Supplementary Information

The Bacterial Metabolome and Metalome Weakens Global RNA Secondary Structure Stability

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Magnesium ion, Metabolites, Chelated magnesium, RNA folding, RNA function, near-cellular condition

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1. Supplementary methods

Isothermal Titration Calorimetry (ITC). Buffers were prepared by dissolving high purity (>95%) salts in purified (18 mΩ) water in volumetric flasks to the final concentrations described in Supplementary Table 1. A standard 1.00 M (±0.01) magnesium chloride solution from Sigma was diluted in buffer, or for high Mg^{2+} syringe concentrations (>15 mM), magnesium chloride hexahydrate was dissolved in buffer and then the concentration was determines with atomic absorption spectroscopy, to ensure accurate magnesium chloride concentrations. Samples were degassed at the experimental temperature using a ThermoVac (MicroCal, LLC) before loading into a VP-ITC MicroCalorimeter (MicroCal, LLC) according to the manufacturer's recommendations with pure (18 mΩ) water in the reference cell. Titration was performed with a 10 µcal/sec reference power and a stirring speed of 310 rpm. Twenty nine total injections (one 2 µL injection followed by twenty eight 10 µL injections) were performed at an injection rate of 0.5 µL/sec with a 150 sec equillibration period following each injection. The first injection was not included in subsequent analysis.

Data were analyzed in R (version 4.1.0) using ITC parsing and non-linear regression fitting functions in MetaboMgITC (https://github.com/JPSieg/MetaboMgITC). Injections where metabolites were >90% saturated with bound Mg^{2+} were not fit in order to minimize the time required for the experiment, thus minimizing the contribution of possible ligand degradation to the fit values (fit values from ITC experiments do not depend on saturation levels from 70% to 100% saturation).

Apparent K_D calculation. Apparent metabolite-Mg^{2+} disassociation constants at pH 7 and an ionic strength of 0.38 M were calculated using MetaboMgITC, which is a compiled database of protonation constants and divalent magnesium binding constants for cellular metabolites (https://github.com/JPSieg/MetaboMgITC), and provides facile functions for calculating Apparent metabolite-Mg^{2+} disassociation constants.

Artificial cytoplasm preparation. Artificial cytoplasms were prepared to maintain a constant monovalent ion concentration of 240 mM Na^+ and 140 mM K^+ and a pH of 7. First, metabolite salts or solutions were dissolved or diluted in purified (18 mΩ) water in volumetric flasks. Second, the pH of each solution was adjusted to 7.0 using concentrated HCl, 10 M NaOH, and 5 M KOH. The amount of Na^+ or K^+ added was recorded. Third, 5 M NaCl and 2 M KCl were added for a final Na^+ and K^+ concentration of 480 mM and 140 mM, respectively. Enough Mg^{2+} (1.00±0.01 M magnesium chloride solution) for a final free Mg^{2+} concentration of 2 mM was added for fluorescence melting curves and rRNA chemical stability assays.Then, the volumetric flask was filled to 10 mL. Lastly, a test quantity of the 2x solution was diluted to a 1x concentration and the pH was tested with a VWR Symphony pH probe. The 2x concentrated artificial cytoplasm was alliquoted, stored at -20 °C, and used within one week to minimize metabolite degradation. Detailed recipes for each artificial cytoplasm are available in supplementary tables 3, 4, 5, and 6.

Determination of Mg^{2+} speciation with HQS fluorescence. 8-Hydroxy-5-quinolinesulfonic (HQS) acid hydrate (98% purity) was purchased from Sigma Aldrich and recrystallized 10 times to further purify and remove trace metal contamination. Purified HQS was diluted into 20 mM MOPS 0.01 mM EDTA 0.001% (g/mL) sodium dodecyl sulfate pH 7.0 buffer to a stock

