Assessing Tolerance to Heavy-Metal Stress in *Arabidopsis thaliana* Seedlings

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Abstract

Heavy-metal soil contamination is one of the major abiotic stress factors that, by negatively affecting plant growth and development, severely limit agricultural productivity worldwide. Plants have evolved various tolerance and detoxification strategies in order to cope with heavy-metal toxicity, while ensuring adequate supply of essential micronutrients at the whole-plant as well as cellular levels. Genetic studies in the model plant *Arabidopsis thaliana* have been instrumental in elucidating such mechanisms. The root assay constitutes a very powerful and simple method to assess heavy-metal stress tolerance in *Arabidopsis* seedlings. It allows the simultaneous determination of all the standard growth parameters affected by heavy-metal stress (primary root elongation, lateral root development, shoot biomass and chlorophyll content) in a single experiment. Additionally, this protocol emphasizes the tips and tricks that become particularly useful when quantifying subtle alterations in tolerance to a given heavy-metal stress, when simultaneously pursuing a large number of plant lines, or when testing sensitivity to a wide range of heavy metals for a single line.

Key words *Arabidopsis thaliana*, Chlorophyll content, Heavy-metal stress tolerance, Lateral root development, Primary root elongation, Root assay, Seedlings, Shoot biomass

1 Introduction

As sessile organisms, terrestrial plants need to acquire their nutrients from the soil solution and therefore their growth and development largely rely on the soil mineral status. One of the most pervasive causes of loss of crop productivity worldwide is the contamination of arable land with heavy metals. Heavy-metal soil contamination as a result of anthropogenic activities occurs in many regions of the world and, depending on environmental as well as societal factors, may pose health risks to both humans and animals when accumulating in food crops. Given the modern agricultural context, the impact that this unfavorable soil condition exerts on crop yields will grow to paramount importance in the years to come. Thus, the elucidation of the physiological and molecular mechanisms underlying plant heavy-metal stress tolerance will be crucial for the use of biotechnology to reclaim farmlands lost to agriculture as well as in phytoremediation strategies — i.e., the use of plants to decontaminate polluted environments — and has been the subject of intense research in the plant biology field [1,2].

Heavy metals — i.e., in a broad sense, potentially toxic metallic elements — such as zinc (Zn), copper (Cu) or iron (Fe) among others, are essential for plant development as they serve as catalytic co-factors or structural motifs in numerous enzymes and other proteins assuming a key role in many basic metabolic processes. Nonetheless, these micronutrients become potentially toxic when present in excess. Conversely, non-essential heavy metals, such as cadmium (Cd), arsenate (As) or

50 cesium (Cs) constitute toxic elements that can adversely affect plant growth even
51 when present in trace amounts in the soil solution. Depending on the chemical and
52 physical properties of the heavy metal in question, heavy-metal toxicity mainly occurs
53 as a result of a propensity to inactivate crucial proteins through blocking of functional
54 groups — the case of Cd and lead (Pb) — or through displacement of essential metal
55 ions from their site of action — the case of Zn –, as well as a result of the ability to
56 disturb cellular homeostasis of other essential elements – the case of As and Cs – or
57 to induce oxidative stress through the generation of reactive oxygen species (ROS) –
58 the case of Cu and Fe [3].

59 To deal with these opposing effects and adjust to environmental fluctuations in
60 their availability, plants have developed a sophisticated and tightly controlled
61 homeostatic network aimed at ensuring an adequate supply of crucial oligo-elements
62 while preventing the toxic build-up of both essential and non-essential heavy metals
63 at the cellular and whole-plant levels [4]. Heavy metals are primarily acquired from
64 the soil solution as ions, which once absorbed into the root epidermis move mostly
65 symplastically through the adjacent cell layers to reach the central stele. After
66 secretion into the stellar apoplast followed by active loading into the xylem vessels,
67 heavy metals are translocated to the shoot via root pressure and the transpirational
68 stream and subsequently transferred to the phloem sap before allocation to aerial
69 organs. Plants adapt to essential heavy-metal shortage supply prevalently by
70 activating cellular heavy metal uptake systems particularly at the root-soil interface
71 [5]. At the whole-plant level, tolerance to heavy-metal excess is achieved mainly
72 through reduced uptake at the root soil interface and through the rearrangement of its
73 tissue partitioning via enhanced sequestration in leaves, whereas within the root both
74 immobilization in the outer cell layers and exclusion from the epidermis contribute to

75 limit heavy metal entry into the root symplasm. At the cellular level, such
76 mechanisms are primarily intended to restrict the cytosolic accumulation of free
77 heavy metal, mainly though extrusion in the apoplasm, chelation with specific ligands
78 and/or vacuolar compartmentalization [6-8].

