 sucking triatomine bugs (vector-borne transmission) and less frequently by oral transmission
67 through the ingestion of food contaminated with triatomine feces containing the parasites[2].
68 Additionally, the parasite can be transmitted via the placenta from chagasic mothers to
69 newborns, through blood transfusions, or during organ transplantation. These vector-
70 independent ways of acquiring the infection and human migration have contributed to the
71 worldwide spread of the disease, evidenced by an increasing number of cases in North America,
72 Europe, and some African, Eastern Mediterranean, and Western Pacific countries[4]. ChD is
73 considered a neglected disease as vector-borne transmission is closely associated with poverty
74 and is a leading public health issue, especially in Latin American low-income countries. Although
75 there are available treatments, mortality due to ChD in Latin America is the highest among
76 parasitic diseases, including malaria[2].
77
78 There are two registered drugs for ChD treatment introduced in the late 1960s and early 1970s:
79 nifurtimox and benznidazole[5]. Both drugs are effective in the acute phase of the disease in adults,
80 children, and congenitally infected newborns, as well as in children with chronic infection, where
81 cure is usually achieved. However, only a few people are diagnosed early enough to be treated
82 in time. According to the latest clinical trials, both drugs have important limitations in adults and
83 were ineffective in reducing symptoms in people with chronic disease; hence, their use in this
84 stage is controversial. Other drawbacks are the prolonged treatment periods required (60–90
85 days) and the frequent, severe adverse effects observed, which lead to discontinuation of
86 therapy in a proportion of infected people[6,7]. It is estimated that fewer than 10% of the people
87 with ChD have been diagnosed, and even fewer have access to treatment, as many affected
88 individuals live in rural areas with no or scarce access to healthcare[8]. These facts highlight the

89 urgent need to find new drugs against *T. cruz*i to allow for more efficient, safe, and applicable-
90 to-the-field treatments, especially for the chronic phase. In this regard, another challenge in the
91 development of more efficacious compounds is the limitation of systems for assessing drug
92 efficacy *in vitro* and *in vivo*[9].
93
94 Although chemical biology and genomic approaches for the identification of potential drug
95 targets have been used in kinetoplastid parasites, the available genomic tools in *T. cruzi* are
96 limited in contrast to *T. brucei* or *Leishmania*. Thus, the screening of compounds with
97 trypanocidal activity is still the most used approach in the search for new chemotherapeutic drug
98 candidates against ChD. Usually, drug discovery in *T. cruzi* must start with testing the effects of a
99 new drug in an *in vitro* assay against the epimastigote stage. For decades, the only way for
100 measuring the inhibitory effects of candidate compounds on *T. cruzi* was manual microscopic
101 counting, which is laborious, time-consuming, and operator-dependent. Moreover, this approach
102 is suitable for assaying a small number of compounds but is unacceptable for high-throughput
103 screening of large compound libraries. Nowadays, many investigations begin with the analysis of
104 a vast number of compounds from different origins that are assayed *in vitro*, testing their capacity
105 for inhibiting parasite growth. Both colorimetric and fluorometric methods have been developed
106 to increase throughput in these assays, improving the objectivity of the screening and making the
107 whole process less tedious[9].
108
109 One of the most widely used colorimetric methods is based on the β-galactosidase activity of
110 transfected parasites first described by Bucknet and collaborators[10]. The β-galactosidase enzyme
111 expressed by the recombinant parasites hydrolyzes the chromogenic substrate, chlorophenol red
112 β-D-galactopyranoside (CPRG), to chlorophenol red, which can be easily measured
113 colorimetrically using a microplate spectrophotometer. Thus, parasite growth in the presence of
114 a variety of compounds can be simultaneously evaluated and quantitated in microtiter plates.
115 This method has been applied to test drugs in epimastigote forms (present in the insect vector),
116 trypomastigotes, and intracellular amastigotes, the mammalian stages of the parasite. Further,
117 several recombinant *T. cruzi* strains transfected with the pBS:CL-Neo-01/BC-X-10 plasmid
118 (pLacZ)[10] to express the *Escherichia coli* β-galactosidase enzyme are already available (and new
119 ones can be constructed), which allows the evaluation of parasites from different discrete typing
120 units (DTUs) that may not behave equally toward the same compounds[10–13]. This method has
121 already been successfully used to evaluate compounds for activity against *T. cruzi* in low- and
122 high-throughput screening[12,13]. Similar approaches have also been used in other protozoan
123 parasites, including *Toxoplasma gondii* and *Leishmania mexicana*[14,15].
124
125 This paper describes and shows a detailed method for an *in vitro* drug screening against all life
126 cycle stages of *T. cruzi* using parasites expressing β-galactosidase. The assays presented here have
127 been performed with a β-galactosidase-expressing *T. cruzi* line obtained by transfection of *T. cruzi*
128 Dm28c strain from DTU I[13] with pLacZ plasmid (Dm28c/pLacZ). Additionally, the same protocol
129 could be easily adapted to other strains to compare the performance between compounds and
130 between *T. cruzi* strains or DTUs.
131
132 **PROTOCOL:**

