Setup of Continuous Exchange *E.coli* Cell-free (CECF) protein expression reactions - For T7 Lysates

Overview

This protocol delineates all necessary steps, chemicals, and solutions to set up a cell-free protein expression reaction in a continuous exchange (dialysis) format. Compared to cell-free reactions in batch format, higher yields can be obtained in dialysis mode, because the reaction is continuously fed through the dialysis membrane, and reaction by-products are removed from the equilibrium. Reaction times can therefore be extended to overnight incubations.
Cell-free reactions have vast opportunities because they can be supplemented with isotope labeled amino acids, fluorescence-labeled amino acids or other additives. For membrane protein expression, CECF reactions can be supplemented with detergents, nanodiscs, or other lipid-containing components such as bicelles or liposomes.
The novel T7 lysates such as Cellfree E.coli lysates HiYield-T7 already contain the T7 RNA polymerase in optimal quantities. Therefore, this component can be omitted when using these lysates.
Cell-free expression should first be optimized in analytical scale (50-100 µL) for parameters such as magnesium concentration, DNA template type and concentration. Optimal conditions can then be linearly scaled up to several mL volume. Please contact us if you have questions or need assistance optimizing a protocol for your application (contact@cube-biotech.com).

Equipment

- ☐ Micropipettor and pipette tips
- ☐ Thermomix / shaking incubator for 500 rpm and 30°C incubation
- ☐ Ultrasonic device (Optional)
- ☐ Dialysis chambers and feeding mix reservoirs, depending on reaction scales. Alternatively home-built dialysis chambers combined with dialysis membrane, cutoff 12-14 kDa. See (1) for details.

1. Analytical reaction scale for screening, alternative suppliers:

Reaction volume	Dialysis chamber	Feeding mix reservoir
50-100 µl	Scienova Xpress Micro Dialyzer MD 100 (12-14 kDa, 10-100 µL) e.g. cat.no. 40077	96-well Deep-well block with 2 ml volume
50-100 µL	Thermo Pierce Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 0.1mL, e.g. cat.no. 69570	1.5 ml microcentrifuge tube
50-250 µL	Merck Milipore D-tube Dialyzer Mini, 12-14 kDa MWCo, cat.no. 71505	(included)

2. Scale-up reactions, alternative suppliers

Reaction volume	Dialysis chamber	Feeding mix reservoir
50- 300 µl	Scienova Xpress Micro Dialyzer MD 300 (12-14 kDa, 50-300 µL) e.g. cat.no. 40790	96-well Deep-well block with 2 ml volume
200-2000 µl	Thermo Pierce Slide-A-Lyzer MINI Dialysis Device, 10K MWCO, 2mL, cat.no. 88404	50 ml centrifuge tube (e.g. Falcon)
1000-3000 µL	Merck Milipore D-tube Dialyzer Maxi, 12-14 kDa MWCo, cat.no. 71510	(included)