concentrations of 100 mM (determined with UV absorbance using $\varepsilon_{326\ nm} = 260\ M^{-1}cm^{-1}$). HQS was then diluted to a final concentration of 50 μM in 240 mM Na^+ 140 mM K^+ in the same buffer and variable concentrations of Mg^{2+} and metabolites. Absorbance scans were performed on a Hewlet Packert OLIS 8452A Diode Array Spectrometer. Fluorescence emission scans were performed on an HORIBA Jobin Yvon Fluoromax-3 Spectrofluorometer with a 355 nm excitation. Mg^{2+} titrations with metabolites were performed on a Biotek Cytation 3 plate reader with and excitation of 355 nm and emission of 500 nm. Triplicate solutions, with and without metabolite chelators, were prepared with 0, 0.5, 1, 1.5, 2, 3, 5, 10, 15, 20, 30, 40, 50, 60, 80, and 150 mM magnesium chloride. The fluorescence of control experiments with 10 mM EDTA was compared to the 0 mM magnesium chloride condition to check for divalent metal contamination in the artificial cytoplasms.

Data analysis was performed in R (version 4.1.0) to determine free Mg^{2+} concentrations. First, the fluorescence in the absence of metabolite chelators were fit to equation 1 using the *nls* package in base R, in order to determine the inverse molar apparent association constant for HQS binding to Mg^{2+} (K_{HQS}).

$$I = (I_{max} - I_{min}) \frac{K_{HQS} Mg_{free}}{1 + K_{HQS} Mg_{free}} + I_{min} \quad (1)$$

Mg_{free} is the free Mg^{2+} concentration in the sample, which is equal to the total concentration of magnesium chloride in the absence of chelators. I_{max} and I_{min} is the intensity of the Mg^{2+}-HQS complex and the intensity of free HQS respectively. Then, the fluorescence for all samples were normalized using I_{max} and I_{min} to produce a normalized fluorescence intensity I_{norm}.

$$I_{norm} = \frac{(I - I_{min})}{I_{max} - I_{min}} \quad (2)$$

Free Mg^{2+} concentrations for each sample were calculated with equation 3 using I_{norm} and K_{HQS}.

$$Mg_{free} = \frac{I_{norm}}{K_{HQS}(1 - I_{norm})} \quad (3)$$

The total Mg^{2+} concentration (Mg_{total}) required to obtain 2 mM free Mg^{2+} in the presence of chelators was approximated numerically using linear polynomials in the form:

$$Mg_{free} = \sum_{i=0}^{N} A_i Mg_{total}^{i} \quad (4)$$

Where N is the order of the polynomial and A is a constant determined by linear regression. The free Mg^{2+} concentration as a function of the total Mg^{2+} were fit to 1st, 2nd, 3rd, 4th , 5th , 6th, 7th, 8th, 9th and 10th order polynomials. Fits were evaluated by calculating the Akaike information criterion (AIC) with the *model.sel* function in the *MuMIn* R package, in order to determine which polynomial best described the data using the minimum number of terms. The polynomial with the lowest AIC was then solved numerically to calculate the the total Mg^{2+} required to generate 2 mM free Mg^{2+} in the presence of metabolites, with a tolerance of 0.0001 mM.

