bio-protocol

Published: Sep 05, 2022

Incorporation of a Chemically Diverse Set of Non-Standard Amino Acids into a Gram-Positive Organism

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Abstract

The incorporation of non-standard amino acids (nsAAs) within proteins and peptides through genetic code expansion introduces novel chemical functionalities such as photo-crosslinking and bioconjugation. Given the utility of *Bacillus subtilis* in fundamental and applied science, we extended existing nsAA incorporation technology from *Escherichia coli* into *B. subtilis*, demonstrating incorporation of 20 unique nsAAs. The nsAAs we succeeded in incorporating within proteins conferred properties that included fluorescence, photo-crosslinking, and metal chelation. Here, we describe the reagents, equipment, and protocols to test for nsAA incorporation at a small scale (96-well plate and culture tube scales). We report specific media requirements for certain nsAAs, including two variants for different media conditions. Our protocol provides a consistent and reproducible method for incorporation of a chemically diverse set of nsAAs into a model Gram-positive organism.

Keywords: *Bacillus subtilis*, Genetic code expansion, Synthetic biology, Non-standard amino acid, Noncanonical amino acid, Unnatural amino acid, Translational control

This protocol was validated in: Nat Commun (2021), DOI: 10.1038/s41467-021-25691-4

Background

For decades, non-standard amino acids (nsAAs) have been used to introduce chemistries that are not ordinarily found in biological systems through genetically encoded site-specific incorporation within target proteins in live cells. When incorporated within proteins, nsAAs have been used for bioconjugation (Chin et al., 2002b; Seitchik et al., 2012), biocontainment (Mandell et al., 2015; Rovner et al., 2015), photo-crosslinking (Chin et al., 2002a, 2002b), fluorescence (Wang et al., 2006), and biomaterial production (Israeli et al., 2020). Akin to the ribosomally mediated process of translation for standard amino acids, nsAAs require a unique aminoacyl-tRNA synthetase and tRNA pair that exhibits minimal cross talk with other cellular components for codon recognition and addition of the nsAA to the growing polypeptide chain. The systems used to enable this for nsAAs are referred to as orthogonal translation systems (OTS), and they usually contain an aminoacyl-tRNA synthetase (AARS) and tRNA from an evolutionarily distant microbial species, such as the archaea *Methanocaldococcus jannaschii* or *Methanomethylophilus alvus* (Dumas et al., 2015; Beranek et al., 2019). Most commonly, the OTS is engineered to have a CUA anticodon for incorporation of the nsAA at the amber stop codon, UAG. This technology has been developed primarily in *Escherichia coli* and mammalian cell lines, thus limiting its use to a handful of organisms (Dumas et al., 2015). Extensive characterization of nsAAs technology in these species has led to discoveries of specific enzyme mechanisms, protein–protein interactions, and protein structures (Xie et al., 2004; Ai et al., 2011; Zhao et al., 2020).

Bacillus subtilis is a prime target for expansion of nsAA incorporation technology due to its broad utility as a Gram-positive rhizobacterium model in applied and fundamental research. *B. subtilis* has been used to study a variety of biological phenomena, including asymmetric cell division, biofilm formation, and sporulation (Losick et al., 1986; Kearns et al., 2005; McKenney et al., 2013; Bisson-Filho et al., 2017), which could be further investigated through incorporation of functional nsAAs such as those for post-translational modifications or photo-crosslinking. Having been conferred the status *Generally Regarded As Safe* (GRAS) by the US FDA, *B. subtilis* has also been used as a probiotic and vaccine vector for plants, animals, and humans (Cutting, 2011; Oh et al., 2020). Such applications could benefit from the site-specific incorporation of nsAAs within proteins for augmented capabilities or for the implementation of safeguards such as synthetic auxotrophy. *B. subtilis* is commercially used to produce antibiotics, cosmetic small molecules, and proteins (Westers et al., 2004; Stein, 2005; Su et al., 2020; Park et al., 2021). Given the variety of potential applications for *B. subtilis* in fundamental and applied science, we must continue to advance the tools available to scientists working with this organism.

Recent work from our groups demonstrated the potential application of site-specific nsAA incorporation within proteins in *B. subtilis,* including validation of theoretical protein–protein interactions, and tuning of cell wall biosynthesis with nsAA titration (Stork et al., 2021). Our study also provided preliminary evidence of the portability of this technology from *E. coli* to *B. subtilis*. Thus, the method presented here describes the application of these technologies, but it also establishes a fundamental base upon which the community can continue to build new technologies.

