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Work instruction for preparation of CFPS precursor solutions based on Maltodextrin as Energy source

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ABSTRACT

This protocol details the procedures for the preparation of individual cell-free protein synthesis (CFPS) precursor solutions according to Yang et al., 2012, Caschera et al., 2014 and Arce et al, 2021 with some modifications.

If volumes smaller or larger volumes than 2000-4000μL are desired, the number of reagents and chemicals must be scaled down or up accordingly.The precursor solutions have typically been prepared using the following protocols in the following order:

1)Preparation of 10x Energy Mix solution

2)Preparation of 50 mM 19 Amino Acid Mix stock.

3)Preparation of a 2.5x Reaction Buffer

4)Perform cell-free reactions based on Maltodextrin as energy source

*# Equipment Note*Using a heated stir plate with temperature control along with a pH strips and/or pH meter will be helpful in preparing the CFPS precursor solutions.

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Preparing 10x Energy Mix solution

1 The following protocol describes the preparation of **~ 4mL** (3.9526 mL) of **10x Energy Solution.**

The energy solution contains polycations, cofactors, and the nucleotide triphosphates required for cell-free protein synthesis. It is recommended to prepare all stock solution to economize reagents.The procedures to make stock solutions are detailed in sections 1.1 to 1.8. Appropriate volumes of the concentrated precursor solutions are then added along with the appropriate masses or volumes of the other chemical components to form the 10 x Energy solution, detailed in section 1.9.

A	B	C	D	E	F
Reagent	**Formula Weight (g/mol)**	**Stock concentration**	**Concentration in 10X Energy Solution**	**Concentration in CF reaction**	**Required Volume (µL) of Precursor Solution**
HEPES pH8 Sigma H3375	238.3	2000mM	510 mM	51 mM	**1000**
Nucleotide Mix ATP: Sigma A8937-1G CTP: Sigma C1506-250MG GTP: Roche 10106399001 UTP: Sigma 94370-1G	ATP: 583.36 CTP: 527.12 GTP: 567.1 UTP: 586.12	155 mM A,G 140 mM C,U	15mM A,G 14 mM C,U	1.5 mM A,G 1.4 mM C,U	**396**
MRE600 E.coli tRNA (tRNA) Roche 10109541001	N/A	50mg /mL	2.02 mg/mL	202 µg/mL	**160**
NAD Sigma N6522-250MG	663.4	175mM	3.39 mM	0.339mM	**76.6**
CoA Sigma C4282	767.5	65.1 mM	2.63 mM	0.263 mM	**160**
cAMP Sigma A9501-1G	329.22	650mM	7.56 mM	0.756 mM	**46**
Folinic Acid, Calcium Salt Sigma F7878-100MG	529	33.8 mM	0.68 mM	0.068 mM	**80**
900 mM Spermidine Solution Sigma 85558-5G	145.25	900mM	7.71 mM	0.77 mM	**34**
Maltodextrin Sigma 419672-100G	N/A	240 mg/mL	121.44 mg/mL	12.144 mg/mL	**2000**

1.1 Preparation of 2000mM HEPES (pH 8) precursor solution.

The following protocol describes the preparation of **50 mL** of **2000 mM HEPES pH 8**

1. Weigh 23.82 g in a 100 mL beaker
2. Add 25-35 mL of MQ water, adjust pH with 5M KOH and then make up 50mL.
3. Filter the solution with a 0.22mm filter and store at room temperature

1.2 Preparation of Nucleotide mix solution stock (155mM A,G; 140 mM C,U).

The following protocol describes the preparation of **1.5 mL** of **Nucleotide mix stock solution.**

1. Add 140 mg of ATP, 133mg of GTP, 111 mg of CTP and 123 mg of UTP in a 1.5 mL test tube.
2. Add 300 -500µL of MQ water and 353µL of 15%(v/v) KOH to adjust pH at 7.5.
3. Then make up to 1.5 mL and store at -80°C

1.3 Preparation of 175 mM NAD stock solution.

The following protocol describes the preparation of **1.0 m L** of **NAD stock solution**

1. Weigh 116.1 mg of NAD in a 1.5 mL test tube.
2. Add MQ water up to 1.0 mL, it is not necessary adjust the pH.
3. Store at -80°C

1.4 Preparation of 650 mM cAMP stock solution.

The following protocol describes the preparation of **4.0 m L** of **cAMP stock solution**

1. Weigh 855.95 mg of cAMP in a 1.5 mL test tube.
2. Add 2000 µL of MQ water, adjust to pH 8 with ~ 1100µL of 15%(v/v) KOH (use pH strips to follow the pH)
3. Fill up to 4.0 mL with MQ
4. Store at -80°C

1.5 Preparation of 33.8 mM Folinic Acid stock solution.

The following protocol describes the preparation of **1.5 m L** of **Folinic acid stock solution**

