Chapter 24

Quantitation of Protein Translation Rate In Vivo with Bioorthogonal Click-Chemistry

Borja Belda-Palazón, Alejandro Ferrando, and Rosa Farràs

Abstract

The development of novel bioorthogonal reactives that can be used to tag biomolecules in vivo has revolutionized the studies of cellular and molecular biology. Among those novel reactive substances, amino acid analogs can be used to label nascent proteins, thus opening new avenues for measuring protein translation rates in vivo with a limited manipulation of the sample. Here, we describe the use of Click-chemistry to tag and separate newly synthesized proteins in mammalian cells that can be used, coupled with western analysis, to estimate the translation rate of any protein of interest.

Key words Click-chemistry, Bioorthogonal amino acid, Translation rate, Affinity purification, Western blot

1 Introduction

The comprehension of the mechanisms that control the translation process is fundamental for the global understanding of the gene expression in every organism. Proteins are not equally translated and the differences in translation efficiencies have been shown to be involved in the phenotypic divergence of species [1]. Moreover, the changing of the rate at which the ribosome translates an mRNA can alter the behavior of the newly synthesized protein with consequences on protein homeostasis. Therefore, the analysis of protein translation rates over diverse cell growth conditions, such as inhibitor or drug treatments are critical aspects to be studied in cell biology [2]. Early studies on protein turnover relied on isotopic labeling of amino acids, typically labeled with [^{35}S]-Methionine and pulse-chase experiments after blocking protein synthesis. Novel techniques based on mass-spectrometry proteomics allow the determination of protein turnover of large number of proteins after pulse labeling with amino acids that incorporate stable isotopes as it has been shown for human cells [3]. In spite of the power of these global approaches, individual studies of protein

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turnover at relative low cost may be required in many cases. The recent development of "click"-chemistry with bioorthogonal chemical reactives which serve to selectively tag biomolecules such as proteins, DNA, RNA, lipids, glycans, etc., have provided unvaluable tools to carry out these type of studies [4–6].

Here, we focus on the use of the bioorthogonal noncanonical amino acid L-Azidohomoalanine (AHA), which is a methionine analog, for the metabolic labeling of the newly synthesized proteins in human cells [7]. The newly synthesized AHA-containing proteins can click-react with modified biotin-alkyne groups forming stable bioconjugates. Next, the biotinylated de novo synthesized proteins are affinity purified by using a Streptavidin-Agarose chromatography for their subsequent western blot analysis. Eventually, different AHA time-incubation periods are needed to observe and quantify the AHA incorporation to determine the translation rate of the protein of interest.

In conclusion, this method can be used to specifically compare the translation rate for the proteins of interest among different experimental conditions by analyzing the incorporation rate of the bioorthogonal noncanonical amino acid AHA into newly synthesized proteins.

2 Materials

Unless otherwise indicated prepare all solutions using sterile ultrapure 18 MΩ water and store all the reagents at room temperature.

2.1 Cell Culture Media

1. 6 Wells cell culture plates: growth area 9.5 cm^2 per well (*see* **Note 1**).
2. DMEM Mixture F-12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, and 10 μg/mL streptomycin. To prepare DMEM/F12 *see* **Note 2**.
3. DMEM Methionine free (DMEM-Met), supplied with 10% FBS, 10 U/mL penicillin, and 10 μg/mL streptomycin. To prepare DMEM-Met *see* **Note 2**.
4. 50 mM L-Azidohomoalanine (AHA) stock solution (Click-iT® AHA, Life Technologies): add 387 μL of DMSO to 5 mg of Click-iT® AHA (MW = 258.16) and mix well. Store at −20 °C.
5. Phosphate-Buffered Saline (PBS). Store at 4 °C.
6. Sterile cell scraper.

2.2 Protein Extraction and Quantification

1. 1× Protein Extraction Buffer (PEB): 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% IGEPAL® CA-630 (Sigma-Aldrich), 0.5% sodium deoxycholate (DOC), 2 μg/mL leupeptine, 2 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). To prepare 1 mL of 1× PEB, mix 50 μL of 1 M Tris–HCl pH 8.0 (*see* **Note 3a**),

30 µL of 5 M NaCl (*see* **Note 3d**), 10 µL of 10% SDS (*see* **Note 3b**), 10 µL of 100% IGEPAL® CA-630, 50 µL of 10% DOC (*see* **Note 3e**), 1 µL of 2 mg/mL leupeptine, 1 µL of 2 mg/mL aprotinin, and 1 µL of 1 M PMSF. Bring the volume to 1 mL with water and store at 4 °C.