Existing technology for nsAAs incorporation in *Bacilli* species has been limited to a specific, single nsAA for a specific purpose (Scheidler et al., 2020; Tian et al., 2020). Our method presents the first demonstration of incorporation of a broad spectrum of nsAAs within proteins in *B. subtilis* for a variety of functions, including bioconjugation, photo-crosslinking, and fine-tuned control of protein expression. We demonstrated the incorporation of 20 unique nsAAs with six different orthogonal translation systems. In particular, we were able to incorporate 13 unique nsAAs with a single OTS system providing a platform strain for a diverse range of applications. This method also demonstrates the best overall nsAA incorporation with an upwards of 60% of native protein produced, thus far the best reported in *B. subtilis*. Some existing limitations to this system include background incorporation of the nsAA at natural stop codon sites and apparent limitations of the incorporation level in rich media. Despite those, this method provides an opportunity to introduce new tools to chemical biology and *B. subtilis* communities.

Materials and Reagents

Consumables

1. 14 mL culture tubes (Fisher Scientific, Fisherbrand™, catalog number: 149566B)
2. Petri dishes (Fisher Scientific, Fisherbrand™, catalog number: FB0875713)
3. Multichannel reservoir (Fisher Scientific, Biotix™, catalog number: 12111089)
4. Deep well 96-well plates (Fisher Scientific, Fisherbrand™, catalog number: 12566611)
5. Black walled 96-well microplates (Fisher Scientific, Greiner-Bio, catalog number: 07-000-166)
6. Breathable microplate seal covers (Fisher Scientific, Andwin Scientific, catalog number: NC1660916)
7. 1 mL cuvettes (Fisher Scientific, Fisherbrand™, catalog number: 14955128)
8. PCR strips (Fisher Scientific, BrandTech, catalog number: 14380941)
9. Serological pipettes (Fisher Scientific, Basix, catalog number: 14-955-235)
10. 15 mL conical tubes (Fisher Scientific, Basix, catalog number: 14955237)
11. Inoculation loops/toothpicks (Loops: Fisherbrand™, catalog number: 22-363-602)

***B. subtilis* strains are available here:** https://bgsc.org/search.php?Search=bDS
Associated annotated DNA sequences are available in the supplemental data to the original Nature communications publication: https://static-content.springer.com/esm/art%3A10.1038%2Fs41467-021-25691-4/MediaObjects/41467_2021_25691_MOESM6_ESM.zip

Non-standard Amino Acids

1. L-4,4-Biphenylalanine (BipA) (Peptech, catalog number: AL506)
2. L-4-Azidophenylalanine (pAzF) (abcr-GmbH, catalog number: AB308874)
3. Coumarin-nsAA (CouAA) (Sigma-Aldrich, catalog number: 792551)
4. L-4-Benzoylphenylalanine (BpA) (Peptech, catalog number: AL660)
5. L-4-Boc-lysine (boc-K) (Chem Impex Int'l, catalog number: 00363)
6. L-5-Hydroxytryptophan (5OHW) (Sigma-Aldrich, catalog number: 107751)
7. L-4-methyl-phenylalanine (4MeF) (Peptech, catalog number: AL096)
8. L-4-propargyloxy-phenylalanine (pPrF) (Combi-Blocks, catalog number: QW-3179)
9. L-4-amino-phenylalanine (4AmiF) (Peptech, catalog number: AL305)
10. L-4-aminomethyl-phenylalanine (4AmiMeF) (Peptech, catalog number: AL300)
11. L-benzenepentanoic acid, alpha-amino (BzpA) (Peptech, catalog number: AL514)
12. L-4-Nitro-phenylalanine (pNitroF) (Peptech, catalog number: AL061)
13. L-4-cyano-phenylalanine (pCNF) (Fisher Scientific, catalog number: AAH63572MD)
14. L-4-Fluoro-phenylalanine (pFF) (Peptech, catalog number: AL021)
15. L-4-Iodo-phenylalanine (pIF) (Peptech, catalog number: AL261)
16. L-4-Acetyl-phenylalanine (pAcF) (Peptech, catalog number: AL624)
17. L-4-methoxy-phenylalanine (4MeOF) (Alfa aesar, catalog number: H63096)
18. L-2-Naphthylalanine (NapA) (Peptech, catalog number: AL121)
19. L-bipyridyl-phenylalanine (biPyrA) (No longer commercially available)
20. L-4-tert-Butyl-tyrosine (tBut-Y) (Sigma-Aldrich, catalog number: 533130)