1. Weigh 26.82 mg in a 1.5 mL test tube.
2. Add MQ water up to 1.5 mL
3. Store at -80°

1.6 Preparation of 65.1 mM mM of CoA stock solution.

The following protocol describes the preparation of **1 m L** of **Folinic acid stock solution**

1. Weigh 50 mg of CoA in a 1.5 mL test tube.
2. Add MQ water up to 1.0 mL
3. Store at -80°

1.7 Preparation of a 900 mM Spermidine precursor solution

The following protocol describes the preparation of **500 µL** of **900 mM spermidine** from a 5.0 g bottle of spermidine solution). According with SIGMA supplier the product: 85558-5G has a concentration of **6400 mM.**

1. Add 382 µL autoclaved MQ water in a 1.5 Eppendorf tube and then add **70.3 µL** of 6400mM spermidine

2. The pH of the solution should be approximately 13.

3. Leave the solution on ice for immediate use or aliquot in Eppendorf tubes.If you choose to aliquot, then flash freeze using liquid nitrogen and store at -80 °C.

1.8 Preparation of a 240 mg/mL Maltodextrin solution

The following protocol describes the preparation of **10 mL** of **240 mg/mL Maltodextrin solution**

1. Weigh out 2.4 g of maltodextrin in a in a 15 mL tube.

2. Dissolve and fill with MQ water to 10 mL

3. Make aliquots and store at -80 °C.

1.9 Preparation of the 10x Energy solution using precursor solutions

1. Determine the desired final volume for the 10x Energy solution (3.9526 mL).Determine the required quantity of each of the liquid and solid reagents (Table provides the required volumes for preparing 3.9526 mL of a 10x Energy solution based on stock solutions).

2. Add the required volumes of each of the components according to the table.

3. Determine the pH of the solution and record the value.The pH should be approximately 7.8-8.0

4. Aliquot 100 µL for reagent testing.Aliquot the remaining solution into multiple 1.5 Eppendorf tubes, flash freeze with liquid nitrogen, and store at -80 °C.

Preparing (50mM of each amino acid) 19 Amino Acid Mix stock

2 The following describes the preparation of **100 mL** of **50mM 19 Amino Acid Mix**

This is the precursor solution of amino acids that will be used by the CFPS reaction. *Glutamate is omitted* from this precursor solution because it will be already present in the form of potassium glutamate in the in the 2.5x Reaction Buffer. 1 L-Amino Acid kit (Sigma LAA21-1KT) is enough to prepare 100mL of 25X 19 Amino Acid mix. Additionally, you need L-Arginine (Sigma 11009), L-Cysteine, (30089), L-Histidine (53319) since these amino acid versions are not contained into the kit.

Since the content kit can vary, be sure that you have the correct amino acid version of each, sometimes they are replaced by the "hydrochloride" amino acid form.

A Order	B Reagent	C Cat No	D Formula Weight (g/mol)	E Quantity (g)	F pH After Addition	G Dissolution Time (sec)
1	L-Arginine	11009	174.2	**0.8710**	10.65 – 10.68	30
2	L-Valine	V0500	117.1	**0.5855**	8.99 – 9.20	180
3	L-Tryptophan	T0254	204.2	**1.0210**	8.66 – 8.71	300
4	L-Phenylalanine	P2126	165.2	**0.8260**	8.42 – 8.46	300
5	L-Isoleucine	I2752	131.2	**0.6560**	8.31 – 8.34	480
6	L-Leucine	L8000	131.2	**0.6560**	8.22 – 8.25	480
7	L-Cysteine	30089	121.2	**0.6060**	7.80 – 7.84	120
8	L-Methionine	M9625	149.2	**0.7460**	7.73 – 7.75	240
9	DL-Alanine	A7627 or 05129	89.09	**0.4455**	7.76 – 7.77	30
10	L-Asparagine	A0884	132.1	**0.6605**	7.64 – 7.71	60
11	L-Aspartic Acid	A9256 or 11189	133.1	**0.6655**	5.35 – 5.41	180
12	L-Glycine	G7126	75.07	**0.3754**	5.34 – 5.42	60
13	L-Glutamine	G3126	146.1	**0.7305**	5.34 – 5.40	120
14	L-Histidine	H8000 or 53319	155.2	**0.7760**	6.46 – 6.54	240
15	L-Lysine Monohydrochloride	L5626	182.6	**0.9130**	6.47 – 6.54	60
16	L-Proline	P0380	115.1	**0.5755**	6.49 – 6.47	120
17	DL-Serine	S4500 or 84959	105.1	**0.5255**	6.49 – 6.47	30
18	L-Threonine	T8625	119.1	**0.5955**	6.50 – 6.54	30
19	L-Tyrosine	T3754	181.2	**0.9060**	NA	NA

2.1 1.Obtain each of the reagents and set up the stir plate. Place a stir bar into a 250 mL beaker. Add 87.5% (87.5mL) of the final solution volume, autoclaved MQ water to the beaker.

