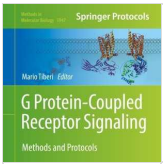

Sep 22, 2021

Cell-Free Protein Synthesis

In 1 collection

Anne Zemella[1], Theresa Richter[1,2], Lena Thoring[1], Stefan Kubick[1]

[1]Cell-free and Cell-based Bioproduction, Fraunhofer Institute for Cell Therapy and Immunology (IZI), Branch Bioanalytics and Bioprocesses (IZI-BB), Potsdam, Germany;

[2]University of Potsdam, Potsdam, Germany

dx.doi.org/10.17504/protocols.io.bqnwmvfe

Springer Nature Books

Satyavati Kharde
Springer Nature

ABSTRACT

This is part 3.3 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

dx.doi.org/10.17504/protocols.io.bqnwmvfe

EXTERNAL LINK

https://link.springer.com/protocol/10.1007/978-1-4939-9121-1_4

PROTOCOL CITATION

Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick 2021. Cell-Free Protein Synthesis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bqnwmvfe

COLLECTIONS

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

KEYWORDS

Cell-free protein synthesis, G protein-coupled receptor, Protein modification, Non-canonical amino acids, Amber suppression, Confocal laser scanning microscopy

CREATED

Dec 12, 2020

LAST MODIFIED

Sep 22, 2021

OWNERSHIP HISTORY

Dec 12, 2020	Lenny Teytelman	protocols.io
Jul 05, 2021	Emma Ganley	protocols.io
Jul 06, 2021	Emma Ganley	protocols.io
Aug 24, 2021	Satya K	
Aug 26, 2021	Satyavati Kharde	Springer Nature
Aug 26, 2021	Satyavati Kharde	Springer Nature
Sep 22, 2021	Satya K	
Sep 22, 2021	Satyavati Kharde	Springer Nature

PROTOCOL INTEGER ID

45494

PARENT PROTOCOLS

Part of collection

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

Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick (09/22/2021). Cell-Free Protein Synthesis. https://dx.doi.org/10.17504/protocols.io.bqnwmvfe

MATERIALS TEXT

2.3 Materials for Cell-Free Protein Synthesis and Fluorescent Labeling of Modified Adora2a

1. Coding sequence for the modified adenosine A_{2A} receptor (Uniprot: P29274, amino acids 1-340, with and without amber stop codon at nucleotide sequence coding for P215) with a receptor C-terminally fused Nanoluciferase (Fig. 1). Flanking sequences containing 5' regulatory sequences (T7 RNA polymerase promotor, Cricket paralysis virus (CRPV) IGR IRES sequence (Genbank accession no. AF218039, nucleotides 6025-6216)) and 3' regulatory sequences (T7 terminator), cloned with the coding sequence into a plasmid (BioCat or Thermo Fisher Scientific).
2. 1.5 ml reaction tubes.
3. CHO lysate prepared as described [19, 20] (*see* **Note 1**).
4. 10× translation mix: 300 mM HEPES-KOH (pH 7.6), 2250 mM KOAc, 2.5 mM spermidine, 1 mM of each canonical amino acid (Merck) and 39 mM $Mg(OAc)_2$.
5. 5× energy: 100 mM creatine phosphate, 1.5 mM GTP, 1.5 mM CTP, 1.5 mM UTP, 8.75 mM ATP and 0.5 mM $m^7G(ppp)G$ cap analogue.
6. 100 µM Polyguanylic acid (polyG, IBA).
7. T7 RNA polymerase (f.c. 1 U/µl).
8. ^{14}C-leucine.
9. 100 µM Bodipy-TMR-lysine-tRNACUA (BP-CUA, biotechrabbit).
10. 100 µM Bodipy-TMR-lysine-tRNAGAA (BP-GAA, biotechrabbit).
11. 100 µM eAzFRS.
12. 100 µM tRNATyrCUA.
13. 100 mM p-propargyloxy-L-phenylalanine (pPa, Iris Biotech).
14. 5 mM Copper(II) sulfate ($CuSO_4$).
15. 2.5 mM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, Iris Biotech).
16. 80 mM Sodium ascorbate (NaAsc).
17. Phosphate-buffered saline (PBS).
18. 100 µM Sulfo-Cy5-azide (Lumiprobe).