Additional Reagents

1. IPTG (Thermo Scientific™, catalog number: FERR0392)
2. Glucose (Thermo Scientific™, catalog number: AA1109036)
3. Glutamate (Acros Organics, catalog number: 156211000)
4. Ammonia sulfate (Sigma-Aldrich, catalog number: A4418)
5. Magnesium chloride, anhydrous (Sigma-Aldrich, catalog number: M8266)

6. Calcium chloride, dihydrate (Sigma-Aldrich, catalog number: 223506)
7. Manganese chloride (Sigma-Aldrich, catalog number: 416479)
8. Zinc chloride, anhydrous (Fisher Scientific, catalog number: AA1235722)
9. Thiamine-HCl (Sigma-Aldrich, catalog number: T1270)
10. Hydrochloric acid (10N) (RICCA Chemical Company, catalog number: 3770-32)
11. Iron (III) chloride, anhydrous (Fisher Scientific, catalog number: AAA1628122)
12. Potassium phosphate (monobasic) (Sigma-Aldrich, catalog number: P9791)
13. MOPS (free acid) (Fisher Scientific, TCI America, catalog number: M0707)
14. Chloramphenicol (Sigma-Aldrich, catalog number: C0378)
15. Kanamycin sulfate (Sigma-Aldrich, catalog number: K4000)
16. Phosphate buffered saline (tablet form) (Sigma-Aldrich, catalog number: P4417)
17. Sodium hydroxide (Acros Organics, catalog number: 134070010)

Media

1. LB Broth, Lennox (Fisher Scientific, Fisher BioReagents™, catalog number: BP9722500)
2. LB Agar, Lennox (Fisher Scientific, Fisher BioReagents™, catalog number: BP9745500)
3. S750 (1 L) (see Recipes)
4. ammoniaS750 (1 L) (see Recipes)
5. 10× S750 salts (1 L) (see Recipes)
6. 100× metals (500 mL) (see Recipes)

Equipment

Note: Equipment specifications are largely recommendations as this protocol has been validated for a series of different instruments.

1. -80 °C freezer
2. Incubator (Thermo Scientific, catalog number: 51-028-065HPM) or Shaking Incubator (Infors-HT Multitrons)
3. Microplate readers (protocol performed on both the Molecular Devices SpectraMax i3X or BioTek H1M Synergy)
4. Spectrophotometer (Thermo Scientific, catalog number: 840301000)
5. Plate shaker (Thermo Scientific, catalog number: 88882005)
6. Rotor drum (FisherBrand, catalog number: 14-251-251)
7. Pipettes (Fisher Scientific, Eppendorf Research Plus)
8. Pipettor (Fisher Scientific, Eppendorf EasyPet 3, catalog number: 12-654-105)
9. Vortex mixer (Scientific Industries, Inc. Vortex-Genie 2, catalog number: SI-0236)

Software

1. Excel
2. Software associated with plate reader used

Procedure

A. S750 protocol for tubes and 96-well plates

1. Remove glycerol stock that contains desired *B. subtilis* strain from -80 °C freezer. Streak cells from the glycerol stock onto an LB agar plate. Antibiotics may be useful for confirming strain identity but are not

necessary as incorporation constructs are stable integrations. Incubate overnight at 37 °C. See Note 1 for essential controls that should be included in every experiment.

2. On the next day, pick desired colonies from the LB plate and transfer to 1 mL of S750 media in culture tubes. Mix vigorously by pipetting up and down to break up biofilms and promote suspension growth. These serve as the seed cultures for the experiment, without induction of heterologous gene expression. See Note 2 for optional media conditions.
3. Grow in a 37 °C shaking incubator or rotor drum for 4–6 h, to an OD between 0.2 and 0.7.
4. Prepare experimental media (which will serve to induce heterologous gene expression and nsAA incorporation) by adding the desired IPTG and nsAA to S750. IPTG final concentration is 1 mM, which is 1,000× of a 1 M stock solution. IPTG is typically added to all cultures including nsAA, except in the case of titration; for double titration see Figure 1. nsAA final concentration is usually 100 µM for most tyrosine-derived nsAAs. 1 mM should be used for boc-K, 5OHW, and CouAA. Similarly, other concentrations can be used for titration (see Figure 1 for an example). Different nsAAs will exhibit different titration dynamics due to the differences in binding to their cognate synthetases.
    a. For a 96-well plate format, a suggested higher throughput strategy is to prepare the master mix media unique to each nsAA in 15 mL tubes, then transfer to reservoir and distribute 300 µL with a 1 mL multichannel into a 2 mL deep well plate. In our experience, culture volumes above 300 µL do not aerate well in the deep 96-well plate format. See Figure 2 for the suggested media layout.
    b. For single culture tubes, use no more than 1 mL media per tube for aeration reasons.

