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Solutions for CFPS (Version 1.1)- Haseloff Lab V.2

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We use this protocol and it's working

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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., 2012.500 µL of the 4x premix is sufficient to perform 2mL of CFPS.If volumes smaller or larger volumes than 500 µL 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 a Magnesium Glutamate Solution
2)Preparation of a 10x Salt Solution
3)Preparation of a 25x Nucleotide Mix
4)Preparation of a 25× 19 amino acid mix
5)Preparation of a 25x PEP solution
 # 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.

NOTE :

The protocol described here is an adaptation from these papers:

- **Adam D. Silverman**, Nancy Kelley-Loughnane, Julius B. Lucks, and Michael C. Jewett (2019).*Deconstructing Cell-Free Extract Preparation for in Vitro Activation of Transcriptional Genetic Circuitry*. ACS Synthetic Biology, 403-414. DOI: 10.1021/acssynbio.8b00430.

- **Andriy Didovyk**, Taishi Tonooka, Lev Tsimring, and Jeff Hasty. (2017).*Rapid and Scalable Preparation of Bacterial Lysates for Cell-Free Gene Expression*. ACS Synthetic Biology, 2198-2208. DOI: 10.1021/acssynbio.7b00253.

- **Yang WC,** Patel KG, Wong HE, Swartz JR.(2012). *Simplifying and streamlining Escherichia coli-based cell-free protein synthesis*.Biotechnol Prog. 28(2):413-420. DOI:10.1002/btpr.1509.

Please note that some steps were taken literally as they appear in these sources, and it is highly recommended to read these papers before starting.

Our adaptations includes: changes in reagents and volumes.

Attachments

PDF

V1.1 Solutions for C...

268KB

Troubleshooting

1: Preparing Magnesium Glutamate Solution

1 The following protocol describes the preparation of **22 mL**of **1000 mM magnesium** glutamate solution. Magnesium glutamate is kept as a separate addition because the magnesium concentration for maximum protein production varies from one lot of cell extract to another. The typical optimal magnesium glutamate concentration for a CFPS is in between 10-20 mM.Therefore, additional magnesium glutamate may need to be added to reach the optimum concentration depending on the particular extract that is used.

	Reagent	Formula Weight (g/mol)	Main Precursor Concentration	Concentration in CFPS	Required Quantity (g)
	L-Glutamic Acid, Hemimagnesium Tetryhydrate (Magnesium Glutamate)	388.6	1000 mM (as default 100x)	TBD (as default 10mM)	8.55

1)Determine the desired final volume of the magnesium glutamate solution based on the mass (22 mL). Determine the required quantity of magnesium glutamate (8.55 g).

2)Obtain an appropriately sized mixing vessel and place it on a stir plate.

3)Place a stir bar in the mixing vessel and set the heating temperature to 37 °C.

4)Add autoclaved MQ water to the vessel (75% of the final volume).

5)Set the stir rate to a high mixing rate but avoid splashing.

6)Weigh out the required quantity of magnesium glutamate in a weigh boat and pour the quantity into the mixing vessel.

7)Allow the magnesium glutamate to dissolve at 37 °C for 10 minutes with stirring.

8)Determine the volume of the solution using a pipette or graduated cylinder.

9)Add autoclaved MQ water to obtain the desired final volume. Allow the solution to stir at room temperature for 1– 2 minutes until fully homogeneous.

10)Determine the pH of the solution and record the value.The pH should be approximately 6.4 - 6.5.

11)Aliquot 50 µL into an Eppendorf tube for subsequent reagent testing.

12)Aliquot the remaining solution into 1.5 Eppendorf tubes, flash freeze using liquid nitrogen and store at -80 °C.

2. Preparing 10x Salt Solution

2 The following protocol describes the preparation of **100 mL** of **10x Salt Solution.**

	Reagent	Formula Weight (g/mol)	Main Precursor Concentration	Concentration in CFPS	Required Quantity (g)
	L-Glutamic Acid, Monopotassium Salt (Potassium Glutamate)	203.2	1750 mM	175 mM	35.56
	L-Glutamic Acid, Ammonium Salt (Ammonium Glutamate) MPBiomedicals 180595	164.2	100 mM	10 mM	1.64
	Potassium Oxalate, Monohydrate	184.2	27 mM	2.7 mM	0.5

1)Determine the desired final volume of the 10x salt solution (100 mL). Determine the required quantity of potassium glutamate, ammonium glutamate, and potassium oxalate.

2)Obtain an appropriately sized mixing vessel and place it on the stir plate. Place a stir bar in the mixing vessel.

3)Add autoclaved MQ water (45% of the desired final volume). Set the stir rate to a high mixing rate but avoid splashing. Set the stir plate heating to 37 °C.

4)Weigh out the required quantity of potassium glutamate and add into the beaker. Subsequent reagents can be added immediately.