79 Deciphering the steps in plant heavy-metal stress tolerance and identifying the
80 genetic determinants mediating heavy-metal uptake, translocation, chelation and
81 detoxification largely benefit from molecular genetic studies in the plant model
82 *Arabidopsis thaliana*. In the presence of excessive amounts of a given heavy metal,
83 *Arabidopsis* seedlings develop pleiotropic toxicity symptoms, generally including
84 shoot growth retardation, leaf chlorosis and remodeling of the root system
85 architecture, i.e. inhibition of primary root elongation and altered lateral root
86 development. The easiest and most commonly employed method to reliably appraise
87 the extent of damage caused by heavy-metal stress in *Arabidopsis* seedlings
88 remains the root assay [9,10], which was initially adapted to heavy-metal
89 susceptibility assessment by Howden and Cobbett in 1992 [11]. Root growth can be
90 rapidly scored, and the assay requires relatively little specific equipment, providing
91 both qualitative and quantitative data. It typically involves vertically-oriented growth of
92 seedlings on solid media imposing or not a given rhizotoxic stress and scoring
93 followed by comparison of primary root elongation in exposed versus non-exposed
94 seedlings. Such an experiment can at first seem quite straightforward but often turns
95 out to be rather challenging to interpret, in particular when a large number of lines of
96 interest must be simultaneously compared, when a wide range of heavy metals has
97 to be tested, or when subtle alterations between genotypes need to be reproducibly
98 quantified. In addition, most of the reports using this assay focus merely on primary
99 root elongation and disregard evaluation of other susceptibility indicators, such as

100 shoot growth, photosynthesis performance or lateral root development. The present
101 chapter does not aim solely at describing the root assay itself but rather to provide a
102 precise practical application of the method, which is particularly helpful to accurately
103 and fully evaluate *Arabidopsis* heavy-metal stress tolerance by measuring all
104 standard phenotypic parameters in a single experiment. Using this combined
105 approach, we were able to assign a role in ion rhizotoxicity tolerance to three
106 *Arabidopsis* transporters from the Major Facilitator Superfamily. In particular, we
107 reported that Pht1;9 function confers oversensitivity to As [12], while activity of the
108 ZIF2 and ZIFL2 carriers promotes tolerance to Zn and Cs toxicity, respectively
109 [13,14].

110

111 ## 2 Materials

112

113 ### *2.1 Plant Material*

114 1. Good-viability *Arabidopsis* seeds of the appropriate genotypes along with seeds
115 of the corresponding wild type(s)
116 2. Seeds of previously reported tolerant and/or sensitive heavy-metal stress lines
117 may also be used as controls (*see* **Note 1**)

118

119 ### *2.2 Reagents and Solutions*

120 1. MS (Murashige and Skoog, [15]) medium (or equivalent) freshly prepared before
121 use: 1x MS basal salt mix, supplemented with 0.1 g L^{-1} *myo*-inositol and 0.5 g L^{-1}
122 2-(N-morpholino)ethanesulfonic acid (MES), pH adjusted to 5.7 with KOH 1M,
123 solidified with 0.8% ultrapur agar and autoclaved (*see* **Note 2**).
124 2. Sterilization solution freshly prepared before use: 50% [v/v] sodium hypochlorite
125 and 0.02% [v/v] Triton X-100 in sterile distilled water

126 3. Appropriate antibiotics for seedling selection when mutant or transgenic seed
127 batches are not derived from homozygous plants
128 4. Stock solutions of the heavy metals to be tested (Table 1)
129
130 ### *2.3 Consumables*

131 1. Eppendorf tubes (1.5 and 2 mL)
132 2. Fine forceps
133 3. Square Petri dishes (11.5 cm).
134 4. Razor blades.
135 5. Micropore tape (3 M).
136
137 ### *2.4 Equipment*