Fluorescence melting curves. 5´-FAM labeled and 3´-BHQ1 RNA were ordered from Integrated DNA Technology (IDT) and HPLC purified by ITD or in house. For in house HPLC, RNA peaks were manually collected on a Water's AQUINITY Arc UPLC system equipped with a Waters Xbridge C18 2.3 μm 4.6x150 mm column. 5 nmole of RNA were injected to a 60 °C preheated column and separated for 5 minutes with 100% 0.1 M aqueous Triethylamine acetate (TEAA), followed by a 25 min transition to 100% 0.1 M TEAA 20% acetonitrile, followed by 5 minutes of 100% 0.1 M TEAA, and then a 5 min transition back to 100% aqueous 0.1 M TEAA, at a flow rate of 1 mL/min. Samples were dried under vacuum, and resuspended in 20 mM MOPS 0.01 mM EDTA 0.001% (g/mL) sodium dodecyl sulfate pH 7.0 buffer. Samples were dialyzed to at least a 1/3125 dilution using either 3 kDa Amicon Ultra 0.5 mL Centrifugal Filter Devices or an 8 well Life Technologies Microdialysis System equipped with a 1 kDA dialysis membrane (SpectraPore). Dialyzed RNA concentrations were determined using the at absorbance at 260 nm and extinction coefficients provided by IDT. Samples were stored at -20 °C until the day of the experiment. Samples were prepared in triplicate, where FAM-RNA were diluted to 200 nM and BHQ1-RNA were diluted to 100, 150, 200, 400, 800, and 1000 nM. The 2xconcentrated salts or artificial cytoplasms were diluted into the same samples to achieve a final 1x concentration. Samples were heated to 90 °C for 1.5 min, cooled at at room temperature for 20 min, transfered into a qPCR plate, sealed with transparent qPCR film, and centrifuged for 2 min. Fluorescence emission as a function of temperature was quantified using an Applied Biosystems Step 1 Plus qPCR instrument. Samples were heated from 20 to 80 °C at a ramp rate of 0.5 °C per minute, recording fluorecence at 0.5 °C increments. Normalized FAM emission (using ROX as a passive reference dye) was exported from the Applied Biosystems software and analyzed in R (version 4.1.0). The thermodenaturation point (T_M) was identified by finding the maximum of the first derivative of emission (smoothed by 8 datapoints, or 4 °C) as a function of temperature.

rRNA chemical stability assays. 16S ribosomal RNA (rRNA) were purified from E. coli strain BW25113 and 5´-P32 labeled. Starter cultures were precultured in LB media at 37 °C for ~16 hr. Then the cells were 0.5% seeded into a 30 mL LB culture and grown in a 37 °C shaker waterbath until the OD_{600} reached 0.6 OD. Cells were treated with 30 μL of 20 mg/mL chloramphenicol and snap cooled in ice water. The culture was centrifuged at 12,000 xg for 5 min at 4 °C, the media was decanted, and total RNA were extracted with TRIzol (Thermo Fisher Scientific). 16S rRNA were purified from total RNA by electroluting (ELUTRAP Whatman) excised 16S rRNA bands from a SYBR gold stained 1% agarose gel. Electroluted 16S rRNA were ethanol precipitated in >70% ethanol containing 250 mM NaCl buffer for 30 min on dry ice, spun down for 30 min at 16,000 xg at 4 °C, dried at room temperature, and resuspended in water. Then, 10 pmol of 16S rRNA were 5´-P23 labeled by incubating the 16S rRNA in 0.5 μM γ-P32-ATP with 1.25 U/μL T4 Polynucleotide kinase (PNK) with 1xPNK buffer (New England Biolabs). Kinased products were excised from a denaturing (8.3 M urea) 3% (g/mL) acrylamide 0.5 % (g/mL) agarose gel containing 1xTBE, crush and soaked overnight in TEN_{250} 4 °C, ethanol precipitated, and resuspended in 20 mM MOPS 0.01 mM EDTA 0.001% (g/mL) sodium dodecyl sulfate pH 7.0 buffer. Lastly, samples were dialyzed to at least a 1/3125 dilution using 3 kDa Amicon Ultra 0.5 mL Centrifugal Filter Devices and stored at -20 °C.

For degradation assays, 5,000 cpm/μL RNA was incubated in triplicate at 25, 31, 37, 43, and 49 °C on a PCR block. Time points were taken at 0 min, 10 min, 20 min, 40 min, 1 hr, 2 hr, 6 hr, and 12 hrs by quenching 10 μL of reaction in 10 μL of 95% formamide containing 50 mM

EDTA pH 8.5 buffer with trace xylene cyanol and bromophenol blue, and stored at -20 °C . Then, 5 µL of sample were ran on a preran 4 °C 3% acrylamide 0.5% agarose 1xTBE gel at 200 V for 5 hours. The gel was dried, exposed on a phosphoimager cassette, and scanned with a Typhoon Phosphoimager. Gels images were quantified with imageJ (https://imagej.nih.gov/ij/index.html) and quantifications were fit to single exponential kinetics in R (version 4.1.0) with the base R *nls* package.

2. Supplementary Figures

Supplementary Figure 1

3. Supplementary Tables

Supplementary Table 1. Samples in the syringe and cell for isothermal titration calorimetry experiments.