Materials

Chemical	Suggested supplier	Suggested cat.no.
E.coli lysate, for cell-free protein expression	Cube Biotech	21001, 21011
1,4-Dithiotreitol (DTT)		
Acetyl phosphate lithium potassium salt (ACP)	Sigma-Aldrich	A0262
Acetic Acid		
Adenosine 5'-triphosphate (ATP)	Roche	10127523001
Amino acids for cell-free expression	Sigma-Aldrich	LAA21-1KT
Complete protease inhibitor cocktail	Roche	04693116001
Cytidine 5'-triphosphate di-sodium salt (CTP)	Sigma-Aldrich	30320
Dipotassium hydrogen phosphate (K2HPO4)		
Ethylenediaminetetraacetic acid (EDTA)		
Folinic acid calcium salt	Sigma-Aldrich	47612
Guanosine 5'-triphosphate di-sodium salt (GTP)	Sigma-Aldrich	51120
2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethane sulfonic acid (HEPES)		
Magnesium acetate tetrahydrate (Mg(oAc)2)		
Magnesium chloride hexahydrate (MgCl2)		
Sodium azide (NaN3)		
PEG 8,000	Carl Roth	0263.1
Phosphoenol pyruvic acid monopotassium salt (PEP)	Sigma-Aldrich	860077
Potassium acetate (KoAc)		
Potassium chloride (KCl)		
Potassium dihydrogenphosphate (KH2PO4)		
Pyruvate kinase (PK)	Roche	10128155001
RiboLock RNase inhibitor	Thermo Scientific	#EO0381
Sodium chloride		
Tris base		
tRNA E.coli MRE 600	Roche	10109541001
Uridine 5'-triphosphate tri-sodium salt (UTP)	Sigma-Aldrich	94370
Template DNA		
Optional: Positive control template DNA		
Recommended: Suitable affinity chromatography matrix or magnetic beads	Cube Biotech	depending on protein affinity tag used
SDS-PAGE/ Western Blot equipment and antibody		
Potassium hydroxide (KOH)		
Sodium hydroxide (NaOH)		
For membrane protein expression:		
Pre-assembled nanodisc	Cube Biotech	Pre-assembled nanodiscs or assembly kits with various scaffold protein/ phospholipid variations
Detergents, e.g. Decylmaltoside, dodecylmaltoside	Cube Biotech	16009, 16013
Detergents, e.g. Brij series		

A. Solutions and buffers

The following stock solutions need to be prepared in advance. They can be stored until use.

Single component stock solutions

Component	Stock concentration	Molecular weight (g/mol)	Amount needed for stock	Volume	Comment	Storage
1,4 Dithiothreitol (DTT)	500 mM	154.2	154 mg	2 mL	sterile filter	-20°C
Li^+, K^+ Acetyl phosphate (ACP)*	1 M	184.1	386.2	2 mL	set pH 7.0 with 20 µL KOH 10M	-20°C
Complete protease inhibitor	50x	-	1 tablet	1 mL		-20°C
Folinic acid (Ca^{2+})*	10 mg/mL	511.5	10 mg	1 mL	sterile filter. Corresponds to 19.6 mM	-20°C
Magnesium acetate, $Mg(OAc)_2$	1 M	214.4	10.72 g	50 mL	sterile filter	-20°C
Potassium acetate, $KOAc^+$	10 M	98.15	49.075 g	50 mL	dissolve at 37°C sterile filter	-20°C
Polyethylene glycol (PEG) 8000	40% (w/v)		20 g	50 ml	dissolve at 37°C	-20°C
Phosphoenolpyruvate, K^+ (PEP)	1 M	206.1	412.2	2 mL	set pH 7.0 with 470 µL KOH 10M	-20°C
Pyruvate kinase	10 mg/mL					4°C
Sodium azide (NaN_3)	10% (w/v)	65.01	100 mg	1 mL	sterile filter	-20°C
Tris-acetate pH 8.2	1 M	121.14	6.05 g	50 mL	set pH to 8.2 with acetic acid	room temp
tRNA E.coli	40 mg/mL		40 mg	1 mL	sterile filter	-20°C
*Concentration is very high, possibly difficult to solubilize. Mix well, maybe use ultrasound.						

HEPES/EDTA 24 x Stock

Component	Stock concentration	Molecular weight (g/mol)	Amount needed for stock	Volume	Comment
HEPES	2.5 M	238.3	28.596 g	48 mL	Set pH 8.0 with 6480 µL KOH stock
EDTA	0.5 M	292.3	292.3 mg	2 mL	mix with HEPES-KOH solution
Instructions: Dissolve HEPES and set pH by addition of KOH. Measurement of pH is not required. Add EDTA solution to obtain the 24 x HEPES/EDTA stock solution. Sterile filter, store at -20°C.					