138 1. A climate-controlled growth cabinet set to long-day (16-h light, 22°C/8-h dark,
139 18°C) or under continuous light (20-h light, 22°C/4-h dark, 18°C) conditions with
140 60% relative humidity and cool-white light (~100-120 µmol m^{-2} s^{-1})
141 2. Space at 4°C (room or fridge)
142 3. Laminar flow chamber
143 4. Fume hood
144

145 ## 3 Methods

146 The pipeline of the whole process is presented in **Fig. 1**.
147 1. Under sterile conditions, prepare square Petri dishes containing equivalent
148 amounts of control MS medium or selective MS medium in case seed selection is
149 required. Allow the plates to dry before closing them in order to avoid any
150 condensation on the lid.

151 2. Surface-sterilize the seeds by incubating them 10 min in sterilization solution (in
152 Eppendorf tubes) under constant and vigorous shaking followed by four rinses
153 with sterile distilled water.
154 3. Under sterile conditions, immediately spread evenly the seeds with the help of a
155 tip onto the appropriate control plates (*see* **Note 3**) in 1-4 rows starting 1.5 cm
156 from the top of the plate without spacing between the seeds (*see* **Note 4**). Allow
157 the plates to dry until the water containing the seeds has totally evaporated. Seal
158 the plates using Micropore tape and wrap them together in aluminum foil (*see*
159 **Note 5**).
160 4. Incubate the plates vertically (*see* **Note 6**) at 4°C for 3 days to break seed
161 dormancy.
162 5. After stratification, remove the aluminum foil and incubate the plates vertically
163 (*see* **Note 7**) in the controlled-growth cabinet. Let the seeds germinate and the
164 seedlings grow until root lengths reach roughly 1.0-1.2 cm (maximum 1.5 cm). In
165 our hands, this corresponds to about 4-6 days depending on the light conditions.
166 6. At this point, careful visual inspection of all plates is essential to ensure that the
167 pre-defined experiment design is still feasible. First, it is crucial to ensure that the
168 number of seedlings capable of being transferred is sufficient: seedlings of all the
169 genotypes to be concomitantly tested must be at the same developmental stage,
170 in particular with roots of similar length (less than 0.2 cm variation) and the plates
171 must be free of fungal or bacterial contaminations. We typically transfer 16
172 seedlings per genotype per condition onto two different plates, each
173 accommodating two genotypes in parallel, i.e. two sets of 8 seedlings. The
174 appropriate controls should not be forgotten, i.e. transfer also of i) each genotype
175 to a control plate to ensure that the phenotypic parameters to be measured are

176 not altered under control conditions (and later normalize heavy-metal stress
177 effects), and ii) seedlings from the wild-type background to each of the analyzed
178 conditions. For studies where a single mutant or transgenic line is being
179 compared to the corresponding wild type, the easiest way to ensure a valid
180 comparison is to grow the wild-type seedlings on the same plate as the genotype
181 under evaluation in order to avoid any effects of plate to plate variability.
182 Alternatively, when a relatively high number of distinct genotypes in the same
183 background need to be tested, 2-3 repetitions of wild-type seedling transfer in
184 between the genotypes of interest may be acceptable to avoid extensive
185 measurements.
186 7. Prepare square Petri dishes containing equivalent amounts of control MS
187 medium and MS medium supplemented with the heavy metal(s) to be tested.
188 Allow the plates to dry completely before closing them to avoid any condensation
189 on the lid. Heavy-metal containing medium can be easily prepared by
190 incorporating an appropriate amount of heavy-metal stock solution into previously
191 autoclaved medium (*see* **Note 8**). The concentrations of the different heavy
192 metals that we routinely test for the *Arabidopsis* ecotype Columbia (Col-0) are
193 described in Table 1, but the appropriate concentrations should be empirically
194 established depending on the accessions employed and the nature of the lines to
195 be analyzed (tolerant or sensitive when compared to wild type). For a first
196 screen, it is recommended to test a full range of heavy-metal concentrations.
197 8. Under sterile conditions, gently transfer seedlings using regularly disinfected
198 forceps to the new plates by carefully allowing the root tip to touch the medium
199 and, at an angle of approximately 30°, delicately sliding the root over the medium
200 surface until the hypocotyl-root junction reaches a line drawn at 1.5 cm from the

201 top of the plate. This way, the roots will be straight and contact with the medium
202 surface will be maximized (*see* **Note 9**). Maintain a regular spacing between the
203 seedlings. When the transfer is finalized, seal the plates, mark the position of the
204 root tips directly on the bottom of the plate, and incubate plates vertically in the
205 controlled-growth cabinet with roots pointing downward (*see* **Note 10**).