Ligand	Temperature (°C)	Syringe	Cell
ATP	25, 31, 37	15 mM $MgCl_2$, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)	0.1 mM ATP, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)
ATP	43, 49	15 mM $MgCl_2$, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)	0.2 mM ATP, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)
GTP	25, 31, 37, 43, 49	15 mM $MgCl_2$, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)	0.2 mM GTP, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)
UTP	25, 31, 37, 43, 49	15 mM $MgCl_2$, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)	0.2 mM UTP, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)
dTTP	25, 31, 37, 43, 49	15 mM $MgCl_2$, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)	0.2 mM dTTP, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)
Glucose 6-P	25	100 mM $MgCl_2$, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)	5 mM Glucose 6-P, 240 mM NaCl, 140 mM KCl, 10 mM Sodium HEPES (pH 7.0)

Supplementary Table 2. Fit results from isothermal titration calorimetry analysis of Mg^{2+} binding to selected ligands.

Metabolite	Temp. (°C)[a]	K'[b] (1/M)	K_D'[c] (mM)	dG[c] (kcal/mol)	dH[b] (kcal/mol)	dS[c] (Kcal/mol/K)	%Saturation[d]
ATP	25.07 (±0.00)	3,940 (±30)	0.254 (±0.002)	-4.91 (±0.00)	1.89 (±0.00)	0.27 (±0.00)	89.81 (±0.04)
ATP	31.03 (±0.01)	4,340 (±40)	0.230 (±0.002)	-5.06 (±0.01)	1.90 (±0.01)	0.22 (±0.00)	89.78 (±0.08)
ATP	37.03 (±0.01)	3,600 (±200)	0.280 (±0.01)	-5.05 (±0.03)	1.83 (±0.04)	0.19 (±0.00)	89.7 (±0.5)
ATP	43.02 (±0.01)	7,000 (±300)	0.142 (±0.008)	-5.57 (±0.03)	1.30 (±0.02)	0.16 (±0.00)	89.4 (±0.4)
ATP	49.02 (±0.01)	6,100 (±100)	0.164 (±0.003)	-5.58 (±0.01)	1.48 (±0.01)	0.14 (±0.00)	89.9 (±0.1)
dTTP	25.03 (±0.01)	4,980 (±30)	0.201 (±0.002)	-5.04 (±0.01)	2.17 (±0.01)	0.29 (±0.00)	89.89 (±0.09)
dTTP	31.02 (±0.01)	5,590 (±70)	0.179 (±0.002)	-5.22 (±0.01)	2.21 (±0.01)	0.24 (±0.00)	89.6 (±0.1)
dTTP	37.02 (±0.01)	6,200 (±100)	0.163 (±0.003)	-5.38 (±0.01)	2.20 (±0.01)	0.20 (±0.00)	90.0 (±0.2)
dTTP	43.04 (±0.01)	8,600 (±200)	0.116 (±0.003)	-5.70 (±0.01)	1.49 (±0.01)	0.17 (±0.00)	89.8 (±0.2)
dTTP	49.02 (±0.01)	5,400 (±50)	0.186 (±0.002)	-5.50 (±0.01)	2.49 (±0.01)	0.16 (±0.00)	89.76 (±0.08)
GTP	25.19 (±0.00)	4,200 (±100)	0.237 (±0.008)	-4.95 (±0.02)	1.42 (±0.02)	0.25 (±0.00)	89.9 (±0.3)
GTP	31.02 (±0.00)	4,400 (±200)	0.230 (±0.01)	-5.07 (±0.03)	1.48 (±0.02)	0.21 (±0.00)	89.9 (±0.4)
GTP	37.02 (±0.01)	5,000 (±200)	0.200 (±0.008)	-5.25 (±0.03)	1.43 (±0.02)	0.18 (±0.00)	90.0 (±0.4)
GTP	43.02 (±0.01)	4,900 (±300)	0.210 (±0.01)	-5.34 (±0.03)	1.43 (±0.03)	0.16 (±0.00)	89.7 (±0.5)
GTP	49.04 (±0.01)	6,100 (±300)	0.165 (±0.008)	-5.58 (±0.03)	1.24 (±0.02)	0.14 (±0.00)	89.8 (±0.5)
UTP	25.03 (±0.00)	3,610 (±40)	0.277 (±0.002)	-4.85 (±0.01)	1.76 (±0.01)	0.26 (±0.00)	89.36 (±0.09)
UTP	31.03 (±0.00)	4,100 (±60)	0.244 (±0.003)	-5.03 (±0.01)	1.75 (±0.01)	0.22 (±0.00)	89.9 (±0.1)
UTP	37.03 (±0.01)	4,200 (±70)	0.238 (±0.004)	-5.14 (±0.01)	1.69 (±0.01)	0.18 (±0.00)	89.8 (±0.2)
UTP	43.03 (±0.01)	3,800 (±100)	0.265 (±0.007)	-5.17 (±0.02)	1.51 (±0.02)	0.16 (±0.00)	89.8 (±0.3)
UTP	49.02 (±0.01)	10,900 (±500)	0.092 (±0.004)	-5.95 (±0.03)	0.99 (±0.01)	0.14 (±0.00)	89.0 (±0.4)
Fructose 1,6-BP							
Fructose 1,6-BP							
Fructose 1,6-BP							
Fructose 1,6-BP							