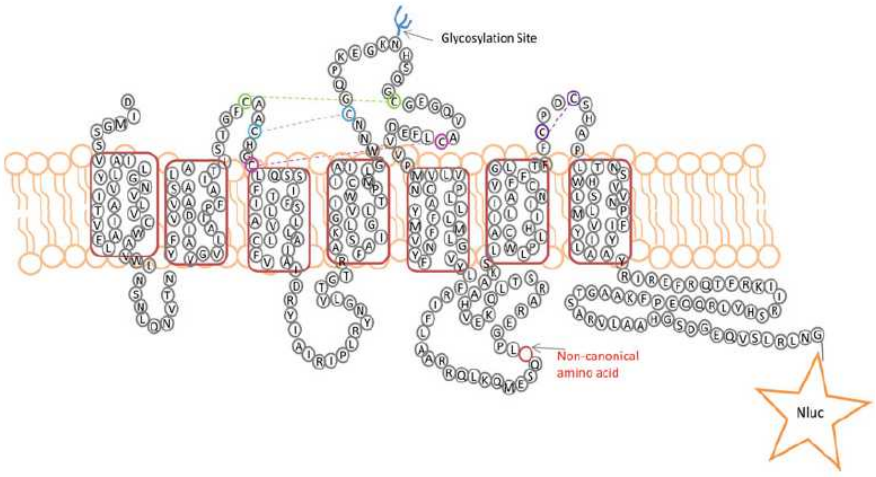

Fig. 1 Schematic illustration of the Adora2a receptor with a C-terminally fused Nluc. Arrows indicate the glycosylation site and the position of the non-canonical amino acid. Disulfide bridges are indicated by the colors green, blue, pink, and purple

SAFETY WARNINGS

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

3.3 Cell-Free Protein Synthesis

1 Thaw all required components for cell-free protein synthesis **On ice** (*see* **Note 8**).

2

Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick (09/22/2021). Cell-Free Protein Synthesis. https://dx.doi.org/10.17504/protocols.io.bqnwmvfe

The cell-free protein synthesis is performed in a coupled mode where transcription and translation reaction take place in one vessel. A standard reaction is composed of [M]**40 % CHO lysate** , [M]**10 Micromolar (µM) polyG** , [M]**1 x translation mix** , and [M]**1 x energy mix** . Add plasmid at a concentration of [M]**40 Nanomolar (nM)** (*see* **Note 9**). Add [M]**1 U/µl T7 RNA polymerase** for the transcription reaction and ^{14}C-leucine (specific radioactivity of 66.67 dpm/pmol) for further analysis of de novo synthesized proteins. Fill the reaction with water to the final volume of **50 µl** .

3.3.1 Fluorescent Labeling with Bodipy-TMR-Lysine

3 Thaw the Bodipy-TMR-lysine-tRNACUA (BP-CUA) and Bodipy-TMR-lysine-tRNAGAA (BP-GAA) **On ice** and keep it dark (*see***Note 10**).

4 3h

Pipette [M]**2 Micromolar (µM) precharged tRNA** to the cell-free reaction and incubate the prepared cell-free protein synthesis reaction by **27 °C** for **03:00:00** and shaking at **600 rpm** (*see***Note 11**). Cover the thermomixer with a lid or aluminum foil to prevent the reaction of any light.

5 15m

Take a **5 µl** aliquot of the translation mixture for SDS-PAGE. Centrifuge the translation reaction at **16000 x g, 4°C, 00:15:00** . Take **5 µl** of the supernatant and resuspend the pellet (microsomal fraction) in an equal volume PBS in comparison to the volume of the translation reaction. This step is required for the analysis of the localization of the synthesized protein. The expectations of the incorporation of the different Bodipy-tRNAs are described in Table 1 (*see***Note 12**).