Amino acid stock solution, 25 mM each

Amino acid		Molecular weight (g/mol)	Amount needed for stock (mg)
R	Arginine	174.2	217.8
N	Asparagine	150.1	187.5
A	Alanine	89.09	111.4
D	Aspartate	133.1	166.4
E	Glutamate	185.2	231.5
Q	Glutamine	146.1	182.6
G	Glycine	75.07	93.8
H	Histidine	155.16	194.0
I	Isoleucine	131.2	164.0
L	Leucine	131.2	164.0
F	Phenylalanine	165.2	206.5
P	Proline	115.1	143.9
K	Lysine	146.19	182.7
S	Serine	105.1	131.4
W	Tryptophane	204.2	255.3
T	Threonine	119.1	148.9
V	Valine	117.1	146.4
C	Cysteine	175.63	219.5
M	Methionine	149.2	186.5
Y	Tyrosine	181.2	226.5
Instructions: Weigh all components and resuspend them together in 50 mL ddH_2O. As the solution is very concentrated, it will not become clear. Store at -20°C. Mix well before use, maybe use ultrasound.			

„6 Amino Acid mix", 16.67 mM each

Amino acid		Molecular weight (g/mol)	Amount needed for stock (mg)
R	Arginine	174.2	145.2
D	Aspartate	133.1	110.9
E	Glutamate	185.2	154.4
W	Tryptophane	204.2	170.2
C	Cysteine	175.63	146.4
M	Methionine	149.2	124.4
Instructions: Weigh all components and resuspend them together in 50 mL ddH_2O. As the solution is very concentrated, it will not become clear. Store at -20°C. Mix well before use, maybe use ultrasound.			

NTP 75x Stock

Component	Stock concentration	Molecular weight (g/mol)	Amount needed for stock	Volume	Comment	Storage
ATP	360 mM	605.2	435.7 mg	2 mL	Set pH 7.0 with 233 µL 5 M NaOH, sterile filter	4°C
GTP	240 mM	567.1	272.2 mg	2 mL	Set pH 7.0 with 72 µL 5 M NaOH, sterile filter	4°C
CTP	240 mM	527.1	253.0 mg	2 mL	Set pH 7.0 with 144 µL 5 M NaOH, sterile filter	4°C
UTP	240 mM	550.1	264.0 mg	2 mL	Set pH 7.0 with 66 µL 5 M NaOH, sterile filter	4°C
Instructions: Mix equal volumes of all four stock solutions to obtain a 75x NTP stock. Store at -20°C.						

B. Reaction mixes and master mixes

Reaction mixes and master mixes should be prepared fresh and used up the same day.

Feeding Mix Buffer (S-30 C Buffer), 10 ml stock

Component	Stock concentration	Amount needed	Final concentration
Tris-acetate, pH 8.2	1 M	100 µL	10 mM
Magnesium acetate, $Mg(OAc)_2$	1 M	140 µL	14 mM
Potassium acetate, KOAc	10 M	60 µL	60 mM
DTT	500 mM	10 µL	0.5 mM
Instructions: Add all components and fill up with water to 10 mL. Add DTT immediately before use.			

Reaction Master Mix, 5 mL: For 1 mL reaction mix and 16 mL feeding mix.
Prepare enough master mix for the number of reactions to be set up the same day.

Component	Stock concentration	Final concentration in cell-free reaction	Volume
6 Amino acid mix (RCWMDE)	16.7 mM	1 mM	1020 µL
Amino acid mix	25 mM	0.5 mM	340 µL
Li^+, K^+ Acetyl phosphate (ACP)	1 M	20 mM	340 µL
Phosphoenolpyruvate, K^+ (PEP)	1 M	20 mM	340 µL
75 x NTP Mix	90 mM ATP; 60 mM G/C/UTP	1.2 mM ATP; 0.8 mM G/C/UTP	226.7 µL
1,4 Dithiothreitol (DTT)	500 mM	2 mM	68 µL
Folinic acid (Ca^{2+})	10 mg/mL	0.1 mg/mL	170 µL
Complete protease inhibitor	50 x	1 x	340 µL
HEPES/EDTA buffer	24 x	1 x	623.3 µL
Magnesium acetate, $Mg(OAc)_2$	1 M	16 (11.1) mM§	274 µL
Potassium acetate, KOAc	4 M	270 (110) mM§	382.5 µL
PEG 8000	40% (w/v)	2% (w/v)	850 µL
Sodium azide (NaN_3)	10% (w/v)	0.05% (w/v)	85 µL
		Total	**5.0595 mL**