2.Place the temperature probe into the water close to the edge of the beaker about halfway into the water (without having the probe touching the beaker). Set the heating temperature to 37 °C. Allow the water to reach temperature. Elevated temperatures at even 45 °C for extended time will decrease solution activity.

3.Set the stir rate to 600 rpm. If the final volume is being scaled up, the stir rate should be set so that the bottom of the vortex forms just above the stir bar.

4.Weigh out the required quantity of each of the reagents in Table.**Add each of the reagents, one at a time according to the order of addition in Table.** After dissolution of each reagent, measure the pH (using pH strips). Do not add the subsequent reagent until the previous is fully dissolved. Use the “approx. dissolution time” and “approx. pH” as a guide. Repeat this step until tyrosine addition. After threonine has dissolved, allow the solution to mix for an additional 5 minutes.

5.Turn off heating on the heating/stir plate.

6.Pour the solution into a graduated cylinder to determine the volume. Add autoclaved MQ water to bring the total volume to 100 mL (approximately 5% of the final volume will be needed).

 # Optional. - Sterile filtering the solution may decrease the activity of the final mixture by removing undissolved amino acids. The solution should appear clear and colourless and relatively free of particulates. Freezing the solution at this point should not cause any precipitation.

7.Pour solution back into the beaker and turn on stirring to 600 rpm. While mixing, add the required quantity of tyrosine to the solution and allow the suspension to mix for 5 minutes.**Note:** the tyrosine will not fully dissolve and will remain in suspension.

8.While still mixing, aliquot 100 µL for subsequent reagent testing.

9.While still mixing, aliquot the remaining solution into multiple 1.5 Eppendorf tubes.Flash freeze the aliquots using liquid nitrogen and store in the -80 °C freezer.

Preparing 2.5x Reaction Buffer

3 The following protocol describes the preparation of **2 mL** of **2.5x Reaction Buffer**

It is recommended to prepare all stock solution to economize reagents.The procedures to make stock solutions are detailed in sections 3.1 to 3

A	B	C	D	E
Component	**Stock Concentration**	**Concentration in 2.5x Buffer concentration**	**Concentration in CF reaction**	**Required Volume (µL) of Precursor Solution**
Energy Solution	10X	2.5X	1.0 X	**500**
Amino Acid Solution	17mM	8.5 mM	3.4 mM	**1000**
PEG-8000 Promega V3011	40%	5%	2.0 %	**250**
HMP Fisher Scientific S/4160/53	30 mg/mL	1.5 mg/mL	0.6 mg/mL	**100**
Potassium Glutamate Sigma G1501-500G	3.5 M	0.14 M	0.056M	**80**
Magnesium Glutamate Sigma 49605-250G	175 mM	6.475 mM	2.590 mM	**74**

3.1 Preparation of 17 mM Amino Acid (AA) solution.

The following protocol describes the preparation of **2 mL** of **17mM AA solution**

1. Add 680 µL of 50mM 19 Amino Acid Mix stock
2. A then make up 2mL with MQ water sterile
3. Store in the -80 °C freezer

3.2 Preparation of 30 mg/ mL HMP solution.

The following protocol describes the preparation of **10 mL** of **30mg/mL HMP solution**

1. Weigh out 300 mg in a 15 mL tube.
2. Then make up 10mL with MQ water sterile
3. Prepare 1 mL aliquots in 1.5 test tubes and **boil them for 5-10 min**.
4. Let them cool down on the bench until they reach room temperature.
5. Store in the -80 °C freezer.

**Note: If you are planning to use a previous prepared30 mg/ mL HMP solution, it is highly recommended you boil the solution 5 min before use it to prepare 2.5X Reaction buffer.*

3.3 Preparation of 3.5 M of potassium glutamate solution.

The following protocol describes the preparation of **10 mL** of **3.5M potassium glutamate solution.**

1. Weigh out 7.11 g of potassium glutamate in a 15 mL tube.
2. Then make up 10mL with MQ water sterile
3. Prepare 1 mL aliquots in 1.5 test tubes and storein the -80 °C freezer.

3.4 Preparation of 175 mM of magnesium glutamate solution.

The following protocol describes the preparation of **10 mL** of **175 mM of magnesium glutamate solution.**

1. Weigh out 680 mg of magnesium glutamate in a 15 mL tube.
2. Then make up 10 mL with MQ water sterile
3. Prepare 1 mL aliquots in 1.5 test tubes and storein the -80 °C freezer.

Performing cell-free reactions based on Maltodextrin

4 To set up a single cell-free reaction of 12µL, thaw the aliquots of each precursor solution and pipette the required volume following the order in the next table. *# Cell-free extract must be added always at the end.*

A	B
Precursor solution	**Required quantity (µL)**
Cell-Extract	4
2.5X Reaction Buffer	4.8
DNA (60nM)	1
MQ water	2.2
Final Volume	12

Literature/ References

5 (Yang et al., 2012)
(Caschera F and Noireaux, 2014)
(Arce et al., 2021)

Change history

6 None so far