A	B	C	D
Construct	**Precharged tRNA**	**Expectation**	
		Synthesis	**Fluorescence**
Adora2a	BP-GAA	Full-length protein	Highly fluorescently labeled protein with statistical incorporation of ncAA
Adora2a	BP-CUA	Full-length protein	The full-length protein is not labeled with the fluorescent dye and the ncAA is not incorporated.
Adora2a_amb	BP-CUA	Full-length protein and partial termination product	The full-length protein is fluorescently labeled. The fluorescence signal has a lower intensity in comparison to the statistically labeled Adora2a due to the site-specific incorporation of the ncAA at one defined position. Termination product is not labeled.
Adora2a_amb	BP-GAA	Termination product	The termination product is highly fluorescently labeled due to the statistical incorporation of the ncAA. No full-length product is visible.

Table 1
Expectations of cell-free protein synthesis and fluorescent labeling of different Adora2a constructs with Bodipy-tRNA directed to phenylalanine codons (GAA) or directed to the amber stop codon (CUA)

6 An example of the performance of the fluorescent labeling of Adora2a with or without incorporated non-canonical

Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick (09/22/2021). Cell-Free Protein Synthesis. https://dx.doi.org/10.17504/protocols.io.bqnwmvfe

amino acid determined by in-gel-fluorescence and autoradiography, and the measurement of the reporter protein are shown in Fig. 2. In-gel-fluorescence and autoradiography (Fig. 2a, c) showed the expected results as described in Table 1. The reporter protein assay (Fig. 2b) is based on the activity of a receptor C-terminally fused Nanoluciferase (Nluc). Nluc activity can only be measured after the translation of the full-length fusion protein. The Adora2a synthesized by using the DNA construct without an amber stop codon (Adora2a + BP-GAA/ BP-CUA) resulted in a fusion protein with a detectable Nluc activity. The fusion protein translated from the amber stop codon DNA construct (Adora2a_amb + BP-CUA) showed a fivefold reduced Nluc activity. This result implicates a rather low incorporation efficiency of the precharged tRNA addressing the amber stop codon. The combination of Adora2a_amb with BP-GAA showed no luciferase activity since only the termination product was translated. Protein yields were calculated by scintillation counting and resulted in approximately **[M]20 µg/ml** in translation mixture. In the microsomal fraction and the supernatant, approximately **[M]6 µg/ml** and **[M]14 µg/ml** were detected, respectively. A similar Adora2a distribution was obtained by in-gel-fluorescence, luciferase activity, and autoradiography. Previously reported distributions of membrane proteins are comparable to the here described distribution [7]. A comparable protein yield was calculated for the termination product.

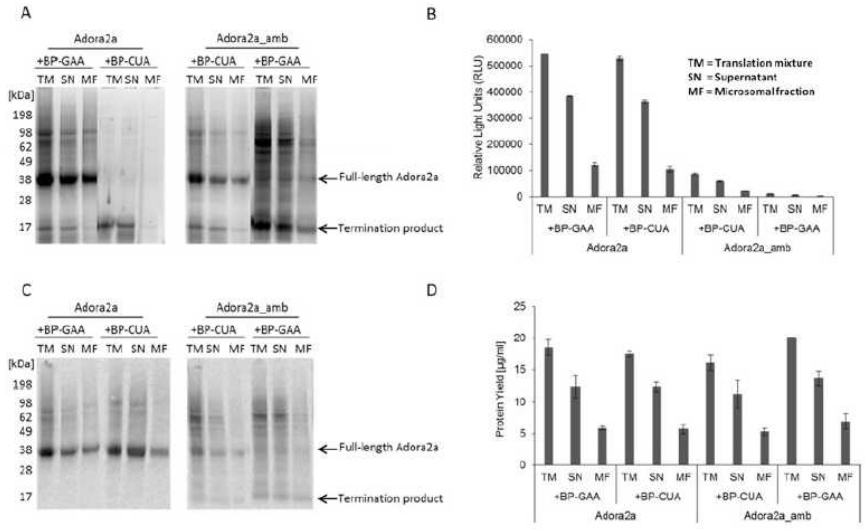

Fig. 2 Analysis of fluorescent labeling by using precharged tRNAs. (**a**) In-gel-analysis of Adora2a by statistical and site-specific incorporation of a fluorescently labeled amino acid. The translation mixture (TM) was fractionated into supernatant (SN) and microsomal fraction (MF). (**b**) Analysis of the translation of a reporter protein (Nluc) fused to the C-terminus of Adora2a by measuring the luminescence of the Nluc. (**c**) Autoradiographic analysis of de novo synthesized Adora2a by incorporation of 14C-leucine during the cell-free synthesis reaction. (**d**) Determination of protein yield of de novo synthesized Adora2a by scintillation counting

3.3.2 Analysis of Ligand- Dependent Conformational Change Using a BRET Assay

7 Site-specifically label the Adora2a_amb using the precharged tRNA Bodipy-TMR-lysine-tRNACUA as described in section 3.3.1 above, steps 3–5.