206 9. After approximately 7 days (*see* **Note 11**) of growth on new media, primary root
207 elongation can be scored by marking the new position of the primary root tips
208 (*see* **Note 12**).

209 10. After a further incubation period, i.e. just before the longest root of one genotype
210 reaches the bottom of the plate, lateral root development can be recorded by first
211 marking the new position of the primary root tips and then scanning the plates
212 from their bottom side (*see* **Note 13**).

213 11. Immediately following scanning or after a further incubation period in the control-
214 growth cabinet (*see* **Note 11**), seedlings can be assessed concomitantly for
215 shoot biomass and chlorophyll content. Shoot biomass is determined by
216 measuring the fresh weight of two pooled plant shoots (*see* **Notes 13** and **14**).
217 Immediately after weighing, place the two plant shoots together in the bottom of a
218 2-mL Eppendorf tube and add 1 mL of 80% acetone. Once shoot biomass
219 measurements are complete, incubate all the tubes overnight in the dark under
220 gentle but continuous agitation. The following day, measure spectroscopically the
221 absorbance of the acetone solution at 647 nm and 660 nm.

222 12. Even before the end of the experiment, the primary root elongation and lateral
223 root development parameters can be quantified for each seedling on scanned
224 images using an image analysis software, such as ImageJ
225 (http://rsb.info.nih.gov/ij/index.html). Primary root elongation is evaluated by

226 measuring the exact distance between the initial and the corresponding mark.
227 Lateral root density is evaluated by counting the number of lateral roots
228 (excluding adventitious roots) and normalizing to the total length of the
229 corresponding primary root, determined by measuring the exact distance
230 between the hypocotyl-root junction and the final mark. Total lateral root length is
231 evaluated by adding up the length of each lateral root of a given seedling. Total
232 chlorophyll content is determined according to the method and equation (Total
233 chlorophyll = $18.71A_{647nm} + 7.15A_{660nm}$) of MacKinney [16] and expressed on a
234 fresh weight basis (*see* **Note 15**). Finally, the average value of each of the
235 analyzed parameters under a given heavy-metal stress is normalized to the
236 corresponding average value in the non-stress condition, typically using n = 16
237 for primary root elongation, n = 8 or 16 for lateral root development parameters,
238 and n = 8 for shoot biomass and chlorophyll content. To ascertain that tolerance
239 to a given heavy-metal stress is affected, similar results need to be obtained in at
240 least three independent experiments (*see* **Note 16**).
241

242 ## 4 Notes

243
244 1. Best results will be produced if seed batches have been obtained from plants
245 cultured simultaneously.
246 2. Before use, it is imperative that all soap, detergent and other cleaning fluids be
247 completely removed from glassware, as even slight traces of such compounds in
248 the medium will interfere with the assay. Glassware should be rinsed thoroughly
249 3 to 6 times with sterile distilled water before preparing the medium. Preparing
250 the medium with sterile distilled water and avoiding storage of the plates before
251 use significantly help preventing fungal and bacterial contaminations.

252 3. The root assay can be carried out by germinating the seeds directly onto heavy-
253 metal containing plates or by transferring seedlings grown beforehand on control
254 medium plates to heavy-metal containing plates. From our experience, the most
255 informative is the transfer method described here, as it excludes a possible effect
256 of heavy-metal stress on germination rate (which can be easily scored in an
257 independent assay by measuring the germination rates of exposed versus non-
258 exposed seedlings) while being relevant when seedling selection on medium
259 supplemented with antibiotics is required prior to transfer.
260 4. Sowing the seeds at high density so that they touch each other on the plate
261 rather than leaving space between them will greatly improve synchronization of
262 seedling growth right after seed germination, in particular at the root level. In
263 addition, this will favor straight growth of the roots and prevent their curling or
264 curving. Another essential point when sowing the seeds is to avoid as much as
265 possible scratching the medium surface with the tip to minimize root growth
266 inside the medium rather than on its surface and thus prevent their subsequent
267 transfer.
268 5. Spreading only one genotype per plate will prevent any cross-contamination
269 between the lines to be tested. Alternatively, in the case of preliminary small-
270 scale tests, two genotypes can be spread onto a single plate divided vertically.
271 The number of seedlings amenable to transfer is often limiting, so be sure to
272 plate enough seeds. The number of plates to be prepared depends on the
273 germination rate of the seed batch and on the kind of screening to be performed
274 (i.e., the type and range of heavy-metal concentrations to be tested).
275 Nevertheless, we recommend preparing at least two plates per genotype in case
276 fungal or bacterial contaminations appear.