2. Bradford Reagent (Sigma-Aldrich). Store at 4 °C.
3. 2 mg/mL Quick Start Bovine Serum Albumin (BSA) Standard (Bio-Rad). Store at −20 °C.
4. Phosphate-Buffered Saline (PBS). Store at 4 °C.
5. Refrigerated centrifuge 5430 R (Eppendorf) and its fixed-angle rotors: F-35-6-30 for 15 or 50 mL tubes and FA-45-30-11 for 1.5 or 2 mL tubes.
6. 96 Wells microtiter plate.
7. Microplate reader.
8. Microplate centrifugator.

2.3 Click-It Reaction

1. 4 mM Biotin-PEG4-alkyne (Sigma-Aldrich) stock solution: add 2.732 mL of DMSO to 5 mg Biotin-PEG4-alkyne (MW = 457.58) and mix well. Store at −20 °C.
2. Click-iT® Protein Reaction Buffer Kit (Life Technologies), includes contents of 2× concentrate Click-iT® reaction buffer (Component A), 40 mM Copper (II) sulfate ($CuSO_4$; Component B), Click-iT® reaction buffer additive 1 (Component C), Click-iT® reaction buffer additive 2 (Component D). Fully dissolve the Component C and Component D in 500 µL and 540 µL of distilled deionized (DDI) water, respectively. Store all the reagents at −20 °C.
3. Sterile ultrapure 18 MΩ water.
4. End over end rotator.

2.4 Purification of Biotinylated Proteins

1. PD MiniTrap G-25 (GE Healthcare).
2. 1× Equilibration Buffer (EB): 1% IGEPAL® CA-630 (Sigma-Aldrich) and 0.1% SDS in PBS. To prepare 200 mL of 1× EB, mix 2 mL of 100% IGEPAL® CA-630, 2 mL of 10% SDS (*see* **Note 3b**), and 196 mL of PBS.
3. Centrifugable and autoclavable 1 mL columns Mobicol classic and filters of 10 µm pore size (MoBiTec GmbH).
4. Streptavidin-Agarose (Sigma-Aldrich) stored a 4 °C
5. 1× Washing Buffer (WB): 1% IGEPAL® CA-630 in PBS. To prepare 200 mL of 1× WB, mix 2 mL of 100% IGEPAL® CA-630 and 198 mL of PBS.
6. 5× Laemmli Buffer (LB): 0.25 M Tris–HCl pH 6.8, 10% SDS, 0.5 M dithiothreitol (DTT), 30% (w/v) sucrose, 0.5% (w/v)

bromophenol blue (BPB). To prepare 1 mL of 5× LB, mix 250 µL of 1 M Tris–HCl pH 6.8 (*see* **Note 3a**), 300 mg of sucrose, 5 mg of BPB, 76 mg of DTT (MW = 154.25), and 100 mg of SDS. Bring the volume to 1 mL with water and dissolve well. Store at −20 °C.

7. Uncooled Benchtop Centrifuge.
8. Refrigerated centrifuge 5430 R (Eppendorf) and its fixed-angle rotors: F-35-6-30 for 15 or 50 mL tubes and FA-45-30-11 for 1.5 mL tubes.
9. 1.5, 2 and 15 mL polypropylene tubes and tube adapters.
10. Thermoblock for 1.5 mL tubes.