Fructose 1,6-BP											
UDP-GlcNAC											
UDP-GlcNAC											
UDP-GlcNAC											
UDP-GlcNAC											
UDP-GlcNAC											
Glucose-6P											
Glucose-6P											
Glucose-6P											
Glucose-6P											
Glucose-6P											
6P-gluconic acid											
6P-gluconic acid											
6P-gluconic acid											
6P-gluconic acid											
6P-gluconic acid											

[a]Mean temperature of the cell during the experiment. Error repesents the standard deviation over the whole experiment.

[b]Non-linear regression to a Wiseman-isotherm fit parameters the *nls* package in base R. Error represents standard error in fit parameters calculated from the residuals by *nls*.

[c]Calculated from fit parameters dH and K. Errors were propagated from standard errors in dH, K, and temperature using error propagation rules.

[d]Calculated saturation of metabolite ligands by Mg^{2+}, by analytically solving the association constant equilibrium expression for total ligand, total Mg^{2+}, and K, after the last injection was that was fit .Errors were propagated from standard errors the fit K using error propagation rules.

Supplementary Table 3. Recipe for a 2x stock solution of the strong chelator artificial cytoplasm. Prepare in a 10 mL volumetric flask.

Step 1 mass reagents into a 10 mL volumetric flask							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Adenine triphoshate disodium salt hydrate	Sigma-Aldrich A2383	≥99%	551.14	19.26	385 µL of 500 mM	38.52	
Uridine triphoshate trisodium salt	Alpha-Azar J63427	≥98%	550.09	16.58	332 µL of 500 mM	49.74	
Guanosine triphoshate disodium salt	Sigma-Aldrich G8877	≥95%	523.18	9.74	195 µL of 500 mM	19.48	
Deoxy thymidine triphoshate disodium salt	Thermo R0171	≥99%	solution	9.24	924 µL of 100 mM	18.48	
MOPS	Sigma-Aldrich M3183	≥99.5	209.26	20	41.9 g	0	
EDTA	IBI scientific IB70182	≥99%	372.24	0.01	1 µL of 100 mM tetrasodium solution	0.04	
SDS	JT Baker 4095-04	≥99%	288.38	0.035 (0.001%)	10 µL of 35 mM (1%)	0.035	
Step 2 Add acid or base to to pH 7.0							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Hydrochloric acid	Millipore Sigma HX0603	NA	NA	NA	concentrated 36.5-38.0%		
Sodium hydroxide	Millipore Sigma Sx0593	≥97%	40.00	NA	µL of 10 M		
Potassium hydroxide	JT Baker 3140-11	≥85%	56.11	NA	µL of 5 M		
Step 3 Calculate how much Na^+ and K^+ was added							
					Sum	Na^+ (mM)	K^+ (mM)
					Total monovalent intermediate (mM)		
Step 4 Add NaCl and KCl for a total final monovalent concentration of 480 mM Na^+ and 280 mM K^+							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Sodium chloride	Dot Scientific DSS23020	≥99%	58.4		µL of 5 M		
Potassium chloride	Millipore Sigma P9541	≥99%	74.55		µL of 2 M		
Step 5 Calculate how much Na^+ and K^+ was added							
					Total monovalent final (mM)		
Step 6 If applicable add $MgCl_2$ to a final concentration of 2 mM free Mg^{2+}							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Magnesium chloride	Sigma-Aldrich M1028	≥99%	95.21		µL of 1 M		
Step 7 Fill to 10 mL							
Step 8 Check 1xConcentrated pH							
						Final pH	