8

Resuspend the microsomal fraction of the Adora2a_amb in PBS. **5 µl** aliquots of resuspended Adora2a_amb were mixed with **5 µl adenosine** in PBS with final concentrations of **[M]0 Micromolar (µM)** , **[M]100 Micromolar (µM)** , **[M]1000 Micromolar (µM)** , and **[M]5000 Micromolar (µM) adenosine** (*see* **Note 13**).

9 **10 µl samples** were applied for the luminescence and fluorescence measurement. In a first step, the luminescence of the Nluc was detected using an OD2 filter. In a second step, the fluorescence of the coupled Bodipy dye, excited by the Nluc emission was detected.

Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick (09/22/2021). Cell-Free Protein Synthesis. https://dx.doi.org/10.17504/protocols.io.bqnwmvfe

10 As a *control* **5 µl** *of the resuspended microsomal fraction of* the full-length Adora2a protein without any fluorescent label is treated with the same concentrations of adenosine (**step 8**) to determine background fluorescence caused by the broad emission spectrum of the Nluc and possible interactions of adenosine with the Nluc.

11 The BRET ratio is calculated as follows:

$$BRET_{ratio} = \frac{Fluorescence\ (sample)}{Luminescence\ (sample)} - \frac{Fluorescence\ (control)}{Luminescence\ (control)}$$

12 Herein, the calculated BRET ratio (Fig. 3) showed a change in the relation of the fluorescence of the Bodipy in comparison to the Nluc luminescence after the addition of different adenosine concentrations. Only a minimal increase of the BRET ratio can be seen after addition of higher adenosine concentrations (above **[M]100 Micromolar (µM)**) indicating that at lower concentrations all receptors are occupied with adenosine. The result indicates a conformational change of the helix III and the connected third intracellular loop that is expected for the Adora2a.

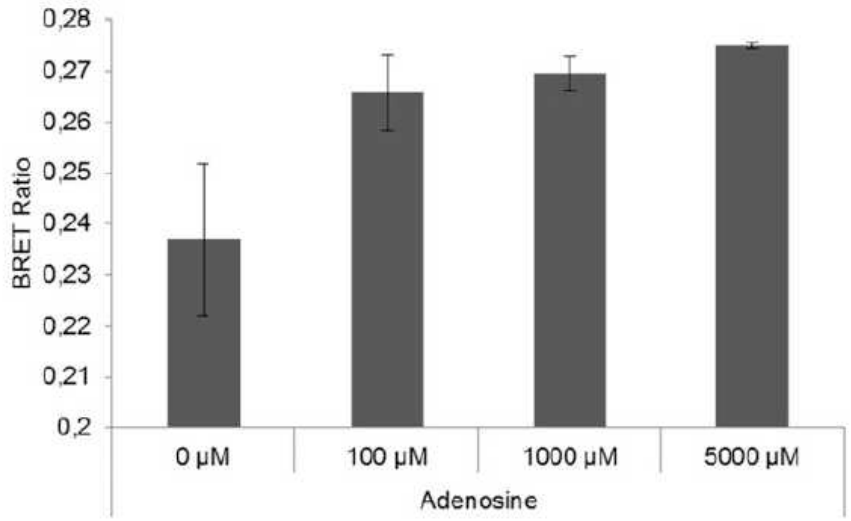

Fig. 3 BRET signal of the fluorescently labeled Adora2a_amb after adenosine binding. Cell-free synthesized and fluorescently labeled Adora2a_amb was subjected to luminescence and fluorescence analysis in presence of different adenosine concentrations (0–5000 µM). The ratio of the Bodipy fluorescence to the luminescence of the Nluc was calculated and subtracted by a control ratio

3.3.3 Site-Specific Incorporation of a Non-canonical Amino Acid with Subsequent Fluorescent Labeling and Microscopic Analysis

4h

13 Additional components are required for the recharging of the suppressor tRNA and a subsequent incorporation of a non-canonical amino acid. Therefore, add the p-propargyloxy-L-phenylalanine, tRNATyrCUA, and eAzFRS in a specific order (Table 2) *(see* **Note 14**).

Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick (09/22/2021). Cell-Free Protein Synthesis. https://dx.doi.org/10.17504/protocols.io.bqnwmvfe

A	B	C
Order	**Components**	**Final concentration**
1	Water	
2	PolyG	10 µM
3	Translation mix	1×
4	pPa	2 mM
5	DNA-template	40 nM
6	Lysate	40%
7	tRNATyrCUA	3 µM
8	eAzFRS	3 µM
9	^{14}C-leucine	66.67 dpm/pmol
10	T7-RNA-Polymerase	1 U/µl
11	Energy mix	1×

Table 2
Pipetting order of a standard cell-free reaction with orthogonal components for the incorporation of non-canonical amino acids

14 3h

Incubate the prepared cell-free reaction at **27 °C** for **03:00:00** by gentle shaking at **600 rpm** . Keep the reaction in the dark (*see* **Note 15**).

15

The membrane protein is translocated and integrated into the microsomal membrane during the cell-free reaction. Therefore, separate the microsomal fraction by centrifugation at **16000 x g, 4°C, 00:15:00** . Resuspend the pellet fraction in PBS. Use an equal volume of the cell-free reaction for resuspension.

16 1h

For the labeling reaction prepare the labeling mix as follows: combine **200 Micromolar (µM)** **$CuSO_4$** with **600 Micromolar (µM)** **THPTA** , **5 Milimolar (mM)** **NaAsc** , PBS and a final concentration of **3 Micromolar (µM)** **Sulfo-Cy5-azid** to a final volume of **5 µl** . Add **5 µl** of the resuspended microsomal fraction to the labeling mix. Incubate the labeling reaction at **Room temperature** for **01:00:00** . Keep the reaction dark (*see* **Note 16**).

17 15m

Centrifuge the labeling reaction for **16000 x g, 4°C, 00:15:00** . Discard the supernatant and resuspend the pellet in **10 µl** **PBS** . This step removes excess fluorescent dye to decrease the background signal in the subsequent fluorescent analyses.

18 The analysis of the synthesis of the full-length protein by using the Adora2a construct with or without an amber stop codon was performed by autoradiography and a reporter protein assay (Fig. 4). The autoradiography shows for both synthesis reactions a similar band pattern (Fig. 4a). Interestingly, the incorporation of pPa led to a comparable band signal as obtained for the full-length protein translated from the DNA construct without an amber stop codon. This result implicates a high incorporation efficiency of the non-canonical amino acid. In addition, no termination product is detected in the autoradiograph. The high incorporation efficiency is the basis for a further coupling reaction to a fluorescent dye. In addition, this result is supported by the Nluc assay (Fig. 4b). The measured luciferase activity of the

Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick (09/22/2021). Cell-Free Protein Synthesis. https://dx.doi.org/10.17504/protocols.io.bqnwmvfe

suppression product reaches up to 80% of the luciferase activity of the full-length product. The protein yield and protein distribution (Fig. 4c) is comparable to the previously described results (Fig. 2, see section 3.3.1 above "Fluorescent Labeling with Bodipy-TMRLysine", **step 6**).