277 6. We have observed that performing the stratification step with Petri dishes set up
278 already in a vertical position promotes synchronization of seedling root growth.
279 7. Petri dishes must be incubated vertically but with a slight inclination — i.e., a
280 forward shift of ~2 cm at the base of the dish — so that the seeds (and later the
281 seedlings) face the light source. This will greatly favor the straight growth of the
282 roots and is highly facilitated by the presence of gridded shelves (specific from
283 the growth cabinet or, alternatively, from a fridge) holding the plates
284 approximately at half their height. All the plates from a single experiment must be
285 similarly inclined and if possible positioned on the same shelf so that they are
286 exposed to the same amount of light.
287 8. Interpretation of the results can be erroneous if special care is not taken to
288 ensure that the genotypes of interest are exposed to the exact same severity of
289 heavy-metal stress as the wild-type control and thus uniform composition of the
290 medium between plates is essential. We recommend preparing all the plates of a
291 given concentration from the same heavy metal stock solution.
292 9. Transfer of the seedlings is the most critical step of the protocol. Its success
293 depends largely on intact seedlings and any damage needs to be strictly
294 avoided. Initially, it may take some practice of the transfer procedure to achieve
295 quick transfer and correct positioning without wounding the seedlings, particularly
296 squashing at the hypocotyl region. We strongly advise to delicately lift the
297 seedling shoot using the fine forceps as a lever rather than closing them. If
298 correct positioning is not achieved at a first attempt, make the seedling root slide
299 again but never touch the root in order to preserve its integrity. To minimize
300 dehydration of the seedlings, keep the lid of the initial and receiver plates as
301 closed as possible during the transfer procedure. Any clearly wounded or dry

302 seedling should be discarded. We highly recommend checking root integrity, in
303 particular root tip intactness, of each transferred seedlings under a dissection
304 microscope at the first transfer attempts, while routinely ensuring that 24 hours
305 after transfer the roots have recovered and resumed steady-state growth even
306 under heavy-metal stress (although at a slower rate than under control
307 conditions).

308 10. Some studies indicate the inversion of the plates after transfer, so that the roots
309 are pointed upward, to facilitate evaluation of primary root growth without having
310 to mark root tip positions. However, we believe this method is only amenable to
311 qualitative assessment of root elongation upon exposure to heavy-metal stress
312 and largely privilege continuous growth as it allows the full extent of primary root
313 elongation and lateral root parameters to be accurately measured, while
314 eliminating possible effects of agravitropic behavior of the lines under evaluation.

315 11. One advantage of this method is that, as long as each specific trait is
316 simultaneously quantified for all the genotypes under study, some slight
317 variability in incubation times can be tolerated.

318 12. Susceptibility to heavy-metal stress will not necessarily follow a linear
319 progression, particularly regarding primary root elongation. As scoring this
320 parameter is a non-invasive method, we highly recommend marking the position
321 of the root tips at 2-days intervals, at least in a first screen.

322 13. When recording phenotypical data, particular attention should be paid to the
323 water frequently accumulating inside the plates. Water at the bottom of the plates
324 can disturb seedling root position and it is crucial that they remain in place for
325 later measurement from scanned images, while water condensed on the lid can
326 easily wet seedling shoot and lead to highly erroneous conclusions. Keep the

