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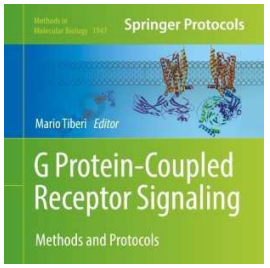
Preparation of Enhanced Orthogonal Aminoacyl-tRNA-Synthetase



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Abstract

This is part 3.1 of the "A Combined Cell-Free Protein Synthesis and Fluorescence-Based Approach to Investigate GPCR Binding Properties" collection of protocols: **https://www.protocols.io/view/a-combined-cell-free-protein-synthesis-and-fluores-bqntmven**

Collection Abstract: Fluorescent labeling of de novo synthesized proteins is in particular a valuable tool for functional and structural studies of membrane proteins. In this context, we present two methods for the site-specific fluorescent labeling of difficult-to-express membrane proteins in combination with cell-free protein synthesis. The cell-free protein synthesis system is based on Chinese Hamster Ovary Cells (CHO) since this system contains endogenous membrane structures derived from the endoplasmic reticulum. These so-called microsomes enable a direct integration of membrane proteins into a biological membrane. In this protocol the first part describes the fluorescent labeling by using a precharged tRNA, loaded with a fluorescent amino acid. The second part describes the preparation of a modified aminoacyl-tRNA-synthetase and a suppressor tRNA that are applied to the CHO cell-free system to enable the incorporation of a non-canonical amino acid. The reactive group of the non-canonical amino acid is further coupled to a fluorescent dye. Both methods utilize the amber stop codon suppression technology. The successful fluorescent labeling of the model G protein-coupled receptor adenosine A2A (Adora2a) is analyzed by in-gel-fluorescence, a reporter protein assay, and confocal laser scanning microscopy (CLSM). Moreover, a ligand-dependent conformational change of the fluorescently labeled Adora2a was analyzed by bioluminescence resonance energy transfer (BRET).

For **Introduction and Notes**, please see: https://www.protocols.io/view/a-combined-cell-free-protein-synthesis-and-fluores-bqntmven/guidelines

Materials

2.1 Materials for Preparation of Enhanced Orthogonal Aminoacyl-tRNA-Synthetase

1. Coding sequence for the modified tyrosyl-tRNA-synthetase (eAzFRS, including the mutations Thr37, Ser182, Ala183, and Arg265 [11, 12] and a C-terminal Strep-Tag) from *E.coli*.
2. *E.coli* expression system (RTS 500 *E.coli* HY Kit, biotechrabbit).
3. 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)
4. Gravity flow Strep-Tactin® superflow mini-column (0.2 ml).
5. Strep-Tactin® Purification Buffer Set: 10× Washing Buffer (1 M Tris–Cl, pH 8.0, 1.5 M NaCl, 10 mM EDTA), 10× Elution Buffer (1 M Tris–Cl, pH 8.0, 1.5 M NaCl, 10 mM EDTA, 25 mM Desthiobiotin) and 10× Regeneration Buffer (1 M Tris–Cl, 1.5 M NaCl, 10 mM EDTA, 10 mM HABA (hydroxyl-azophenyl-benzoic acid)).
6. Zeba™ Spin Desalting Columns (7 K MWCO, 0.5 ml).
7. Amicon® Ultra Centrifugal Filters (10 K device, 0.5 ml).
8. Synthetase storage buffer: 50 mM HEPES pH 7.6, 10 mM KOAc, 1 mM $MgCl_2$, 4 mM DTT.
9. Thermomixer with a microtiter plate adapter and a RTS 500 adapter.

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

3.1 Preparation of Enhanced Orthogonal Aminoacyl-tRNA-Synthetase

20h

1 For prokaryotic cell-free synthesis, the eAzFRS gene should be cloned into a vector containing a T7 promotor, ribosomal binding site, and T7 terminator such as pIX3.0, pIVEX2.3d, and pIVEX2.4d vectors or alternatively containing a T5 promotor such as pQE2 vectors as used in this protocol. eAzFRS is synthesized in a cell-free system using an *E. coli* lysate in a dialysis mode.
A typical 1.1 ml reaction is composed of 0.525 mL E.coli lysate , 0.225 mL reaction mix , 0.27 mL amino acids without methionine , 30 µL methionine , 11 µL IPTG for the induction of the protein expression pQE2 vector, 39 µL template containing 110 µg plasmid DNA .

2 The surrounding feeding mixture contains 7990 µL feeding mix , 110 µL IPTG , 2650 µL amino acids without methionine and 300 µL methionine (*see* **Note 2**).

3 Fill the reaction solution into the reaction compartment (marked through the red lid).

4 Fill the feeding mix into the feeding chamber (marked through the colorless lid).

5 Insert the prepared chamber into the RTS 500 adapter in a thermomixer. The reaction time is 20:00:00 at 30 °C and a shaking speed of 900 rpm . 20h

6 For the separation of aggregated proteins from soluble eAzFRS a centrifugation step at 16000 x g, 4°C, 00:10:00 is recommended.

7 Equilibrate two Strep-Tactin columns with 400 µL 10× washing buffer and add 500 µL supernatant of the cell-free reaction to each column.

8 After the supernatant has completely entered the column, wash each column 5× with 200 µL washing buffer (*see* **Note 3**).

9 Elute the protein 6× with 100 µL elution buffer and collect the fractions.

10 Elution fractions containing the target protein are pooled.

11 Regenerate the column with 3× 1 mL 1× regeneration buffer and remove the regeneration buffer 2× with 800 µL 1× washing buffer. Store the column in 2 mL washing buffer at 4 °C.

12 The combined elution fractions are applied to Zeba™ Spin Desalting Columns to exchange the elution buffer of the strep-tag purification to a synthetase storage buffer. Therefore, remove the storage solution of the Zeba™ Spin Desalting Column by centrifugation at 1500 x g, 00:01:00. Add 300 µL synthetase storage buffer to the resin bed and centrifuge at 1500 x g, 00:01:00. Repeat this **step 2×**.
Place the column in a new collection tube and apply 100 µL pooled synthetase solution to each column. Centrifuge at 2000 x g, 00:02:00 and collect the synthetase.

13 The concentration of the synthetase can be performed with Amicon® Ultra Centrifugal Filters. Add up to 500 µL synthetase solution to the concentrator and centrifuge at 14000 x g, 4°C, 00:10:00. Collect the concentrated sample and determine the concentration by NanoDrop measurement using the molecular mass (48.6 kDa) and the extinction coefficient (54.3) (*see* **Note 4**).

14 The synthetase can be stored at -80 °C after shock freezing in liquid nitrogen.