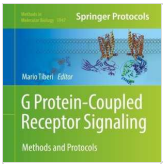

Sep 22, 2021

Preparation of Suppressor tRNA

In 1 collection

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ABSTRACT

This is part 3.2 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

DOI

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EXTERNAL LINK

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COLLECTIONS

A Combined Cell-Free Protein Synthesis and Fluorescence-Based Approach to Investigate GPCR Binding Properties

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MATERIALS TEXT

2.2 Materials for Preparation of Suppressor tRNA

2.2.1 Generation of PCR Product

1. Vector containing the nucleotide sequence of tRNATyrCUA (SupF Gene).
2. tRNATyrCUA-specific forward primer (5′ CgA gCT CgC CCA CCg gAA TTC 3′) and 2′-OMe reverse primer (5′ Tgg Tgg Tgg ggg AAg gAT TCg 3′).
3. 0.2 ml PCR tubes.
4. PCR cycler.
5. Taq DNA polymerase.
6. Taq buffer.
7. dNTPs.
8. 25 mM $MgCl_2$.
9. Agarose gel electrophoresis chambers.
10. Agarose.
11. Rotiphorese 10× TBE buffer.
12. DNA stain.
13. DNA ladder.
14. PCR Purification Kit.

2.2.2 Generation of RNA Transcript

1. T7 RNA Polymerase (f.c. 1 U/µl, Agilent).
2. 5× NTP mix containing 18.75 mM ATP, 18.75 mM CTP, 18.75 mM UTP and 7.5 mM GTP.
3. 5× transcription buffer: 400 mM HEPES-KOH, 0.5 mM Spermidine, 50 mM DTE and 75 mM $MgCl_2$.
4. DNAseI (1 U per µg plasmid DNA).
5. 10× MOPS buffer: 200 mM MOPS, 50 mM NaOAc, 10 mM EDTA, pH 8.0.
6. MOPS sample buffer: 8% (v/v) formaldehyde, 12 ml formamide, 2.4 ml 10× MOPS buffer, 0.05% (v/v) bromophenol blue to a total volume of 24 ml.

2.2.3 RNA Isolation and Folding

1. TRIzol reagent.
2. High Performance Liquid Chromatography (HPLC) grade Chloroform.
3. HPLC grade Isopropyl.
4. 75% Ethanol.
5. Cooled centrifuge.
6. Nanodrop 2000c.

SAFETY WARNINGS

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

3.2.1 Generation of PCR Product

1 For specific and homogenous 3′-ends of the suppressor tRNA, an additional PCR step before transcription reaction is included. Therefore, the reverse primer contains a 2′-OMe-group to prevent unspecific nucleotides at the 3′-end of the tRNA that can be added by the T7 polymerase during transcription reaction. Amplify the template by pipetting in a PCR tube final concentrations of **[M]1 x Taq Buffer** , **[M]0.2 Milimolar (mM) dNTP mix** , **[M]0.5 Micromolar (µM) forward primer** , **[M]0.5 Micromolar (µM) reverse primer** , **[M]2.5 Milimolar (mM) MgCl2** , **[M]0.01 ng/µl plasmid** and **[M]0.04 U/µl Taq DNA polymerase** . Fill the reaction with water to a final volume of **250 µl** (*see* **Note 5**).

3.2.2 Generation of RNA Transcript 6h

2 

Thaw the components for in vitro transcription **On ice** and pipette the reaction at **Room temperature** . Mix

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[M]**1 x transcription buffer** , [M]**1 x NTP mix** , [M]**1 U/µl T7 RNA Polymerase** and [M]**8 ng/µl template DNA**

.

3

Fill the reaction with water to the final volume of **500 µl** .

4 6h

Incubate the reaction for **03:00:00** – **06:00:00** at **500 rpm, 37°C** .

5

Centrifuge the RNA at **12000 x g, 00:01:00** und use the supernatant for the DNAseI treatment (*see* **Note 6**).

6

Add **1 U DNAse I per 1 µg DNA**.

7 10m

Incubate for **500 rpm, 37°C, 00:10:00** .

3.2.3 RNA Isolation and Folding 1h 41m 15s

8

Handle the TRIzol and chloroform reagent with care and use a fume hood.

Add a threefold volume of TRIzol to the transcription reaction and mix carefully.

9 5m

Incubate for **00:05:00** at **Room temperature** .

10 15s

Add **200 µl chloroform** for **1 mL TRIzol** and mix carefully for **00:00:15** by inverting.

11 3m

Incubate for **00:03:00** at **Room temperature** .

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12

Centrifuge at **12000 x g, 4°C, 00:15:00** . Isolate the aqueous phase (*see* **Note 7**).

13

Add **500 µl isopropyl** for **1 mL TRIzol** and mix carefully.

14 3m

Incubate **Overnight** at **4 °C** .

15 1h

Centrifuge at **15000 x g** at least for **01:00:00** at **4 °C** and discard the supernatant.

16 30m

Overlay the pellet with **1 mL 75% ethanol** for **1 mL TRIzol** and incubate for **00:30:00** at **-20 °C** .

17

Centrifuge at **7500 x g, 4°C, 00:10:00** . Discard the supernatant and air dry the pellet.

18

Solve the pellet in water. Measure concentration using a NanoDrop and adjust the concentration to **100 Micromolar (µM)** .

19

Fold the tRNA by slowly decreasing the temperature from **80 °C** to **25 °C** in a PCR cycler. The tRNA can be stored at **-80 °C** after shock freezing in liquid nitrogen.

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