5)Weigh out the required quantity of ammonium glutamate and add into the beaker.

6)Weigh out the required quantity of potassium oxalate and pour into the beaker.

7)Allow the salts to dissolve at 37 °C for 10 minutes on the stir plate.

8)Determine the volume of the solution using a pipette or graduated cylinder.

9)Add autoclaved MQ water to obtain the desired final volume.

10)Allow the salts to dissolve at room temperature. This may take 10 minutes.

11)Obtain a pH reading for the solution once all components are completely dissolved.The pH should be approximately 7.2.

12)Aliquot 1 mL for subsequent reagent testing.

13)Aliquot the remaining solution into multiple 15 mL falcon tubes, flash freeze with liquid nitrogen, and store at -80 °C.

3. Preparing 25x Nucleotide mix

3 The following protocol describes the preparation of **5 mL** of **25x Nucleotide Mix**
The Nucleotide Mix contains polycations, cofactors, and the nucleotide triphosphates and monophosphates required for cell-free protein synthesis.The polycations putrescine and spermidine are difficult to weigh out as solids, so concentrated precursor solutions must be prepared prior to making the 25x Nucleotide Mix. It is recommended to prepare a NAD stock solution to economize this reagent.The procedures to do this are detailed in sections 3.1 , 3.2. and 3.3 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 **25x Nucleotide Mix**, detailed in section 3.4.

	Reagent	Formula Weight (g/mol)	Main Precursor Concentration	Concentration in CFPS	Required Volume (µL) of Precursor Solution
	1000 mM 1,4-Diaminobutane Solution (Putrescine) Sigma/Merck 51799-100MG	88.15	25 mM	1 mM	125

	1500 mM Spermidine Solution (Sigma 85558-5G)	145.25	37.5 mM	1.5 mM	125
	50 mM Nicotinamide Adenine Dinucleotide (NAD)(Sigma N6522-250MG)	663.4	8.3 mM	0.33mM	830
	Reagent	**Formula Weight (g/mol)**	**Main Precursor Concentration**	**Concentration in CFPS**	**Required Mass (g)**
	Adenosine 5′-Triphosphate Dipotassium Salt hydrate(ATP). (Sigma A8937-1G)	601.36	30 mM	1.2 mM	0.0900
	Cytidine 5′-triphosphate disodium salt (CTP). (Sigma C1506-250MG)	527.12	21.5 mM	0.86 mM	0.0566
	Guanosine 5′-triphosphate disodium salt hydrate(GTP). (Roche 10106399001)	567.14	21.5 mM	0.86 mM	0.061
	Uridine 5′-triphosphate trisodium salt dihydrate (UTP) (Sigma 94370-1G)	586.12	21.5 mM	0.86 mM	0.0630
	Coenzyme A Hydrate (CoA). (Sigma C4282)	767.5	6.8 mM	0.27 mM	0.0260
	MRE600 E.coli tRNA (tRNA). (Roche 10109541001)	N/A	4.3 mg/mL	172 µg/mL	215µL*
	Folinic Acid, Calcium Salt. (Sigma F7878-100MG)	511.5	0.9 mg/mL. [1.7595mM]	36 µg/mL. [0.0704mM]	45µL*

* From a stock of 100 mg/mL

3.1 **3.1. Preparation of a 1000 mM 1,4-diaminobutane (Putrescine) precursor solution**

The following protocol describes the preparation of **1.132 mL** of **1000 mM putrescine** from a 0.1 g bottle of 1,4-diaminobutane, otherwise known as putrescine)

1) Determine the final volume for the 1000 mM 1,4-Diaminobutane precursor solution based on the mass (1.132 mL).

2)Add 0.8 mL autoclaved MQ water directly to the bottle of 1,4-diaminobutane.

3) Vortex the bottle vigorously for 1-2 minutes to facilitate dissolution. Pour out the liquid from the bottle into 1.5 mL Eppendorf tube.

4) After adding the Autoclaved MQ water, let the tube sit on ice for 5 minutes.

5) At this point the 1,4-diaminobutane should be completely dissolved or the solid should have dislodged from the original bottle. As needed, more water can be added to dissolve any remaining reagent. However, do not exceed the desired final volume.

6) Once the 1,4-Diaminobutane has dissolved, determine the volume of the solution using a micropipette.

7) Add autoclaved MQ water to obtain the desired final volume.

8) The pH should be approximately 13.

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

3.2 **3.2. Preparation of a 1500 mM Spermidine precursor solution**

The following protocol describes the preparation of **500µL** of **1500 mM spermidine**from a 5.0 g bottle of
spermidine solution). According with SIGMA supplier the product: 85558-5G has a concentration of **6400mM.**

1)Add 382µL autoclaved MQ water in a 1.5 Eppendorf tube and then add **118µL** 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.