Instructions: Mix all components as prepared in previous steps.
§: Subject to optimization. Volumes are calculated for final total concentrations of Mg^{2+} of 16 mM and K^+ of 270 mM. Additional amounts of 4.9 mM Mg^{2+} and 160 mM K^+ are contributed by other compounds. Numbers in parentheses indicate the concentration of ions added at this step. Concentration of Mg^{2+} and K^+ should be optimized for each protein and each new batch of chemicals used. Typical concentrations are in the range of 12-25 mM Mg^{2+} (optimized in 2 mM steps) and 250-350 mM K^+ (optimized in 20 mM steps). Please refer to the datasheet delivered with each batch of cell-free lysate for a guideline of optimal Mg^{2+} and K^+ concentrations.

Reaction Mix, 1 mL
Prepare enough reaction mix for the number of reactions to be set up the same day.

Component	Stock concentration	Final concentration	Volume
Master mix			297.6 µL
Pyruvate kinase	10 mg/mL	0.04 mg/mL	4 µL
tRNA (*E.coli*)	40 mg/mL	0.5 mg/mL	12.5 µL
RiboLock	40 U/µL	0.3 U/µL	7.5 µL
DNA template	200-500 µg/mL§	15-30 µg/mL	60 µL
E.coli lysate	1x	0.35x	350 µL
ddH_2O		ad 1 mL	268.4 µL
		Total	**1 mL**
Instructions: Add all components as prepared in previous steps. Mix by gentle shaking or pipetting. §: Subject to optimization.			

Feeding Mix, 16 mL: For 1 mL Reaction Mix
Prepare enough feeding mix for the number of reactions to be set up the same day.

Component	Stock concentration	Final concentration	Volume
Master mix			4,762.1 µL
Feeding mix buffer	1 x	0.35 x	5,600 µL
Amino acid mix	40 mg/mL	0.5 mg/mL	320 µL
ddH_2O		ad 16 mL	5,317.9 µL
		Total	**16 mL**
Instructions: Add all components as prepared in previous steps. Mix thoroughly by vortexing.			

Optional: GFP Assay Mix, 20 mL
For evaluation of reaction conditions using a positive control vector.

Component	Stock concentration	Molecular weight (g/mol)	Amount needed for stock	Final concentration	Volume
Tris-HCl, pH 7.4	1 M	121.14	12.11 g / 100 mL	20 mM	400 µL
Sodium chloride, NaCl	5 M	58.44	29.22 g / 100 mL	150 mM	600 µL
Instructions: Prepare stock solutions, and set pH of Tris stock with HCl to 7.4.					

Procedure

A. Setup of analytical scale CECF reactions

1. Prepare all stock solutions as listed above, set pH with the given amounts of NaOH or KOH, sterile-filter, aliquot, and store.
2. Prepare a DNA template coding for the protein of interest in a concentration of 0.2-0.5 mg/mL in ddH_2O.
3. Prepare a dialysis reaction container: either assemble a self-built container with a dialysis membrane, or a commercially available reaction container according to the manufacturer's instructions.
4. Prepare the required amount of master mix for the number of reactions to be set up.
5. Reconstitute reaction and feeding mixture in the required amounts.
6. Check dialysis devices for leakage. Briefly incubate the dialysis membrane in the feeding mix before adding the reaction mix.
7. Fill reaction and feeding mixture aliquots into reaction containers according to manufacturer's instructions. When using home-built mini-CECF reactors, ensure that a fresh piece of dialysis membrane is used every time. Always make sure no air bubbles are trapped between the membrane and the solutions.
8. When using a lysate batch and set of chemical stock solutions for the first time, perform a screen of Mg^{2+} and K^+ concentrations using GFP as positive control. For optimization, screen different amounts of DNA template, and, optionally also concentrations of Mg^{2+} and K^+.
9. Incubate the CECF reactions overnight at 30°C with gentle shaking or rolling to ensure efficient substance exchange between reaction and feeding mixture.
10. Spin the mixture at 15,000 rpm for at least 15 min to remove precipitates. Analyze both supernatants and precipitates in SDS-PAGE.
11. Purify proteins from the reaction mixtures using appropriate affinity chromatography matrices.
12. Analyze protein in SDS-PAGE and, optionally, in a Western Blot using an appropriate antibody. For quantification of expressed protein via ^{35}S-Methionine, refer to (1). Measure GFP fluorescence in GFP Assay buffer.