133
134 NOTE: An overview of the entire experimental design is depicted in **Figure 1**.
135
136 [Place **Figure 1** here]
137
138 1. **Preparation of stock solutions**
139
140 1.1. Preparation of media and solutions
141
142 1.1.1. Hemin solution (**Supplemental Table S1**)
143
144 1.1.1.1. Add all the components to a 50 mL centrifuge tube in the order given in the recipe
145 and homogenize by inversion several times.
146
147 1.1.1.2. Sterilize by filtration through a 0.22 µm filter.
148
149 1.1.1.3. Prepare 1 mL aliquots in 1.5 mL microcentrifuge tubes and keep them at -80 °C
150 until use.
151
152 1.1.2. Liver Infusion Tryptose (LIT) Medium (**Supplemental Table S1**)
153
154 1.1.2.1. Weigh all the components and stir to homogenize at room temperature in a 1 L
155 beaker containing at least 700 mL of distilled water.
156
157 1.1.2.2. Adjust the pH to 7.2 and top up the volume to 900 mL in a 1 L graduated cylinder
158 with distilled water; sterilize by filtration or autoclaving (121 °C for 20 min).
159
160 1.1.2.3. Supplement the medium by adding 100 mL of fetal calf serum (FCS), (10% FCS,
161 sterile and heat-inactivated at 56 °C for 45 min), 20 mL of 40% sterile glucose solution (sterilized
162 by autoclaving, 121 °C for 20 min), and 5 mL of hemin solution (final concentration 5 µM) to 900
163 mL of LIT medium.
164
165 1.1.3. Prepare Dulbecco's Modified Eagle Medium (DMEM) from the powder following the
166 manufacturer's instructions.
167
168 1.1.3. Phosphate-buffered saline (PBS) (**Supplemental Table S1**)
169
170 1.1.4.1. Dissolve all solid components by stirring the solution at room temperature in a 1 L beaker.
171
172 1.1.4.2. Adjust the pH to 7.2, level up to 1 L in a 1 L graduated cylinder with distilled water, and
173 sterilize by filtration or autoclaving (121 °C, 20 min).
174
175 1.2. Benznidazole (BZN) stock solutions and dilutions
176