Supplementary Table 4. Recipe for a 2x stock solution of the weak chelator artificial cytoplasm. Prepare in a 10 mL volumetric flask.

Step 1 mass reagents into a 10 mL volumetric flask

Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
L-glutamic acid potassium salt monohydrate	Millipore Sigma G1501	≥99%	203.23	192	0.3902 g		192
Glutathione, reduced, free acid	Cal biochem 3541	≥98%	307.32	33.2	0.102 g		
D-Fructose 1,6-bisphosphate trisodium salt hydrate	Sigma-Aldrich F6803	≥98%	406.06	30.4	304 µL of 1000 mM	91.2	
Uridine 5'-diphospho-N-acetylglucosamine disodium salt	Sigma-Aldrich U4375	≥98%	651.32	18.48	370 µL of 500 mM	36.96	
D-Glucose 6-phosphate dipotassium salt hydrate	Sigma-Aldrich G7375	≥98%	336.32	15.76	158 µL of 1000 mM		31.52
L-aspartic acid monopota-sium	Millipore Sigma A9256	≥98%	133.10	8.46	85 µL of 1000 mM		8.46
L-valine	Sigma Aldrich V0500	≥98%	117.15	8.04	80 µL of 1000 mM		
L-glutamine	Sigma G3126	≥99%	146.14	7.62	75 µL of 1000 mM		
6-Phosphogluconic acid trisodium salt	Sigma-Aldrich P6888	≥95%	342.08	7.54	151 µL of 1000 mM	22.62	
Sodium pyruvate	Sigma P8574	≥99%	110.04	7.32	73 µL of 1000 mM	7.32	
MOPS	Sigma-Aldrich M3183	≥99.5	209.26	20	41.9 g		
EDTA	IBI scientific IB70182	≥99%	372.24	0.01	1 µL of 100 mM tetrasodium solution	0.04	
SDS	JT Baker 4095-04	≥99%	288.38	0.035 (0.001%)	10 µL of 35 mM (1%)	0.035	

Step 2 Add acid or base to to pH 7.0

Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Hydrochloric acid	Millipore Sigma HX0603	NA	NA	NA	concentrated 36.5-38.0%		
Sodium hydroxide	Millipore Sigma Sx0593	≥97%	40.00	NA	µL of 10 M		
Potassium hydroxide	JT Baker 3140-11	≥85%	56.11	NA	µL of 5 M		

Step 3 Calculate how much Na^+ and K^+ was added

						Sum	Na^+ (mM)	K^+ (mM)
					Total monovalent intermediate (mM)			

Step 4 Add NaCl and KCl for a total final monovalent concentration of 480 mM Na^+ and 280 mM K^+

Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Sodium chloride	Dot Scientific DSS23020	≥99%	58.4		µL of 5 M		
Potassium chloride	Millipore Sigma P9541	≥99%	74.55		µL of 2 M		

Step 5 Calculate how much Na^+ and K^+ was added

					Total monovalent final (mM)		

Step 6 If applicable add $MgCl_2$ to a final concentration of 2 mM free Mg^{2+}

Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Magnesium chloride	Sigma-Aldrich M1028	≥99%	95.21		µL of 1 M		

Step 7 Fill to 10 mL

Step 8 Check 1xConcentrated pH

							Final pH

Supplementary Table 5. Recipe for a 2x stock solution of the total chelator artificial cytoplasm. Prepare in a 10 mL volumetric flask.