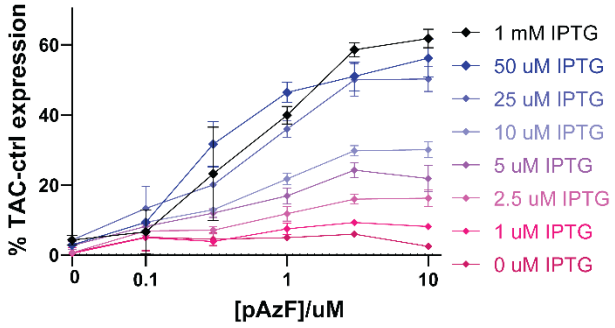

Figure 1. Double titration with nsAAs and transcriptional inducers.
A UAG-mNeongreen construct under the control of a pHyperspank promoter was expressed with variable levels of nsAA and IPTG; a very sensitive induction was possible with low levels of IPTG and titration of nsAA. The signal was normalized to a positive control construct, where a TAC tyrosine codon replaced the UAG stop codon and 1 mM IPTG was provided. For more details, see Data analysis.

Suggested plate layout		Columns				
Rows		1-3	4-6		7-9	10-12
A	WT Py79	+nsAA	-nsAA	Strain/nsAA 7	+nsAA	-nsAA
B	TAC + ctrl			Strain/nsAA 8		
C	Strain/nsAA 1			Strain/nsAA 9		
D	Strain/nsAA 2			Strain/nsAA 10		
E	Strain/nsAA 3			Strain/nsAA 11		
F	Strain/nsAA 4			Strain/nsAA 12		
G	Strain/nsAA 5			Strain/nsAA 13		
H	Strain/nsAA 6			Strain/nsAA 14		

Figure 2. Suggested plate layout for easy plate-filling.
This plate layout is easy to set up with a multichannel pipette with minimal errors.

5. Measure OD of the seed cultures, then dilute them with additional S750 to start the experimental cultures at a theoretical density of 0.002 with desired cells.
   a. For a 96-well plate setup, dilute cultures with S750 in a PCR tube to OD 0.2, then use a multichannel to distribute 3 µL across the plate as desired, to achieve a final OD of 0.002 in 300 µL of experimental media.
   b. For single culture tubes, dilute media appropriately to final OD 0.002. For example, add 4 µL of OD 0.5 seed culture to 1 mL of S750 containing IPTG and nsAA.
6. Incubate tubes/plate overnight, shaking at 37 °C.
   a. For 96-well deep well plates, cover with a breathable cover (see Note 4b). Example data are in Figure 3a.
   b. For clear-bottomed shallow 96-well plates used in time courses, cover with a transparent breathable cover. Shaking should be set to a fast speed, though either orbital or double orbital can be used. Read OD_{600} and fluorescence every 5–10 min. For the mNeongreen reporter, reading at 488/530 is acceptable, though many different filter settings will work for mNeongreen. Example data in Figure 3b.
7. For single culture tubes, grow in a 37 °C shaking incubator or rotor drum.
8. Read endpoint data after overnight growth. Extensively long growth periods will see some decrease of signal, but anything between 14 and 24 h is usually adequate.
   a. For fluorescence readout, dilute culture 1:1 with PBS, mix thoroughly, transfer 200 µL to clear-bottomed plates, and read OD and fluorescence. If the nsAA used is itself fluorescent, such as CouAA, spin down cells and wash them two to three times in PBS before resuspending in PBS for the plate reader experiment. If attempting microscopy, do washes in 1.5 mL tubes, carefully removing as much of the wash as possible in each case, and thoroughly resuspend pellets by pipetting. CouAA's broad fluorescence spectrum grants very high background unless it is sufficiently washed out.
   b. For nanoLuciferase readout, add 100 µL culture to a plate, take ODs, then add 100 µL of Nano-Glo luciferase reagent. The Nano-Glo will lyse cells, making OD measurements taken after addition unreliable.