Note: Both circular plasmid and linear PCR templates can be used, provided they contain T7 promotor and termination sites. These sites can be introduced by a two-step PCR reaction.
In addition, expression tags can be introduced at the translational start site that may help initiate translation. See (1) for reference.

For membrane protein expression: Add detergent (e.g. DM, DDM, Brij-series) or empty nanodiscs (concentration 5-120 µM, subject to optimization) to the reaction mixt Alternatively, membrane proteins can be precipitated from the reaction solution and re-solubilized before purification. Refer to (1) for details.

Note: Depending on reaction volume and expression scale, protein bands may not be visible in SDS-PAGE unless proteins are purified. Affinity magnetic beads, e.g. PureCube Ni-NTA MagBeads, are suitable for low expression rates, as they are easily scalable.

B. Setup of preparative scale CECF reactions

1. After optimizing expression conditions for a given protein in analytical scale, calculate the reaction volume required to obtain the desired amount of recombinant protein.
2. Prepare enough DNA template coding for the protein of interest in a concentration of 0.2-0.5 mg/mL in ddH_2O.
3. Prepare a dialysis reaction container: either assemble a self-built container with a dialysis membrane, or a commercially available reaction container according to the manufacturer's instructions.
4. Prepare the required amount of master mix for the number of reactions to be set up.
5. Reconstitute reaction and feeding mixture in the required amounts.
6. Fill reaction and feeding mixture aliquots into reaction containers according to manufacturer's instructions.
7. Incubate the CECF reactions overnight at 30°C with gentle shaking or rolling to ensure efficient substance exchange between reaction and feeding mixture.
8. Purify proteins from the reaction mixtures using appropriate affinity chromatography matrices.
9. Analyze protein in SDS-PAGE and, optionally, in a Western Blot using an appropriate antibody. For quantification of expressed protein via ^{35}S-Methionine, refer to (1). Measure GFP fluorescence in GFP Assay buffer.

Note: Both circular plasmid and linear PCR templates can be used, provided they contain T7 promotor and termination sites. These sites can be introduced by a two-step PCR reaction.
In addition, expression tags can be introduced at the translational start site that may help initiate translation. See (1) for reference.

For membrane protein expression: Add detergent or empty nanodiscs, as optimized in the small scale reactions, to the reaction mix. Alternatively, membrane proteins can be precipitated from the reaction solution and re-solubilized before purification. Refer to (1) for details.

Note: Depending on reaction volume and expression scale, protein bands may not be visible in SDS-PAGE unless proteins are purified. Affinity magnetic beads, e.g. PureCube Ni-NTA MagBeads, are suitable for low expression rates, as they are easily scalable.

Reaction scale	Reaction mixture volume	Feeding mixture volume
Analytical / Small scale screening	50 µL	700-800 µL
Analytical / Small scale screening	100 µL	1.4-1.6 mL
Medium / first upscale	1 mL	14-16 mL
Large / upscale	3 mL	42-48 mL

References:

1. Roos, C. et al. (2014), High-Level Cell-Free Production of Membrane Proteins with Nanodiscs. From: Alexandrov, K., and Johnston, W.A. (eds.) Cell-Free Protein Synthesis: Methods and Protocols. Methods in Molecular Biology, vol. 1118, Chapter 7.