177 NOTE: The range of BZN concentration used in this work was 2.5 to 80 µM.
178
179 1.2.1. Prepare a stock solution of 1 M BZN by dissolving 13 mg of the drug in 50 µL of
180 dimethylsulfoxide (DMSO). Under aseptic conditions, prepare serial dilutions from this 1 M BZN
181 stock solution at twice the final desired concentration (2x solutions) in a final volume that is
182 adequate for the number of wells to be assayed.
183
184 NOTE: Calculate for 100 µL per well with an excess of 10–20%. The BZN stock solution and all BZN
185 dilutions must be prepared immediately before use in the assay due to the low solubility of the
186 drug in the medium.
187
188 1.2.2. Prepare 2x BZN dilutions of 160, 80, 40, 20, 10, and 5 µM.
189
190 1.2.2.1. Dilute 1 M BZN stock solution at a 100-fold dilution (10 µL of 1 M BZN + 990 µL of
191 medium) to obtain a 10 mM solution in the appropriate medium used for each life cycle stage of
192 *T. cruzi*. Mix continuously to homogenize the suspension.
193
194 1.2.2.2. Dilute 10 mM BZN solution to prepare 320 µM BZN in the appropriate medium: 32 µL of
195 10 mM BZN + 968 µL of medium. Mix continuously to homogenize the suspension.
196
197 1.2.2.3. Dilute 320 µM BZN 2-fold to obtain a concentration of 160 µM (500 µL of 320 µM BZN +
198 500 µL of medium). Mix continuously to homogenize the suspension. Repeat this 2-fold dilution
199 with each resulting solution to obtain 80, 40, 20, 10, and 5 µM solutions.
200
201 1.2.2.4. Dilute DMSO 1,000-fold in the appropriate medium for use as untreated control (100%
202 survival control).
203
204 NOTE: Epimastigotes tolerate up to a 100-fold dilution of DMSO, whereas Vero cells tolerate only
205 up to a 1,000-fold dilution of DMSO. If necessary, a death control with 50% DMSO can be included
206 as the 0% survival condition.
207
208 1.3. Substrate solution
209
210 1.3.1. Dissolve CPRG at 1 mM concentration in distilled water. For a 96-well plate, add 2.4 mg of
211 CPRG to 4 mL of water.
212
213 NOTE: CPRG solution must be prepared immediately before the assay.
214
215 1.4. Lysis solution
216
217 1.4.1. Prepare a 2.5% v/v solution of nonionic, non-denaturing detergent 2-[4-(2,4,4-
218 trimethylpentan-2-yl)phenoxy]ethanol (see the **Table of Materials**) in 1x PBS. Prepare 1 mL of
219 the solution per 96-well plate immediately before the assay.
220

221 **2. Parasite culture preparation**
222
223 2.1. Epimastigote preparation
224
225 NOTE: *T. cruzi* Dm28c/pLacZ line[13] is used throughout this report.
226
227 2.1.1. Grow the β-galactosidase-expressing *T. cruzi* epimastigotes axenically at 28 °C in cell
228 culture flasks with a growth area of 25 cm^2 (T-25 flasks). Maintain the cultures in log phase by
229 subculturing every 48–72 h (in 5 mL) in LIT medium supplemented with 10% FCS (**Supplemental**
230 **Table S1**) and geneticin sulfate (G418) at a final concentration of 200 µg/mL. Quantify parasite
231 growth by cell counting in a Neubauer chamber before subculturing. Securely close the cap and
232 keep the culture flask (not vented) at 28 °C in a vertical position.
233
234 NOTE: G418 ensures pLacZ plasmid selection and maintenance. Log phase cultures have an
235 epimastigote concentration of 1–5 × 10^7 parasites/mL for the Dm28c/pLacZ line.
236
237 2.1.2. Prepare a suspension of 2 × 10^5 epimastigotes/mL from a log phase culture in LIT
238 supplemented with G418 antibiotic. Dispense 100 µL of the epimastigote suspension per well
239 (20,000 epimastigotes in 100 µL of LIT) of a 96-well microplate and make up the final volume to
240 200 µL per well with the medium.
241
242 2.2. Amastigote preparation
243
244 2.2.1. Use spontaneous metacyclic trypomastigotes obtained from an aged epimastigote
245 culture (for 7 days in this protocol) to perform an initial infection in a T-25 flask with 2 × 10^5 Vero
246 cells seeded previously in DMEM supplemented with 2% FCS.
247
248 2.2.1.1. Count the number of metacyclic trypomastigotes in a Neubauer chamber, infect
249 the Vero cell monolayer with a multiplicity of infection (MOI) of 10 in 5 mL of DMEM with 2%
250 FCS, and incubate at 37 °C and 5% CO_2 for 16 h. Wash the remaining trypomastigotes by removing
251 the medium from the flask with a 5 mL sterile pipette, then add 5 mL of 1x PBS and aspirate.
252 Finally, add 5 mL of DMEM with 2% FCS and incubate under the same conditions.
253
254 2.2.1.2. Use the trypomastigotes emerging from the infected Vero cell monolayer to
255 maintain the infection in T-25 flasks with 2 × 10^5 Vero cells in DMEM with 2% FCS, generating a
256 new infected bottle every week.
257
258 NOTE: After 5–7 days, trypomastigotes start to emerge and are visible in the supernatant. Do not
259 add G418 to the trypomastigotes used to infect the cells or the infected cells, as the Vero cell line
260 is not resistant to G418.
261
262 2.2.2. Prepare a suspension of 1 × 10^5 Vero cells/mL in DMEM supplemented with 2% FCS and
263 seed 100 µL of the suspension per well in 96-well tissue culture plates (10,000 cells per well).
264 Incubate overnight (12–16 h) at 37 °C and 5% CO_2 to ensure cell adherence to the bottom of the

