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Evaluation of amino acid consumption in cultured bone cells and isolated bone shafts.
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1 **TITLE:**
2 Evaluation of Amino Acid Consumption in Cultured Bone Cells and Isolated Bone Shafts
3
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17
18 **SUMMARY:**
19 This protocol presents a radiolabeled amino acid uptake assay, which is useful for evaluating
20 amino acid consumption either in primary cells or in isolated bones.
21
22 **ABSTRACT:**
23 Bone development and homeostasis is dependent upon the differentiation and activity of bone
24 forming osteoblasts. Osteoblast differentiation is sequentially characterized by proliferation
25 followed by protein synthesis and ultimately bone matrix secretion. Proliferation and protein
26 synthesis require a constant supply of amino acids. Despite this, very little is known about amino
27 acid consumption in osteoblasts. Here we describe a very sensitive protocol that is designed to
28 measure amino acid consumption using radiolabeled amino acids. This method is optimized to
29 quantify changes in amino acid uptake that are associated with osteoblast proliferation or
30 differentiation, drug or growth factor treatments, or various genetic manipulations. Importantly,
31 this method can be used interchangeably to quantify amino acid consumption in cultured cell
32 lines or primary cells *in vitro* or in isolated bone shafts *ex vivo*. Finally, our method can be easily
33 adapted to measure the transport of any of the amino acids as well as glucose and other
34 radiolabeled nutrients.
35
36 **INTRODUCTION:**
37 Amino acids are organic compounds that contain an amino (-NH$_2$) and carboxyl (-COOH)
38 functional groups with a variable side chain that is specific to each amino acid. In general, amino
39 acids are well known as the basic constituent of protein. More recently, novel uses, and functions
40 of amino acids have been elucidated. For example, individual amino acids can be metabolized to
41 generate intermediate metabolites that contribute to bioenergetics, function as enzymatic
42 cofactors, regulate reactive oxygen species or are used to synthesize other amino acids[1–10]. Many
43 studies demonstrate that amino acid metabolism is critical for cell pluripotency, proliferation,
44 and differentiation in various contexts[3,6,11–17].

45

46 Osteoblasts are secretory cells that produce and secrete the Collagen Type 1 rich extracellular
47 bone matrix. To sustain high rates of protein synthesis during bone formation, osteoblasts
48 demand a constant supply of amino acids. To meet this demand, osteoblasts must actively
49 acquire amino acids. Consistent with this, recent studies reveal the importance of amino acid
50 uptake and metabolism in osteoblast activity and bone formation[15–20].

51

52 Osteoblasts acquire cellular amino acids from three major sources: extracellular milieu,
53 intracellular protein degradation and *de novo* amino acid biosynthesis. This protocol will focus
54 on the evaluation of amino acid uptake from extracellular environment. The most common
55 methods to measure amino acid uptake rely on either radiolabeled (e.g., ^{3}H or ^{14}C) or heavy
56 isotope labeled (e.g., ^{13}C) amino acids. Heavy isotopomer assays can analyze amino acid uptake
57 and metabolism more thoroughly and safely but are more time consuming taking multiple days
58 to complete as it takes a day to prepare and derivatize samples and multiple days to analyze on
59 the mass spectrometer depending on the number of samples[21,22]. By comparison, radiolabeled
60 amino acid uptake assays are not informative about downstream metabolism but are cheap and
61 relatively fast, being able to be completed within 2–3 h from the start of the experiment[23,24].
62 Here, we describe an easily modifiable basic protocol designed to evaluate radiolabeled amino
63 acid uptake in cultured primary cells or cell lines *in vitro* or individual bone shafts *ex vivo*. The
64 application of these two protocols can be extended to other radiolabeled amino acids and other
65 bone associated cell types and tissues.

66

67 **PROTOCOL:**
68 All mouse procedures described herein were approved by the Animal Studies Committees at the
69 University of Texas Southwestern Medical Center at Dallas. The radiation protocol was approved
70 by the Radiation Safety Advisory Committee at the University of Texas Southwestern Medical
71 Center at Dallas.