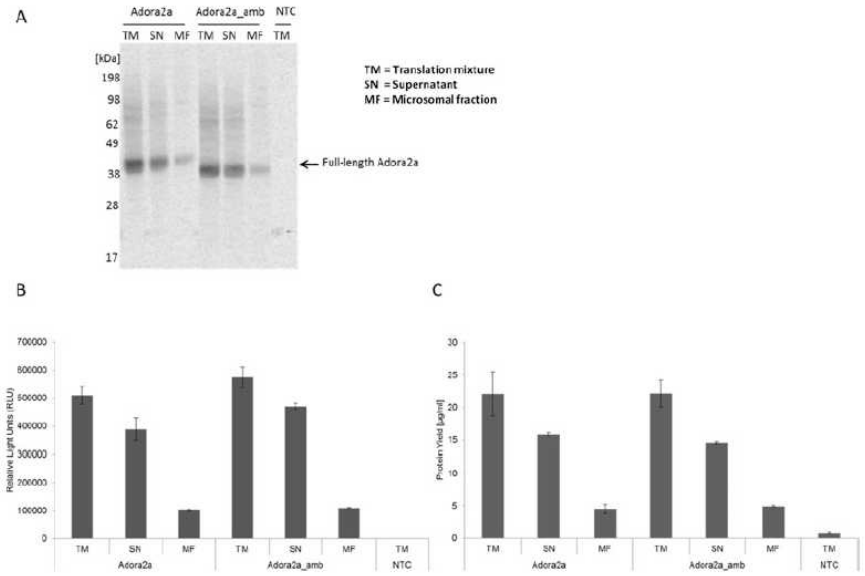

Fig. 4 Analysis of incorporation efficiency using an orthogonal system. (**a**) Autoradiography of synthesized Adora2a after incorporation of ^{14}C-leucine. Translation mixture (TM) was fractionated into supernatant (SN) and microsomal fraction (MF). (**b**) Schematic illustration of subsequent labeling reaction after reaching a high incorporation of the non-canonical amino acid. (**c**) Analysis of the translation of a reporter protein (Nluc) fused to the C-terminus of Adora2a by measuring the luminescence of the luciferase. NTC = no template control. (**d**) Protein yield of synthesized Adora2a, determined by scintillation counting

19 Fluorescently labeled GPCRs should be detectable in microsomal structures (Fig. 5). It is recommended to visualize the labeled sample first by in-gel-fluorescence (Fig. 5, left panel). The synthesis reaction in the presence of all orthogonal components led to a specific band at the expected molecular weight. The control reaction without addition of the modified synthetase resulted in no visible band. The success of the microscopic analysis highly correlates to the quality of the in-gel-fluorescence. High background fluorescence during ingel-analysis often results in unspecific staining of the microsomes. The microscopic analysis clearly shows a difference in the fluorescence intensity of the labeled Adora2a and the unspecific staining of the microsomes (right panel).

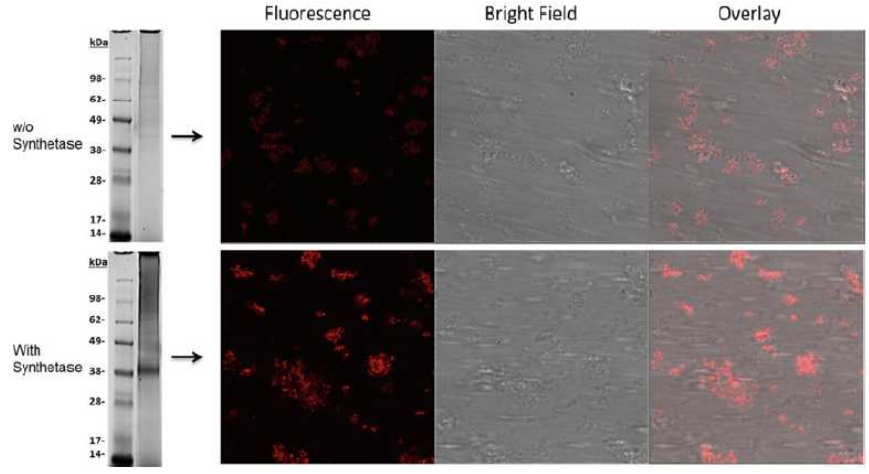

Fig. 5 Fluorescence analysis of labeled Adora2a. Left side: In-gel-fluorescence of synthesized and with Sulfo-Cy5-azide labeled Adora2a in absence or presence of the modified synthetase during the cell-free protein synthesis reaction. Right side: confocal laser scanning microscopy of the control sample (without addition of the modified synthetase during cell-free protein synthesis) and the labeled Adora2a (cell-free protein synthesis in presence of the synthetase)

Citation: Anne Zemella, Theresa Richter, Lena Thoring, Stefan Kubick (09/22/2021). Cell-Free Protein Synthesis. https://dx.doi.org/10.17504/protocols.io.bqnwmvfe