wells.

2.2.3. After the overnight incubation, rinse the Vero cell monolayer three times with 100 µL of sterile 1x PBS. Add *T. cruzi* Dm28c/pLacZ trypomastigotes (obtained from a previous infection in a T-25 flask, step 2.2.1) at an MOI of 10 in 100 µL of DMEM supplemented with 2% FCS per well (100,000 trypomastigotes per well).

2.2.4.Incubate the plates for 6 h at 37 °C and 5% CO_2. After this incubation period, wash the plates twice with 1x PBS, and add 100 µL of DMEM without phenol red supplemented with 2% FCS.

NOTE: After 48 h (2 days post infection), intracytoplasmic amastigotes are visible with an optic microscope. Phenol red, a pH indicator in DMEM and other cell culture media, could interfere with the absorbance measurement of CPRG. If DMEM without phenol red is not available, see alternatives mentioned below in section 3.2.1.

2.3. Trypomastigote preparation

2.3.1. Prepare a suspension of 1×10^6 Vero cells/mL in DMEM supplemented with 2% FCS and seed 800,000 cells in 5 mL of the medium in T-25 flasks. Incubate overnight (12–16 h) at 37 °C and 5% CO_2 to ensure cell adherence.

NOTE: For a T-75 flask, seed 2×10^6 cells in a final volume of 15 mL.

2.3.2. After incubation, rinse two times with 3 mL of sterile 1x PBS. Add *T. cruzi* Dm28c/pLacZ trypomastigotes at an MOI of 10 in 5 mL of DMEM with 2% FCS (8×10^6 trypomastigotes for a T-25 flask).

NOTE: For a T-75 flask, add 20×10^6 trypomastigotes in a final volume of 15 mL of DMEM with 2% FCS.

2.3.3. Incubate overnight (12–16 h) at 37 °C and 5% CO_2. Wash the flask twice with 3 mL of 1x PBS, and add 5 mL of fresh DMEM supplemented with 2% FCS. Incubate at 37 °C and 5% CO_2 for four days.

2.3.4. Check the supernatant for trypomastigotes under an optic microscope. Quantify the trypomastigotes by counting them in a Neubauer chamber. Collect the supernatant in a 15 mL tube and centrifuge at 7,000 × *g* for 10 min at room temperature.