72

73 **1.   Amino acid uptake in cells (Protocol I)**

74

75 1.1.   Plate 5 x 10^4 ST2 cells in each well of a 12-well tissue culture plate. Plate cells in α-MEM
76 containing 10% FBS, 100 U/mL penicillin and 0.1 µg/mL streptomycin (Pen/Strep). Plate extra
77 wells of cells to quantify the cell number per condition for the normalizations in step 1.13.
78 Incubate cells in a humidified cell culture incubator at 37 °C with 5% CO_2.

79

80 1.2.   Culture the cells for 2–3 days until confluent.

81

82 1.3.   On the day of the experiment, prepare the following solutions: 1x Phosphate Buffered
83 Saline (PBS), pH 7.4 and Krebs Ringers HEPES (KRH) buffer, pH 8.0: (120 mM NaCl, 5 mM KCl, 2
84 mM CaCl$_2$, 1 mM MgCl$_2$, NaHCO$_3$, 5 mM HEPES, 1 mM D-Glucose). Prewarm to 37 °C.

85

86 1.4.   Aspirate the medium and wash the cells twice with 1 mL of 1x PBS, pH 7.4.

87

88 NOTE: This protocol is also appropriate for rapidly dividing non-confluent cells. In this case, it is

important to normalize the radioactivity to either absolute cell number or DNA content. In addition, consider increasing the size of the culture plate or flask to increase the overall cell number and cpm values. This is important to determine empirically on an individual basis.

1.5.   Aspirate 1x PBS and wash the cells once with 1 mL KRH.

1.6.   Make 4 µCi/mL L-[3,4-^{3}H]-Glutamine working media by diluting 4 µL of [1 µCi µL^{-1}] L-[3,4-^{3}H]-Glutamine stock per 1 mL of KRH.

NOTE: Before using radioactive materials, please contact the Office of Radiation Safety at your home institution to obtain approvals. All the procedures related to radiation must be performed behind the plexiglass shield.

1.7.   Incubate cells with 0.5 mL of KRH containing 4 µCi/mL L-[3,4-^{3}H]-Glutamine working media for 5 min.

1.8.   Collect the radioactive medium and dispense into the liquid waste container. Wash the cells three times briefly with ice-cold KRH to terminate the reaction. Collect and discard all the washes in the radioactive liquid waste container.

1.9.   Add 1 mL of 1% SDS to each well and triturate 10x to lyse and homogenize the cells. Transfer the cell lysates to 1.5 mL tubes. Discard cell culture plates, serological pipettes, and pipette tips in the solid radioactive waste container.

1.10.   Centrifuge at >10,000 x *g* for 10 min. Transfer the supernatants to scintillation vials containing 8 mL of the scintillation solution. Discard tubes and pipette tips in solid radioactive waste container.

1.11.   Read radioactivity in counts per minute (cpm) using a Scintillation counter. Discard scintillation vials in scintillation vial waste container.

1.12.   Trypsinize, resuspend, and count the cells from the remaining non-radioactive plates of cells (see step 1.1) to estimate the number of cells in the lysed, radioactive cultures. Using a hemocytometer, count the number of cells per non-radioactive well for each experimental condition. Normalize the cpm from step 1.11 to the estimated cell number from the non-radioactive plates.

1.13.   After completion of the experiment, decontaminate the cell culture hood, bench, and all instruments with a radioactivity decontaminant spray. Finally, perform wipe tests to ensure the working area is radiation-free.

2.   Amino acid uptake in freshly isolated bone tissues (Protocol II)

2.1.   Prewarm KRH to 37 °C.

133

134 2.2.   Euthanize a 3-day-old mouse and remove the skin on the arms. Disarticulate the humeri
135 from the shoulder using scissors and dissect out both humeri. Remove all extemporaneous
136 tissues using a scalpel and forceps. Remove the epiphyses from the bone.

137

138 NOTE: In addition to humerii, this protocol can be adapted to neonatal femora, tibiae and
139 calvariae as well as humerii, femora and tibiae isolated from 2- and 4-month-old mice
140 (unpublished data).

141

142 2.3.   Flush out marrow from the bone and weigh the bone shafts to normalize in step 2.14.

143

144 2.4.   Boil one humerus in 1x PBS at 100 °C for 10 min to decellularize the bone. The
145 decellularized boiled bone is used as a negative control.