327 plates as vertical as possible before carefully opening the plate under sterile
328 conditions, removing excess water by gently turning them over and drying the lid
329 with paper. Seal back the plates in case further incubation is needed.
330 14. Still on the agar plates, cut two seedlings at the root-hypocotyl junction with a
331 razor blade and immediately measure their combined weight using a precision
332 weighing scale, while avoiding seedling damage as much as possible. Note that
333 it is essential to be in a calm environment without frequent movements or strong
334 ventilation to avoid quick water loss from the seedling shoots. For the same
335 reason, keep the plates closed between each measurement.
336 15. Even taking particular care during plate preparation and seedling manipulation,
337 contaminations frequently occur. Any contaminated seedling should be
338 eliminated from the data recording, as should those that do not recover quickly
339 after transfer or that suddenly arrest growth for no apparent reason. It is therefore
340 important to follow the plates daily, as fungal and bacterial contaminations
341 usually appear during prolonged incubation times. It should also be noted that
342 shoot biomass and chlorophyll content can be assessed earlier than initially
343 planned, i.e. as soon as a first plant shows signs of contamination, in order to
344 save the experiment.
345 16. Be aware that the root assay is instrumental to determine the level of
346 susceptibility to a given heavy metal, but not when the observed differences are
347 due to altered internal heavy-metal homeostasis or whole-plant heavy metal
348 accumulation. A similar assay to the one presented here, but set up on a larger
349 scale, can be performed to prepare tissue samples for heavy-metal content
350 quantification by methods such as atomic absorption spectroscopy.
351

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358

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406

407 **Figure Legend**

408 **Fig. 1.** Overview of the protocol. Steps are referred to according to the Methods
409 section. Step 1, preparation of the plates; Step 2, surface-sterilization of the seeds;
410 Step 3, sowing of the seeds on control medium; Step 4, stratification of the seeds;
411 Step 5, germination and synchronized growth of the seedlings; Step 6, visual
412 inspection of the plates; Step 7, preparation of the heavy-metal stress and control
413 plates; Step 8, transfer and growth of the seedlings; Step 9, scoring of primary root
414 growth elongation; Step 10, scoring of lateral root development; Step 11, scoring of
415 shoot biomass and chlorophyll content; Step 12, analysis of the data.

Table 1. Heavy-metal stock solutions and concentrations to test for the root assay in the Columbia (Col-0) ecotype of *Arabidopsis thaliana*.

Heavy metal	Cation	Compound	Stock solution		Range
			Concentration	Preparation	
Aluminium	Al^{3+}	$AlCl_3$	0.1 M	0.4 g in 30 ml H_2O	0.5, 0.75, 1, 1.5, 2 mM
Arsenate	AsO_4^{3-}	NaH_2AsO_4	500 mM	0.82 g in 10 ml H_2O	100, 200, 300, 400, 500 μM
Cadmium	Cd^{2+}	$CdCl_2$	30 mM	55 mg in 10 ml H_2O	10, 25, 50, 75, 100 μM
Cobalt	Co^{2+}	$CoCl_2.6H_2O$	100 mM	0.238 g in 10 ml H_2O	25, 50, 75, 100, 150 μM
Copper	Cu^{2+}	$CuCl_2$	30 mM	51.1 mg in 10 ml H_2O	25, 50, 75, 100, 150 μM
Iron	Fe^{2+}	FeSO4	0.1 M	0.278 g in 10 ml H_2O	0.25 mM
Lithium	Li^{2+}	$LiCl_2$	5 M	in 10 ml H_2O	5, 10, 12.5, 15, 20 mM
Manganese	Mn^{2+}	$MnCl_2.4H_2O$	0.5 M	0.990 g in 10 ml H_2O	1, 1.5, 2, 2.5, 3 mM
Nickel	Ni^{2+}	$Ni\ Cl_2.6H_2O$	100 mM	0.238 g in 10 ml H_2O	50, 75, 100, 150, 200 μM
Lead	Pb^{2+}	N_2O_6Pb	0.5 M	1.66 g in 9 ml H_2O + 1 ml HNO_3	0.1, 0.25, 0.5, 0.75, 1 mM
Cesium	Cs^{+}	CsCl	5 M	8.42 g in 10 ml H_2O	1, 2, 3, 4, 5 mM
Thallium	Tl^{3+}	$TlCl_3$	0.33 M	3.1 g in 5 ml HCl 37% + 25 ml H_2O	2.5, 5, 10, 15, 20 μM
Zinc	Zn^{2+}	$ZnSO_4.7H_2O$	100 mM	0.288 g in 10 ml H_2O	100, 250, 500, 750, 1000 μM

All heavy-metal stock solutions are prepared with sterile distilled water.

Figure 1