2.3.5. Discard the supernatant and resuspend the pellet to obtain a concentration of 1×10^6 trypomastigotes/mL in DMEM without phenol red supplemented with 2% FCS. Seed 100 µL of the trypomastigote suspension (100,000 trypomastigotes per well) in a 96-well plate.

NOTE: If DMEM without phenol red is not available, see alternatives below in section 3.2.1.

309 **3. β-galactosidase assay**
310
311 NOTE: Quantitation of β-galactosidase activity is used as an indirect way of determining the
312 number of parasites. It is expected that growth will be inhibited in the presence of a trypanocidal
313 compound, leading to a lower number of parasites compared to the untreated control, which will
314 be reflected in a lower β-galactosidase activity and therefore lower absorbance.
315
316 3.1. Incubate the parasites with BZN.
317
318 3.1.1. Add 100 µL of corresponding 2x BZN solution per well to reach a final concentration of BZN
319 of 80, 40, 20, 10, 5, and 2.5 µM to 100 µL of epimastigote suspension (from step 2.1), Vero cells
320 with amastigotes (2 days post infection) (step 2.2), or trypomastigotes (step 2.3) in a 96-well
321 plate.
322
323 3.1.2. Incubate the epimastigotes at 28 °C for 72 h, and the trypomastigotes or infected Vero cells
324 with amastigotes for 24 h at 37 °C and 5% CO_2.
325
326 NOTE: Each drug concentration should be evaluated at least in triplicate and include control
327 cultures of epimastigotes, trypomastigotes, and infected Vero cells with DMSO (see step 1.2.2.4).
328
329 3.2. Colorimetric reaction
330
331 3.2.1. After the treatment incubation period, if infected Vero cells or trypomastigotes are in
332 DMEM with phenol red, replace the medium with 100 µL of 1x PBS to avoid interference. Perform
333 triplicate blank wells containing only 100 µL of corresponding medium (or 1x PBS as appropriate).
334
335 NOTE: It is not necessary to remove the culture medium for epimastigotes in case of LIT medium
336 or DMEM without phenol red. DMEM with phenol red can still be used; prepare a blank well with
337 DMEM alone to measure the base absorbance and then subtract this value during data analysis
338 (step 3.3.). Schneider's insect medium, which is colorless, is an alternative for epimastigotes.
339
340 3.2.2. Add 40 µL of CPRG substrate solution and 10 µL of the detergent solution to each well,
341 obtaining a final concentration of 200 µM CPRG and 0.1% detergent in a final volume of 250 µL
342 in each well.
343
344 NOTE: The CPRG solution and detergent can be added together in a final volume of 50 µL per
345 well.
346
347 3.2.3. Incubate at 37 °C for 2 h and measure the absorbance at 595 nm in a microplate
348 spectrophotometer.
349
350 NOTE: The expected color change is yellow to reddish-brown upon β-galactosidase enzymatic
351 cleavage (**Figure 2A**). Incubation time can be extended up to 4 h, and the absorbance spectra of
352 chlorophenol red can be read between 570 and 595 nm with similar curve fittings (**Supplemental**