146

147 NOTE: As a quality control, paraffin embed the boiled and non-boiled bones for histological stains
148 to visually confirm that boiling was efficacious in decellularizing the bone.

149

150 2.5.   Equilibrate both humeri in 1 mL of KRH for 30 min in the cell culture incubator at 37 °C.

151

152 2.6.   Make a working solution of 4 µCi/mL L-[2,3,4-³H]-Arginine in KRH by diluting 4 µL of
153 1µCi/µL L-[2,3,4-³H]- Arginine stock per 1 mL KRH.

154

155 NOTE: This protocol is appropriate to evaluate the uptake of whichever radiolabeled nutrient is
156 chosen. L-[2,3,4-³H]-glutamine, L-[¹⁴C_U]-alanine, and L-[2,3-³H]-proline have been tested with
157 similar results. Arginine data is shown to highlight the utility of this protocol.

158

159 CAUTION: All the procedures using radiation must be performed behind the plexiglass shield
160 while using appropriate personal protective equipment (PPE).

161

162 2.7.   Incubate both the experimental and boiled control bone separately in KRH containing 4
163 µCi/mL L-[2,3,4-³H]-Arginine for up to 90 min at 37 °C.

164

165 NOTE: It is important to empirically determine the incubation times for different conditions
166 including the age of the mice, different skeletal elements and the types of radiolabeled amino
167 acids by performing a time course. Saturation mostly occurs after approximately 3–4 h. Uptake
168 should be evaluated at a timepoint in the linear rage of uptake.

169

170 2.8.   Remove the radioactive medium and discard in the liquid radioactive waste container.
171 Wash humeri three times using ice cold 1 mL of KRH to terminate the reaction. Discard all the
172 washes in the liquid radioactive waste container.

173

174 2.9.   Transfer each bone into a 1.5 mL tube. Add 500 µL of RIPA buffer [150 mM NaCl; 5 mM
175 EDTA; 50 mM Tris (pH 8.0); 0.5% NP40 (v/v); 0.5% DOX (w/v); 0.1% SDS (w/v)]. Discard the culture
176 plates, serological pipettes, and pipette tips in a radioactive solid waste container.

177
178 2.10.   Homogenize the humeri by chopping 100 times with scissors in a RIPA buffer.

179
180 2.11.   Sonicate (Amplitude: 35%, Pulse 1 s) the resulting homogenized bone for 10 s.

181
182 NOTE: Sonication is also known to produce aerosols. Sonication steps can be performed in a
183 biological safety cabinet. To reduce aerosol formation, it is important to not overfill the tubes.
184 Sonication of small sample volumes can result in incomplete sonication or sample loss due to
185 foaming. If foaming occurs, centrifuge the sample for 5 min to settle.

186
187 2.12.   Clarify the lysate by centrifugation for 10 min at >10,000 x g at room temperature.
188 Transfer 200 µL of the supernatant to scintillation vials containing 8 mL of the scintillation
189 solution. Discard all solid radioactive waste (e.g., used tubes, pipette tips, plates, etc.) in the solid
190 radioactive waste container.

191
192 2.13.   Read radioactivity (cpm) using the Scintillation counter. Discard used scintillation vials in
193 the radioactive waste container designated for scintillation vials.

194
195 2.14.   Subtract the cpm of boiled bone (basal value) from the cpm of experimental bone.
196 Normalize the radioactivity with bone weight from step 2.3.

197
198 2.15.   Spray the cell culture hood, instruments, and bench with radioactivity decontaminant.
199 Perform wipe tests to confirm the working area is radiation-free.

