

CECF Protocol for 10x 60 µl RM + 900 µl FM Reactions

This pipetting scheme is typical example for an Mg²⁺ ion screen of a new DNA template in the two-compartment (= reaction mixture (RM) + feeding mixture (FM)) CECF configuration. The selected ratio of RM : FM is 1 : 15.

The Mg²⁺ ion concentrations (12, 14, 16, 18, 20 mM) are screened in duplicate reactions.

- Designed either for Mini-CECF reactors (Reckel et al., *Meth. Mol. Biol.* 2010) or for Scienova M100 cartridges.
- **Optional compounds are in blue and not essential but may improve production efficiency.**
- **Critical compounds are in red.**
- Amounts of less critical compounds are rounded.
- Stocks are in water and neutralized if necessary. Some stocks do not dissolve completely and stay turbid. Always vortex before removing aliquots.

Reaction overview:

1st: Prepare master mix MM-F (Table 1), save aliquot MM-F1 for MM-R preparation and then complete MM-F.

2nd: Prepare master mix MM-R (Table 2).

3rd: Prepare 5x 1.5 mL tubes for the RMs and 5x 2 mL tubes for the FMs of the reactions. Complete the RMs and FMs according to Table 3.

4th: Fill 2x 900 µl of each FM and 2x 60 µL of each RM into the appropriate reaction container compartment. Assemble the reaction containers and incubate over night at 30°C with gentle shaking.

Further detailed protocols:

- Rues, R.B., Henrich, E., Boland, C., Caffrey, M., and Bernhard, F. (2016) Cell-free production of membrane proteins in *Escherichia coli* lysates for functional and structural studies. *Meth. Mol. Biol.* **1432**: 1-22. DOI 10.1007/978-1-4939-3637-3_2.
- Henrich, E., Dötsch, V., and Bernhard, F. (2015) Screening for lipid requirements of membrane proteins by combining cell-free expression with nanodiscs. *Meth. Enzymol.*, **556**, 351-369.
- Orbán, E., Proverbio, D., Haberstock, S., Dötsch, V., and Bernhard, F. (2015) Cell-free expression of G-protein coupled receptors. *Meth. Mol. Biol.*, **1261**, 171-95.
- Kai, L., Orbán, E., Henrich, E., Proverbio, D., Dötsch, V., and Bernhard, F. (2015) Co-translational stabilization of insoluble proteins in cell-free expression systems. *Meth. Mol. Biol.*, **1258**, 125-43.
- Roos, C., Kai, L., Haberstock, S., Proverbio, D., Ghoshdastider, U., Ma, Y., Filipek, S., Wang, X., Dötsch, V., and Bernhard, F. (2014) High level cell-free production of membrane proteins with nanodiscs. *Meth. Mol. Biol.*, **1118**, 109-30
- Kai, L., Roos, C., Haberstock, S., Proverbio, D., Ma, Y., Junge, F., Karbyshev, M., Xu, Z., Dötsch, V., and Bernhard, F. (2011) Systems for the cell-free synthesis of proteins. *Meth. Mol. Biol.* **800**, 201-25.
- Schneider, B., Junge, F., Shirokov, V.A., Durst, F., Schwarz, D., Dötsch, V., and Bernhard, F. (2010) Membrane protein expression in cell-free systems. *Meth. Mol. Biol.* **601**, 165-86.
- Reckel, S., Sobhanifar, S., Durst, F., Löhr, F., Shirokov, V.A., Dötsch, V., and Bernhard, F. (2010) Strategies for the cell-free expression of membrane proteins. *Meth. Mol. Biol.* **607**, 187-212.
- Schwarz, D., Junge, F., Durst, F., Frölich, N., Schneider, B., Reckel, S., Sobhanifar, S., Dötsch, V., and Bernhard, F. (2007) Preparative scale expression of membrane proteins in *E. coli* based continuous exchange cell-free systems. *Nat. Protocols* **2**, 2945-57.

Table 1. Master Mix-MM-F:

Compound	Stock	Final concentration	Amount [μl]
20 amino acid mix	25 mM (stays turbid)	1 mM	425
Acetyl phosphate (Li ⁺ , K ⁺ , pH 7.0)	1 M (stays turbid)	20 mM	213
Phospho(enol)pyruvic acid (K ⁺ , pH 7.0)	1 M	20 mM	213
75x NTP mix; pH 7.0 (240 mM each CTP, UTP, GTP and 360 mM ATP)	75x	1x	142
HEPES buffer (K ⁺)	2.4 M	100 mM	460
Folinic acid	10 mg/mL (stays turbid)	0.1 mg/mL	107
Mg(OAc)₂	1 M	7.1 mM	75.4
KOAc	10 M	130 mM	138
DTT	500 mM	2 mM	43
Complete protease inhibitor	50x (1 tablet in 1 mL)	1x	213
NaN ₃	10%	0.05%	53
PEG 8000	40%	2%	531
Total			2613.4
Minus MM-F1 for MM-R preparation:			- 171
Rest			2442.4
+ S30-C buffer			3465
+ additional 20 amino acid mix			396
Final MM-F volume in μL:			6303.4

Table 2. Master Mix-MM-R:

Compound	Stock	Final concentration	Amount [μ l]
MM-F1			171
DNA template	0.75 mg/mL	0.015 mg/mL	14
tRNA (E. coli)	40 mg/mL	0.5 mg/mL	9
Pyruvate kinase	10mg/mL	0.04 mg/mL	3
RiboLock	40 U/ μ L	0.3 U/ μ L	5.5
Total			202.5

Table 3. Screening matrix for Mg²⁺ concentration:

Compound	12 mM ^A	14 mM	16 mM	18 mM	20 mM
RM					
MM-R	37	37	37	37	37
S30 lysate	46	46	46	46	46
100 mM Mg(OAc) ₂	0	2.6	5.2	7.8	10.4
H ₂ O	47	44.4	41.8	39.2	36.6
Total	130	130	130	130	130
FM					
MM-F	1133	1133	1133	1133	1133
100 mM Mg(OAc) ₂	0	36	72	108	144
H ₂ O	667	631	595	559	523
Total	1800	1800	1800	1800	1800

^A, the basic Mg²⁺ concentration is already 12 mM (4.9 mM are added with the S30 lysate and 7.1 mM are added with MM-F)