353 **Figure S1A,B**). Incubation for up to 24 h in the presence of CPRG substrate has shown similar
354 curve fittings (**Supplemental Figure S1C**).
355
356 3.2.3.1. In a microplate spectrophotometer with a monochromator selector, create a new
357 protocol in the equipment software (**Supplemental Figure S2**).
358
359 3.2.3.2. Click **Absorbance** as **detection method | Endpoint** as **read type | Ok** (**Supplemental**
360 **Figure S2A**). Add a **Read Step**, type the selected wavelength, and click **Ok** (**Supplemental Figure**
361 **S2B**).
362
363 3.2.3.3. In the **Plate Layout** section, mark the wells to be read and click **Ok** (**Supplemental Figure**
364 **S2C**). To read the plate, insert it in the tray and click on **Read Plate**. Wait for the values to appear
365 on the screen (**Supplemental Figure S2D**) and export them to a spreadsheet to analyze the
366 results.
367
368 3.3. Data analysis and media inhibitor concentration (IC_{50}) calculation
369
370 3.3.1. Subtract the blank measured value, corresponding to only LIT medium, 1x PBS, or DMEM
371 with or without phenol red plus the CPRG–detergent solution. When testing the trypanocidal
372 activity of colored compounds, measure the absorbance of additional blank controls with LIT or
373 DMEM with each concentration of drug used and then subtract those values from the absorbance
374 values obtained with the parasites at each concentration.
375
376 NOTE: **Supplemental Table S2** shows typical values obtained in this assay with these media
377 without parasites plus CPRG–detergent solution. The differences before and after adding CPRG
378 are significant but do not interfere with the assay with parasites (**Supplemental Table S2**).
379
380 3.3.2. In statistical analysis software, plot the concentration of BZN (in µM) versus the absorbance
381 at 595 nm in an xy table. Transform the BZN concentrations to logarithmic values by clicking on
382 the **Analyze** button, selecting the T**ransform** option **| transform the x-values using x=log(x)**
383 option, and clicking the **Ok** button.
384
385 3.3.3. Obtain the IC_{50} values from the statistical analysis software.
386
387 NOTE: The IC_{50} is defined as the drug concentration that reduces parasite growth by 50%
388 compared to the untreated control and is calculated as the inflection point of the sigmoidal
389 function that fits the curve.
390
391 3.3.3.1. In the statistical analysis software, click on the **Analyze** button, select **Non-linear**
392 **regression (curve fit)** in the **xy analysis list**, and click **Ok**.
393
394 3.3.3.2. In the **model** tab of the **Parameters** window, in the **dose-response–inhibition group** of
395 built-in equations, select the option **dose-response method: log(inhibitor) vs. response –**
396 **Variable Slope (four parameters)**. Leave all the other tabs at default values; click **Ok**.

397
398 3.3.3.3. Click on the **results** section of the statistical analysis software to find the **IC_{50} value**, the
399 **SD**, and the **goodness of the fit**.
400
401 3.3.3.4. Click on the **graph** section to find the **xy graph** of the **logarithmic concentration of the**
402 **drug** versus the **absorbance** values. Look for the curve fit is also graphed in a different color.
403
404 NOTE: A free online IC_{50} calculation tool can be found at https://www.aatbio.com/tools/ic50-
405 calculator.
406
407 **REPRESENTATIVE RESULTS:**
408 Following the protocol described above, β-galactosidase-expressing Dm28c epimastigotes were
409 incubated with 6 concentrations of BZN (2.5, 5, 10, 20, 40, 80 µM) (or compounds of interest) for
410 72 h. After this period, CPRG reagent was added along with detergent, which lyses the cells and
411 releases β-galactosidase. CPRG is cleaved by the β-galactosidase to produce chlorophenol red,
412 leading to a change in color from yellow to reddish (**Figure 2A**). Chlorophenol red was measured
413 by reading the absorbance at 595 nm in a microplate reader after 2 h. An XY table was plotted
414 with the logarithmic concentrations of BZN versus the absorbance at 595 nm. The plot was fitted
415 using non-linear regression (**Figure 2B**). In this particular experiment, the IC_{50} obtained for
416 epimastigotes was 20.59 ± 1.075 µM, similar to that obtained from the literature (**Table I**)[16,17].
417 Representative results using this method for trypomastigotes and amastigotes have been
418 described previously[13].
419
420 **FIGURE AND TABLE LEGENDS:**
421 **Figure 1: Overview of the *in vitro* screening assay of *Trypanosoma cruzi* Dm28c/pLacZ line using**
422 **CPRG as a substrate for the colorimetric reaction.** The assay consists of seeding the parasites
423 (1), incubating them with BZN (2 and 3), and then adding the colorimetric substrate (4). When
424 parasites are lysed, β-galactosidase is released and cleaves CPRG to chlorophenol red; this change
425 in color can be measured spectrophotometrically (5). Data can be analyzed in statistical analysis
426 software to obtain the half inhibitory concentration (IC_{50}) of BZN. Abbreviations: CPRG =
427 chlorophenol red β-D-galactopyranoside; BZN = benznidazole.
428
429 **Figure 2: Calculation of IC_{50} value of benznidazole for epimastigote form Dm28c.** (**A**) A 96-well
430 plate with epimastigotes treated with different BZN concentrations (2.5, 5, 10, 20, 40, and 80
431 µM) before adding CPRG (1), after initial addition of CPRG and detergent (2), and after incubation
432 with CPRG and detergent showing the change of color (3). (**B**) XY-plot of the logarithmic
433 concentrations of BZN versus absorbance (OD) at 595 nm for Dm28c/pLacZ epimastigotes. The
434 plot was fitted using non-linear regression to estimate the IC_{50} value. Each value represents the
435 mean and the standard deviation (error bars) of 6 independent biological replicates. The
436 continuous blue line represents the curve fit. Abbreviations: CPRG = chlorophenol red β-D-
437 galactopyranoside; BZN = benznidazole; OD = optical density; C = control.
438
439 **Table 1: Range of IC_{50} values obtained for epimastigotes, trypomastigotes, and amastigotes**
440 **using this protocol compared with IC_{50} reported in literature[17,18].**