3.3 **3.3. Preparation of a 50mM NAD precursor solution**

The following protocol describes the preparation of **1 mL** of **50mM NAD**.
1)Weigh out 33.17 mg in a in a 1.5 Eppendorf tube, add 700µL autoclaved MQ water and then make up to 1 mL.
2)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.

3.4 **3.4. Preparation of the 25x Nucleotide Mix using the Putrescine and Spermidine precursor solutions**

1)Determine the desired final volume for the **25x Nucleotide Mix solution (5 mL)**.Determine the required quantity of each of the liquid and solid reagents (Table provides the required volumes and masses for preparing 5 mL of a 25x Nucleotide Mix).
2)Add 50% (2.5 mL) of the final volume, autoclaved MQ water to 15 mL Falcon tube
3)Add the required volume of the 1000 mM putrescine precursor solution to the mixing vessel.
4)Add the required volume of the 1500 mM spermidine precursor solution to the mixing vessel.
5)Add the required volume of the 50mM NAD precursor solution to the mixing vessel.
6)Ensure the complete dissolution of the material before proceeding with the next step.
7)Add the required quantity of CoA into the tube.
8)Ensure the complete dissolution of the material before proceeding with the next step.
9)Add the required quantity of ATP into the tube.
10)Add CTP, GTP, and UTP in that order, in a fashion similar to the ATP described above.Ensure the complete dissolution of the material before proceeding with the next step.
11)Add the required volume of Folinic Acid
12)Add the required volume of MRE600 tRNA
13)Determine the volume of the solution using a pipette or graduated cylinder. Record the quantity.
14)Add autoclaved MQ water to obtain the desired final volume.
15)Determine the pH of the solution and record the value.The pH should be approximately 7.4 - 7.6.
16)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.

4. Preparing 25× 19 Amino Acid Mix (50mM of each amino acid)

4 The following describes the preparation of **100 mL** of **25× 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 already be present in the form of potassium glutamate in the previously prepared 10x Salt Solution. 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.

	Order of Addition	Reagent	Catalogue Number	Formula Weight (g/mol)	Quantity (g)	pH After Addition. Approx	Dissolution Time (sec). Approx
	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

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 100µL 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.

5. Preparing 25x PEP solution (833mM PEP)

5 The following protocol describes the preparation of **8 mL** of **833 mM PEP**

	Reagent	Formula Weight (g/mol)	Desired Concentration in 25x Mix	Concentration in CFPS	Required Mass (g)
	Phosphoenolypyruate (PEP). Alfa Aesar B20358-5G	206.14	833 mM	33 mM	1.3728

1)Obtain a 25 mL beaker and place it onto a stir plate. Place a stir bar in the beaker. Set up the pH probe by placing it close to the side of the beaker without it touching the walls or the stir bar.
2)Determine the final desired volume for the 25x PEP (8 mL).Determine the required amount of PEP for the desired volume (1.3728 g).
3)Add the required quantity of PEP into the beaker. If possible, try to keep the PEP chilled on ice. The PEP should not be left out at room temperature for an extended period of time.
4) For each 1g of PEP used, add 1 mL of water to the beaker. If more than one vial of PEP is used, pour the PEP into the mixing vessel. Then use the 1 mL of water to rinse out any empty bottles of PEP. At this point, the PEP should be mostly insoluble. Increasing the pH will be required to dissolve PEP.
5) Adjust the pH of the PEP solution using 5M KOH by placing the pH probe into PEP solution. Slowly add (~2.5mL) 5M KOH until the pH of the solution reaches pH 7.3 ±0.1 at 22 – 25 °C. The pH increases sharply as the pH nears 7.3. # Note: target pH = 7.4.
6)Ensure the complete dissolution of the material before proceeding.
7)Determine the new volume of PEP precursor solution.
8)Determine the volume of the solution using a pipette or graduated cylinder.
9)Add autoclaved MQ water to obtain the desired volume of 25x PEP solution.
10)Aliquot 100 µL for subsequent reagent testing.
11)Aliquot the remaining solution into multiple 1.5 Eppendorf tubes, flash freeze with liquid nitrogen, and store at -80 °C.

6. Literature/ References

6

Citation

Yang WC, Patel KG, Wong HE, Swartz JR (2012)
. Simplifying and streamlining Escherichia coli-based cell-free protein synthesis..
Biotechnology progress.

https://doi.org/10.1002/btpr.1509 LINK

7. Change history

7

GTP molecular weight from GTP was corrected →567.14g/mol.→Suppliers and catalogue numbers added

Citations

Step 6

Yang WC, Patel KG, Wong HE, Swartz JR. Simplifying and streamlining Escherichia coli-based cell-free protein synthesis.
https://doi.org/10.1002/btpr.1509