200
201 **REPRESENTATIVE RESULTS:**
202 Amino acid transport is regulated by many membrane-bound amino acid transporters that have
203 been categorized into distinct transport systems based on numerous characteristics, including
204 substrate specificity, kinetics, as well as ion and pH dependence[25]. For example, glutamine uptake
205 can be mediated by the Na^+-dependent transport systems A, ASC, γ+L and N or the Na^+-
206 independent System L. The Na^+-dependent systems are distinguished by the ability to substitute
207 Li^+ for Na^+ (System N) or sensitivity to the amino acid analogue 2-(methylamino)isobutyric acid
208 (MeAIB) (System A). The purpose of this experiment was to define the amount of glutamine
209 consumption attributable to each transport system. L-{3,4-^{3}H} glutamine uptake was measured
210 in confluent ST2 cells in normal KRH containing 120 mM NaCl, Na^+ free KRH containing 120 mM
211 Choline chloride, normal KRH containing 5 mM MeAIB or Na^+ free KRH containing 120 mM LiCl.
212 Total radioactivity (counts per minute) was normalized to cell number. Na^+ removal led to 90%
213 reduction in glutamine uptake. This indicated that System L accounts for 10% of glutamine uptake
214 whereas 90% of glutamine uptake is Na^+ dependent in ST2 cells (**Figure 2**). The presence of Li^+
215 only increased glutamine uptake by 2% compared to the Na^+ free condition whereas 5 mM MEAIB
216 did not affect glutamine uptake. These data indicate that the majority of glutamine uptake is
217 mediated by Systems ASC and γ + L while System N and System A are responsible for roughly 2%
218 and 0% of Na^+-dependent glutamine uptake, respectively.

219
220 We modified Protocol I to characterize amino acid uptake in bone shafts *ex vivo*. In this

221   experiment we followed Protocol II to characterize arginine uptake kinetics in isolated long bones
222   from 3-day-old (p3) mice. To do this, we first dissected both humeri from p3 mice. The epiphyses
223   were removed, and the marrow was spun out and each humerus was weighed for normalization.
224   The contralateral humerus was designated as a negative control and was boiled to kill cellular
225   activity. The experimental and boiled humeri were then incubated with radiolabeled 4µCi/mL L-
226   [2,3,4-^{3}H]-Arginine for up to 2 h. Arginine uptake increased in a linear manner over the course of
227   this experiment (**Figure 3A**). For comparison, the boiled humeri did not exhibit a dynamic
228   increase of arginine uptake in this experiment, but rather had a basal radioactivity that is
229   attributed to adsorption of the probe in the bone matrix. The normalized and corrected arginine
230   uptake is shown in **Figure 3B**.

231

232   **FIGURE AND TABLE LEGENDS:**

233

234   **Figure 1: Workflow of radiolabeled amino acid uptake assays**. Schematic overview of amino acid
235   uptake assays *in vitro* (Protocol I) and in bones *ex vivo* (Protocol II).

236

237   **Figure 2: Kinetic properties of L-[3,4-^{3}H]-Glutamine uptake in ST2 *in vitro***. (**A**) L-[3,4-^{3}H]-
238   Glutamine uptake assays performed in the presence (+) or absence (-) of sodium (Na$^+$), MeAIB or
239   lithium (Li$^+$) in ST2 cells. (**B**) Percentage of estimated contributions of System A, N, L, or ASC and
240   γ + L to glutamine uptake.

241

242   **Figure 3: *Ex vivo* L-[2,3,4-^{3}H]-Arginine uptake in neonatal humeri**. (**A**) L-[2,3,4-^{3}H]-Arginine
243   uptake performed over a time course of 2 h in live and boiled humeri isolated from 3-day-old
244   C57BL/6 mice. (B) Normalized L-[2,3,4-^{3}H]-Arginine uptake with boiled humeri.

245

246   **DISCUSSION:**
247   The protocol described herein provides a fast and sensitive approach to evaluate amino acid
248   uptake in response to various experimental permutations either *in vitro* or *ex vivo*. When
249   compared to commercially available kits (e.g., Glutamine and Glutamate Determination Kit), this
250   method is much more sensitive, quicker, and less labor intensive[16,17,26]. In our protocol, we
251   evaluate uptake in the Krebs Ringers HEPES buffer. We utilize this media as it is a basal formula
252   that allows for rapid and easy modifications to test a diverse array of conditions to characterize
253   amino acid uptake. For example, to test whether the transport system is Na$^+$ dependent, we can
254   simply replace NaCl with choline chloride to make Na$^+$ free KRH. This flexibility is advantageous
255   to interrogate the various transport systems mediating uptake of individual amino acids. Basal
256   growth media such as alpha-MEM is also appropriate for this assay. In this case, we typically
257   purchase alpha-MEM lacking the individual amino acid in question, which is replaced with the
258   radiolabeled amino acid for the experiment. For example, to evaluate glutamine uptake we use
259   glutamine-free alpha-MEM in primary bone marrow stromal cells[15]. KRH does have some
260   limitations due to its simple composition. KRH is not an ideal option if the goal is to study
261   secondary active transport systems such as symporters and antiporters. For instance, leucine
262   uptake through *Slc7a5/Slc3a2* occurs in exchange for glutamine. In this case, leucine uptake may
263   be underestimated due to the lack of glutamine in KRH; rather, the use of leucine-free alpha-
264   MEM medium would be preferable in this instance. It is also important to note that the use of a