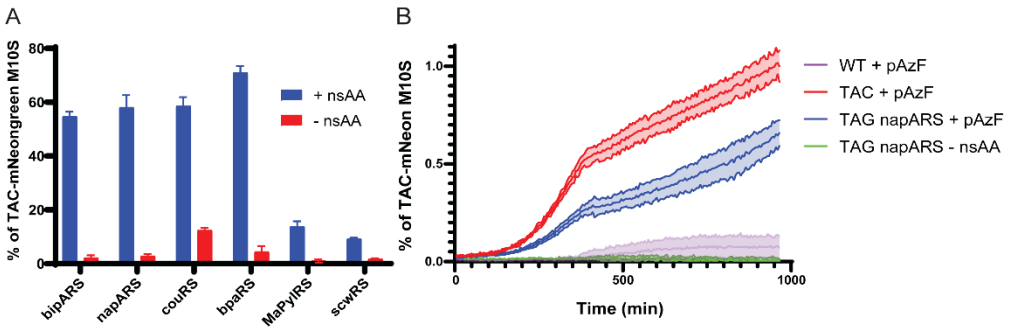

Figure 3. nsAA incorporation data.
(A) Endpoint data for nsAA incorporation, using different nsAAs for different AARSs: BipA for bipARS, pAzF for napARS, CouAA for CouRS, BpA for bpaRS, boc-K for the AbkRS. Data processed as discussed in Data Analysis. Biological triplicates were averaged; error bars represent the standard deviation. (B) Time courses for nsAA incorporation; shaded area is standard deviation among biological triplicates.

B. Variant for incorporation with ammoniaS750 (slower growth, less reliable, more accessible import)

1. When trying to do incorporation of nsAAs that do not import well into the cell (likely due to being large and hydrophobic), import may be improved by using media lacking competing amino acids, such as S750 where ammonia is the sole nitrogen source (see recipes for ammoniaS750).

2. *Bacillus* will grow in this media and incorporation will occur, but significantly more slowly. Replace both the seed culture and experimental media with ammoniaS750, and allocate roughly 50% more growth time, especially for the seed culture, which may take 6–8 h to reach a high enough OD for seeding (minimum OD is approximately 0.2).

C. Variant for incorporation with LB (less reliable, higher background, hindered cellular import)

1. Replace S750 LB starter culture and experimental cultures with LB.
2. Spin down cells and resuspend with PBS prior to measuring fluorescence. Cells do not need to be spun down for luminescence.

Data analysis

Data analysis of nsAA incorporation is straightforward, and results should be interpretable from raw data without manipulation, as shown in Figure 4. However, to achieve final data, follow the steps below. Also see Supplemental Excel file 1 for an example of data processing.

1. Average biological replicates of fluorescence/luminescence data from the WT controls without nsAA, then subtract 90% of that value from the fluorescence/luminescence values of all samples. Since low background synthetases without nsAA tend to produce very little observable background incorporation, if the full WT background is subtracted then the nsAA fluorescence values will often be negative. This complicates graphing and downstream data analysis, such as determination of fold-induction. Since the WT fluorescence is very low, the difference between 90% and 100% is extremely small—usually smaller than the standard deviation between the three WT biological replicates. This results in background-normalized incorporation data.
2. Subtract plate reader background OD (from reading blank media wells; it should be constant per given plate reader) from OD values. This can be anywhere between 0.001 and 0.04 for many plate readers. This results in normalized OD data. Note any especially low OD values and consider data from those wells suspect. Most nsAAs described here should not significantly hinder growth, except for 5OHW, which was observed to decrease final OD.
3. Divide background-normalized incorporation data by the normalized OD data. This results in OD-normalized incorporation data. Skip this step for time course analysis.
4. Divide all averages by OD-normalized fluorescence/luminescence data from the TAC positive control lacking a TAG stop codon. This results in final data as a fraction of protein expression without nsAA incorporation. See Figure 4 and Supplemental Excel file 1for examples.
5. Use standard deviation among three biological replicates to plot error bars.

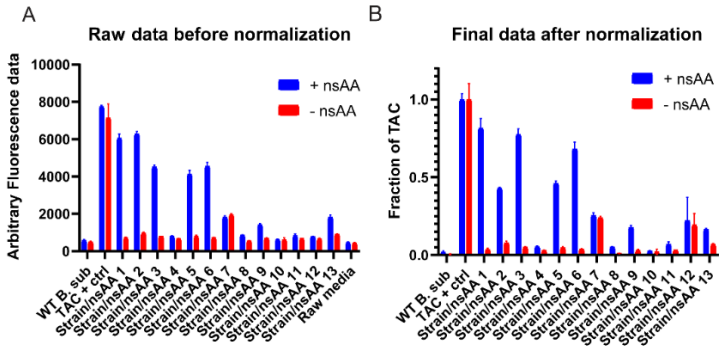

Figure 4. Data analysis example.
(A) Raw fluorescence data from plate reader, with no normalizations. (B) Data processed according to data normalization, with background subtracted, OD normalized, and normalized to maximum possible expression of a reporter lacking a UAG codon.