Step 1 mass reagents into a 10 mL volumetric flask							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Adenine triphoshate disodium salt hydrate	Sigma-Aldrich A2383	≥99%	551.14	19.26	385 µL of 500 mM	38.52	
Uridine triphoshate trisodium salt	Alpha-Azar J63427	≥98%	550.09	16.58	332 µL of 500 mM	49.74	
Guanosine triphoshate disodium salt	Sigma-Aldrich G8877	≥95%	523.18	9.74	195 µL of 500 mM	19.48	
Deoxy thymidine triphoshate disodium salt	Thermo R0171	≥99%	solution	9.24	924 µL of 100 mM	18.48	
L-glutamic acid potassium salt monohydrate	Millipore Sigma G1501	≥99%	203.23	192	0.3902 g		192
Glutathione, reduced, free acid	Cal biochem 3541	≥98%	307.32	33.2	0.102 g		
D-Fructose 1,6-bisphosphate trisodium salt hydrate	Sigma-Aldrich F6803	≥98%	406.06	30.4	304 µL of 1000 mM	91.2	
Uridine 5'-diphospho-N-acetylglucosamine disodium salt	Sigma-Aldrich U4375	≥98%	651.32	18.48	370 µL of 500 mM	36.96	
D-Glucose 6-phosphate dipotassium salt hydrate	Sigma-Aldrich G7375	≥98%	336.32	15.76	158 µL of 1000 mM		31.52
L-aspartic acid monopotassium	Millipore Sigma A9256	≥98%	133.10	8.46	85 µL of 1000 mM		8.46
L-valine	Sigma Aldrich V0500	≥98%	117.15	8.04	80 µL of 1000 mM		
L-glutamine	Sigma G3126	≥99%	146.14	7.62	75 µL of 1000 mM		
6-Phosphogluconic acid trisodium salt	Sigma-Aldrich P6888	≥95%	342.08	7.54	151 µL of 1000 mM	22.62	
Sodium pyruvate	Sigma P8574	≥99%	110.04	7.32	73 µL of 1000 mM	7.32	
MOPS	Sigma-Aldrich M3183	≥99.5	209.26	20	41.9 g		
EDTA	IBI scientific IB70182	≥99%	372.24	0.01	1 µL of 100 mM tetrasodium solution	0.04	
SDS	JT Baker 4095-04	≥99%	288.38	0.035 (0.001%)	10 µL of 35 mM (1%)	0.035	
Step 2 Add acid or base to to pH 7.0							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Hydrochloric acid	Millipore Sigma HX0603	NA	NA	NA	concentrated 36.5-38.0%		
Sodium hydroxide	Millipore Sigma Sx0593	≥97%	40.00	NA	µL of 10 M		
Potassium hydroxide	JT Baker 3140-11	≥85%	56.11	NA	µL of 5 M		
Step 3 Calculate how much Na^+ and K^+ was added							
					Sum	Na^+ (mM)	K^+ (mM)
					Total monovalent intermediate (mM)		
Step 4 Add NaCl and KCl for a total final monovalent concentration of 480 mM Na^+ and 280 mM K^+							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Sodium chloride	Dot Scientific DSS23020	≥99%	58.4		µL of 5 M		
Potassium chloride	Millipore Sigma P9541	≥99%	74.55		µL of 2 M		
Step 5 Calculate how much Na^+ and K^+ was added							
					Total monovalent final (mM)		

Step 6 If applicable add $MgCl_2$ to a final concentration of 2 mM free Mg^{2+}							
Reagent	Vendor (product #)	Purity	Molar mass (g/mL)	2x[Ligand] (mM)	Mass or volume	Na^+ added (mM)	K^+ added (mM)
Magnesium chloride	Sigma-Aldrich M1028	≥99%	95.21		µL of 1 M		
Step 7 Fill to 10 mL							
Step 8 Check 1xConcentrated pH							
						Final pH	

4. Supplementary References (Not updated)

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