265 Geiger counter is not mandatory when working with isotope ^{3}H. However, it is advised to keep
266 the Geiger counter on as a courtesy to alert people that you are working with radiation.
267
268 A critical and mandatory control for the second protocol is the boiling of the contralateral bone.
269 This is necessary as the bone matrix can absorb radioactive amino acids independent of
270 facilitated transport by bone cells. By decellularizing the bone, we can estimate the amount of
271 radiation trapped by bone matrix and use it as the baseline radioactivity to normalize uptake.
272 Another important control is to weigh the bones after removal of the epiphyses and marrow. The
273 weight of the bone is used to normalize uptake relative to the size of the bone shaft. This is
274 important as the size of the bones may vary due to many factors ranging from variability during
275 the removal of the epiphyses to genetic mutations that affect growth. An alternative method is
276 to quantify DNA content of the bone shaft for normalization. In our experience, normalizing DNA
277 content is comparable to bone weight but adds more steps dealing with radioactive materials.
278 Thus, we prefer to normalize to bone weight.
279
280 These protocols can be adapted to cell lines, including ATDC5, MC3T3, 293, or other primary cells
281 such as calvarial osteoblasts, chondrocytes, bone marrow stromal cells, bone marrow
282 macrophages, and osteoclasts. In addition, the protocols are easily adaptable to evaluate other
283 radioactive isotopes (e.g., ^{14}C and ^{35}S) as well as different nutrients, including amino acids,
284 glucose, and fatty acids. Furthermore, protocol II can also be adapted to adult bones and pellet
285 cultures, with the consideration of adjustment to the incubation time. Although this protocol is
286 highly adaptable, it is important to characterize the kinetics of the new isotope labeled molecules
287 or in new cell lines before conducting experiments. The transport kinetics may vary drastically
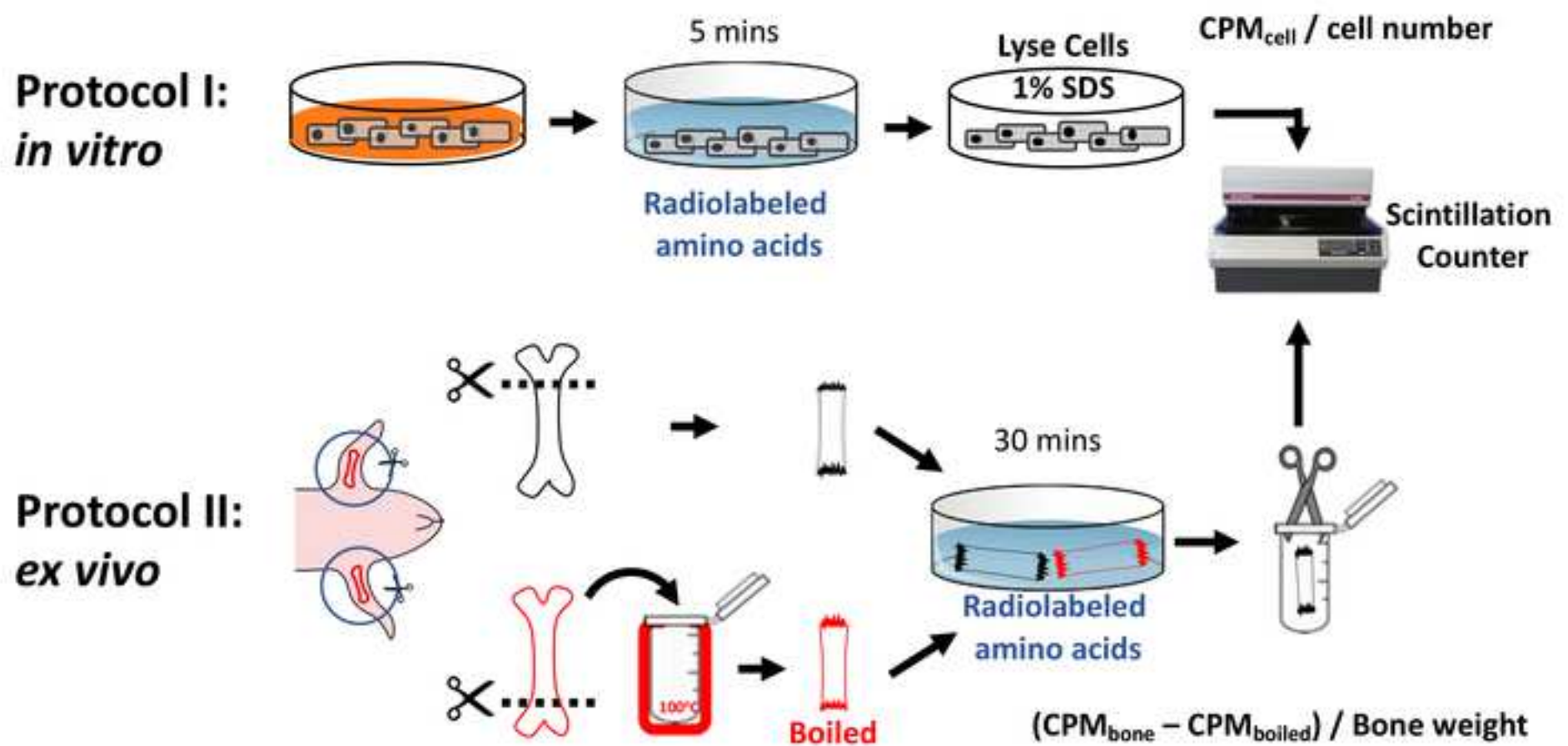288 between different molecules, different isotopes, or different cell types.
289