Notes

1. Always include WT *B. subtilis* (Py79) as a negative control and a TAC-reporter positive control strain to properly normalize experiment-to-experiment signal. The TAC-reporter positive control contains the sense codon TAC, which encodes tyrosine, instead of the nonsense codon TAG at the desired site of nsAA incorporation in the reporter protein. It provides a measure of how much protein expression would be expected if the rate of nsAA incorporation was not a limiting factor and normalizes data across experiments. These strains can be obtained through BGSC (see Materials and Reagents).
2. Adding inducer to the starter cultures is optional but not recommended, as it does not seem to have any effect on final signal. Antibiotic in the starter and experimental cultures is also optional but is unnecessary for stably integrated constructs and can slow growth. Be sure not to add antibiotic to WT controls.
3. Starter cultures
   a. This protocol recommends starting cultures from colonies instead of freezer stocks. It is possible to start cultures from freezer stocks, but starter cultures grown this way grow unreliably and usually take longer to reach an appropriate OD. The same is true for cultures from old plates (>2–3 days). Both of these approaches can be used if needed but ensure that cells have at least gone through two doublings before starting experimental cultures from seed cultures.
   b. Higher ODs than 0.7 do work for starter cultures, but results will not be as reliable.
4. Protocol Variants
   a. The basic 300 µL S750 plate reader endpoint incorporation protocol is extremely reliable when performed correctly. When testing a new synthetase or nsAA, execute that protocol first to demonstrate that incorporation is working before attempting larger cultures, richer media, or other protocol variants.
   b. Because of *Bacillus'* need for aeration, it is better to shake 96-well plates overnight at 1,000 rpm with a 3 mm orbital volume. However, robust incorporation has been observed in 96-well plates with a standard incubator's 250 rpm overnight shaking.
   c. When attempting to purify protein-containing nsAA, the Gram-positive BugBuster kit was found to be very effective for purification of protein from stationary-phase *Bacillus subtilis* grown overnight in S750.
5. Background normalization
   a. In general, it is not necessary to change background subtraction during data analysis based on OD. This is because *B. subtilis* in S750 has relatively low autofluorescence, and after overnight growth almost all cultures will have reached saturation.
   b. However, other media, such as LB, will have higher autofluorescence and potentially more variable ODs. In these cases, it may be necessary to create a linear autofluorescence normalization curve, where several different cultures of WT Py79 at different ODs are measured, then plotted, and a linear autofluorescence curve created. That curve can then be used to subtract autofluorescence based on OD.

Recipes

1. **S750 (1 L)**
   100 mL of 10× S750 salts
   10 mL of 100× metals
   10 mL of 1 M glutamate
   20 mL of 50% glucose
   860 mL of ddH_2O

2. **ammoniaS750 (1 L)**
   100 mL of 10× S750 salts
   10 mL of 100× metals

20 mL of 10% w/v ammonia sulfate
20 mL of 50% glucose
850 mL of ddH_2O

3. **10× S750 salts (1 L)**
   104.7 g of MOPS (free acid)
   13.2 g of ammonia sulfate, anhydrous
   6.8 g of potassium phosphate monobasic, anhydrous
   To 1 L with ddH_2O

4. **100× metals (500 mL)**
   100 mL of 1 M magnesium chloride
   35 mL of 1 M calcium chloride
   2.5 mL of 1 M manganese chloride
   5 mL of 10 mM zinc chloride
   25 mL of 2 mg/mL thiamine-HCl
   1 mL of 1 M HCl
   5 mL of 50 mM iron (III) chloride
   326.5 mL of ddH_2O

Acknowledgments

This work was supported by NSF collaborative research grants MCB-2027074 (to E.C.G.) and MCB-2027092 (to A.M.K.). M.A.J. was partially supported by funding from the Department of Education – Graduate Assistance in Areas of National Need (P200A210065) and from the National Institute of General Medical Sciences of the National Institutes of Health under a Chemistry-Biology Interface Training Grant (T32GM133395).
This protocol is adapted from Stork et al. (2021).

Competing interests

The authors declare the following competing interests: A.M.K. has related financial interests in Nitro Biosciences.

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