441
442 **Supplemental Figure S1: Setup of the microplate reader software for reading absorbance.**
443
444 **Supplemental Figure S2: β-galactosidase activity measurements (optical density at 570 to 595**
445 **nm) using epimastigotes from Dm28c/pLacZ line of *Trypanosoma cruzi* after incubation with**
446 **CPRG at different time points.** Values are expressed as mean and standard deviation of three
447 independent replicates. Colored continuous lines represent the curve fit. (**A**) Incubation with 200
448 µM CPRG for 2 h. (**B**) Incubation with 200 µM CPRG for 4 h. (**C**) Representation of β-galactosidase
449 activity (optical density at 570 nm) at different time points (2, 4, and 24 h). Abbreviation: CPRG =
450 chlorophenol red β-D-galactopyranoside.
451
452 **Supplemental Table S1: Composition and preparation of LIT medium, hemin, and PBS.**
453 Abbreviations: LIT = Liver Infusion Tryptose; PBS = phosphate-buffered saline.
454
455 **Supplemental Table S2: Absorbance readings for LIT and DMEM media without parasites.**
456 Values are expressed as triplicates of each medium; the **media** row contains the mean value of
457 the three replicates; and the **SD** row contains the standard deviation values of the replicates.
458 Abbreviations: SD = Standard Deviation; LIT = Liver Infusion Tryptose; DMEM = Dulbecco's
459 Modified Eagle Medium.
460
461 **DISCUSSION:**
462 This paper describes an assay based on determining the cytoplasmic β-galactosidase activity
463 released due to membrane lysis of *T. cruzi* epimastigotes, trypomastigotes, or infected cells with
464 amastigotes in the presence of the substrate CPRG. We used *T. cruzi* Dm28c/pLacZ parasites, a
465 stable parasite strain obtained after transfection with a β-galactosidase-bearing plasmid
466 constructed by Buckner and co-authors[10]. This assay has been used to search for antitrypanocidal
467 compounds[12,19–21]. The *T. cruzi* Dm28c/pLacZ strain was used to optimize the screening protocol.
468 As summarized in **Figure 1**, this protocol has four major points; the first is parasite seeding in 96-
469 well plates. Epimastigotes and trypomastigotes are counted and seeded from a suspension. In
470 the case of amastigotes, first seed Vero cells to form an adherent monolayer and then infect with
471 trypomastigotes after 48 h. Amastigotes are present inside the cells. Second, prepare dilutions
472 of the drug to be tested in the desired concentrations and incubate with the parasites. Finally,
473 the third step involves adding CPRG and detergent to lyse the cells. CPRG is cleaved upon β-
474 galactosidase release from the parasite cytoplasm, and chlorophenol red is generated and
475 measured spectrophotometrically. The fourth critical step involves data analysis, construction of
476 x-y graphs, and fitting the curve obtained to calculate the IC_{50} values of the drugs of interest.
477
478 An *in vitro* assay for all the life cycle stages of *T. cruzi* is the initial step in studying the effects of
479 a potential new drug. In these assays, the effects are evaluated by microscopic counting, a time-
480 expensive and subjective procedure that is difficult to automate. The substitution of this
481 technique by a colorimetric assay can improve the objectivity of the screening, also making it less
482 time-consuming. The colorimetric plate reading takes only a few minutes, as opposed to the
483 hours required for labor-intensive manual microscopic counting, and facilitates the screening of
484 large libraries of new compounds with potential therapeutic value. Regarding the overall