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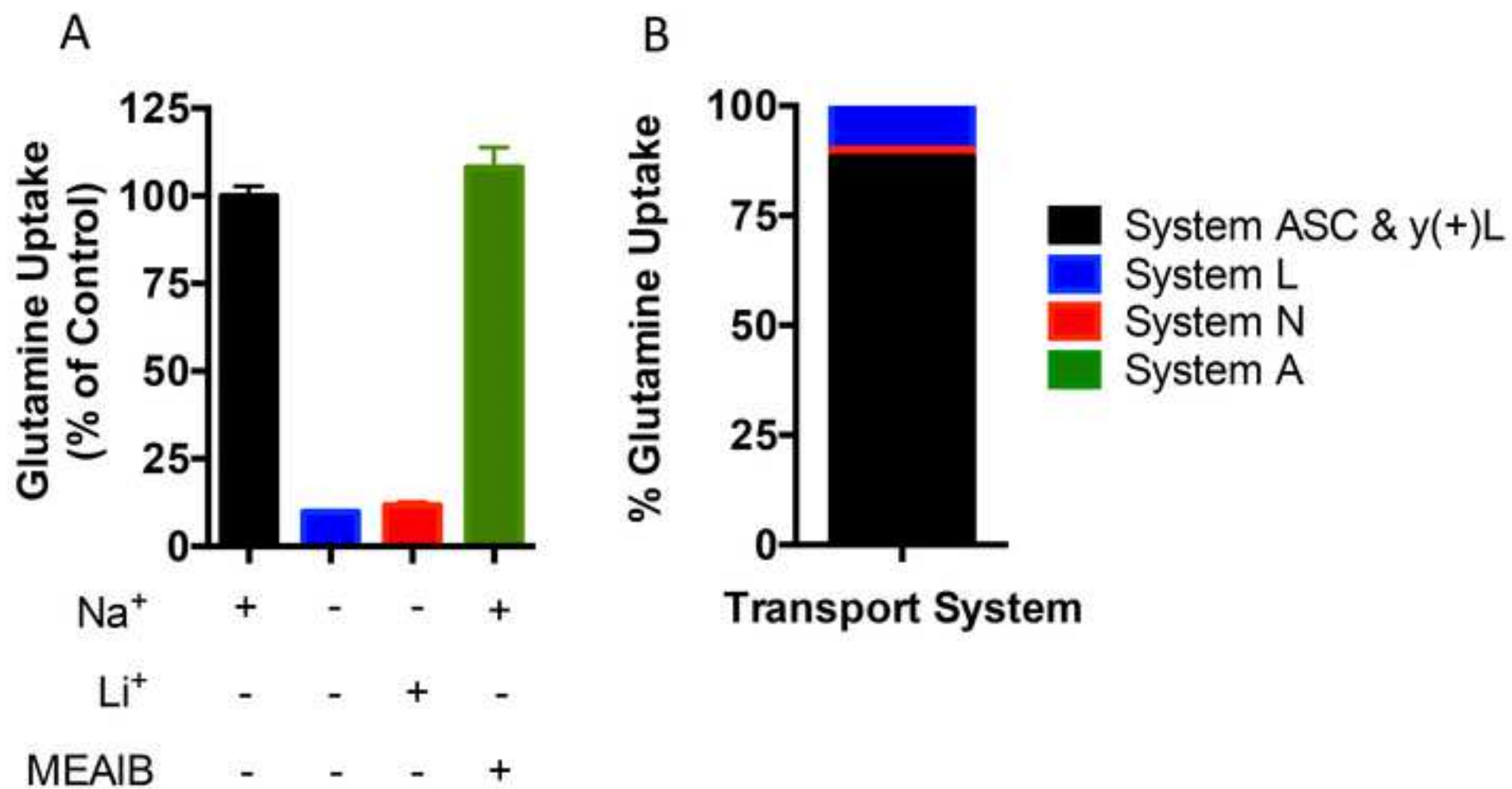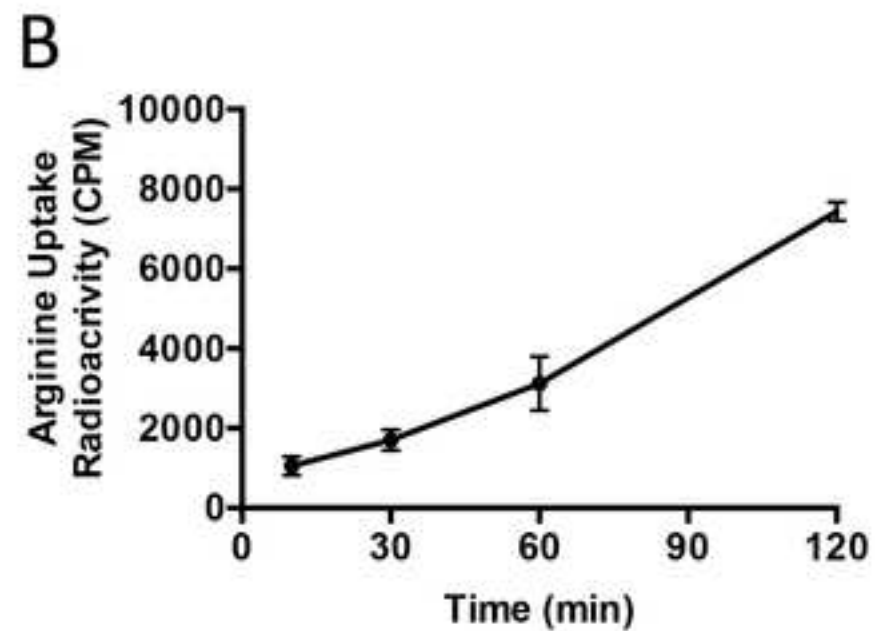355

Figure 1

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Protocol I: in vitro

5 mins

Lyse Cells 1% SDS

CPM_{cell} / cell number

Radiolabeled amino acids

Scintillation Counter

Protocol II: ex vivo

30 mins

Radiolabeled amino acids

Boiled

$(CPM_{bone} - CPM_{boiled})$ / Bone weight

Figure 2

Figure 3

Click here to access/download
Table of Materials
JoVE_Materials-1.xls

We have revised the manuscript according to the comments in the manuscript document. This includes moving the notes under the individual section and adding some additional information about volumes etc. To facilitate re-review, we left the track changes on in the document.