similarity of the β-galactosidase-expressing parasites (Dm28c/pLacZ) compared to the wild-type Dm28c strain, we determined that the parasites were morphologically normal, with similar growth rates and differentiated equally within the life cycle forms. Moreover, the drug testing results obtained comparing the wild-type Dm28c and the Dm28c/pLacZ line quantitated by microscopic counting showed no significant differences[13].

One limitation of this protocol is that colored culture media could interfere with the measured absorbance. DMEM (or RPMI) without phenol red is recommended to overcome this limitation. A blank well with DMEM was successfully used as a basal absorbance value in this protocol. Another limitation is the putative interference when using colored drugs, which could be overcome using media with the compound as a blank or by selecting the absorbance wavelength between 570 and 595 nm to give the lowest interference. CPRG is not altered by the presence of a given drug (colored or not), making this assay quite robust. When using media with phenol red or colored drugs, it is critical to perform blank controls for each condition and then subtract the absorbance value obtained from the measurements with parasites to avoid any interference.

The concentration of CPRG solution reported for *T. cruzi* trypomastigotes and *Toxoplasma gondii* is 100 µM[10,15]. However, the optimal concentration reported for epimastigotes is 200 µM[11]. In this Dm28c/pLacZ line, a 200 µM CPRG solution was used for epimastigotes, trypomastigotes, and amastigotes with reliable results. Regarding CPRG incubation times, the best curve fitting was achieved when epimastigotes were incubated with the substrate solution for 2 h (R^2 = 0.9995). However, the R^2 was very similar (R^2 = 0.9994), and β-galactosidase activity was linear in the range of 6,250–200,000 epimastigotes per well (i.e., 62,500–2,000,000 epimastigotes/mL) at 4 h (**Supplemental Figure S2**). A linear range of 3,150–100,000 trypomastigotes per well (i.e., 31,500–1,000,000 trypomastigotes/mL) was reported for trypomastigotes[13]. To avoid observing large standard deviations, it is important to seed the correct amount of parasites in each well. As volumes are small, it is important to be consistent when counting the parasites before seeding. Further, more than three replicates could be measured for each experimental condition if necessary.

Unlike other colorimetric screening assays[22], subsequent manipulation steps are not necessary here, increasing the reproducibility and reliability of the assay as well as the speed of data collection. This is an easy, quick, and reliable assay suitable for high-throughput drug screening of candidate compounds for ChD treatment and can be applied to other *T. cruzi* strains. The pLacZ plasmid is available upon request from the Bucker lab and could be used to transfect resistant strains of, for example, knockout lines to evaluate the sensitivity of the line to different drugs in different genetic backgrounds. The only critical point to be kept in mind is that the knockout plasmids should have a different antibiotic resistance marker than pLacZ.

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529 (https://smart.servier.com).
530
531 **DISCLOSURES:**
532 The authors have no conflict of interest to disclose.
533
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