2.5 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Mini-Protean® Tetra handcast systems (Bio-Rad) (*see* **Note 4**).
2. Resolving gel: 14% acrylamide–bis-acrylamide (Sigma-Aldrich) (*see* **Notes 5** and **6**), 375 mM Tris–HCl pH 8.8, 0.1% SDS, 0.2% *N,N,N,N′*-tetramethyl-ethylenediamine (TEMED) and 0.08% ammonium persulfate (APS). For 10 mL mix 3.5 mL of 40% acrylamide–bis-acrylamide, 3.75 mL of 1 M Tris–HCl pH 8.8 (*see* **Note 3a**), 100 µL of 10% SDS (*see* **Note 3b**), 2.55 mL of water, 20 µL of 100% TEMED (*see* **Note 6**), and 80 µL of 10% APS (*see* **Notes 3c** and **4**).
3. Stacking gel: 4% acrylamide–bis-acrylamide (Sigma-Aldrich) (*see* **Note 6**), 125 mM Tris–HCl pH 6.8, 0.1% SDS, 0.8% TEMED, and 0.1% APS. For 5 mL mix 0.5 mL of 40% acrylamide–bis-acrylamide, 625 µL of 1 M Tris–HCl pH 6.8 (*see* **Note 3a**), 50 µL of 10% SDS (*see* **Note 3b**), 3.785 mL of water, 40 µL of 100% TEMED (*see* **Note 6**), and 50 µL of 10% APS (*see* **Notes 3c** and **4**).
4. 1× Running buffer (RB): 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3. To prepare a 10× RB stock solution weigh 144.12 g of glycine (MW = 75.07 kDa) and dissolve it in 600 mL of water. Adjust the pH to 8.3 with Tris base, add 100 mL of 10% SDS (*see* **Note 3b**) and bring the volume to 1 L. Prepare working solution 1× RB by mixing 100 mL of 10× RB and 900 mL of water.
5. Molecular weight marker, Precision Plus Protein™ Prestained Standards (Bio-Rad).

2.6 Protein Transfer from SDS-PAGE Gel to Membrane

1. Polyvinylidene fluoride (PVDF) membrane (*see* **Note 7**).
2. Mini Trans-Blot® cell system (Bio-Rad).
3. 1× Transfer Buffer (TB): 6 g/L Tris base, 3.1 g/L boric acid.
4. Extra Thick Blot Paper (7.5 × 10 cm; Bio-Rad).
5. Methanol.
6. Magnetic stirrer and magnetic stir bar.

2.7 Protein Immunodetection by Western Analysis

1. 1× Tris-Buffered Saline (TBS): 20 mM Tris–HCl pH 7.6, 150 mM NaCl. To prepare 1 L of 10× TBS stock solution, mix 200 mL of 1 M Tris–HCl pH 7.6 (*see* **Note 3a**), 300 mL of 5 M NaCl (*see* **Note 3d**), and 500 mL of water. Prepare working solution 1× TBS by mixing 100 mL of 10× TBS and 900 mL of water.
2. 1× Blocking Buffer (BB): 0.05 % Tween 20 and 5 % nonfat dry milk in 1× TBS. To prepare 1 L of 1× BB, weigh 50 g of nonfat dry milk and dissolve it in 500 mL of 1× TBS. Add 500 μL of 100 % Tween 20 and dissolve well. Bring the volume to 1 L with 1× TBS.
3. Primary antibodies against the proteins of interest. In the case example, mouse anti-GAPDH antibodies (Chemicon International). Store at −20 °C.
4. Secondary antibodies conjugated to enzyme, such as horseadish peroxidase (HRP), against the Fc domain of the primary antibodies. In the case example, anti-Mouse IgG (Fab specific)-Peroxidase antibody produced in goat (Sigma-Aldrich). Store at −20 °C.
5. Chemiluminescence detection reagents: Amersham™ ECL Western Blotting analysis system (GE Healthcare). Use SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) if more sensitivity is required. Store the reagents at 4 °C.
6. Platform Rocker.
7. Saran Wrap plastic.
8. X-ray films.
9. Cassette.
10. X-ray film processor.

2.8 Estimation of Protein Synthesis Rate

1. Image scanner.
2. ImageJ software (free download at http://imagej.net/Downloads).

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Figure 1 summarizes the methods used in this protocol.

3.1 Amount of Starting Biological Material

The translation rate of one protein of interest is analyzed in HeLa cells for this case example. Six AHA time-incubation periods are used in order to calculate the translation rate. Therefore in this example the experiment is composed of six samples/cultures grown in a six wells culture plate, with a growth area of 9.5 cm^2 per well.

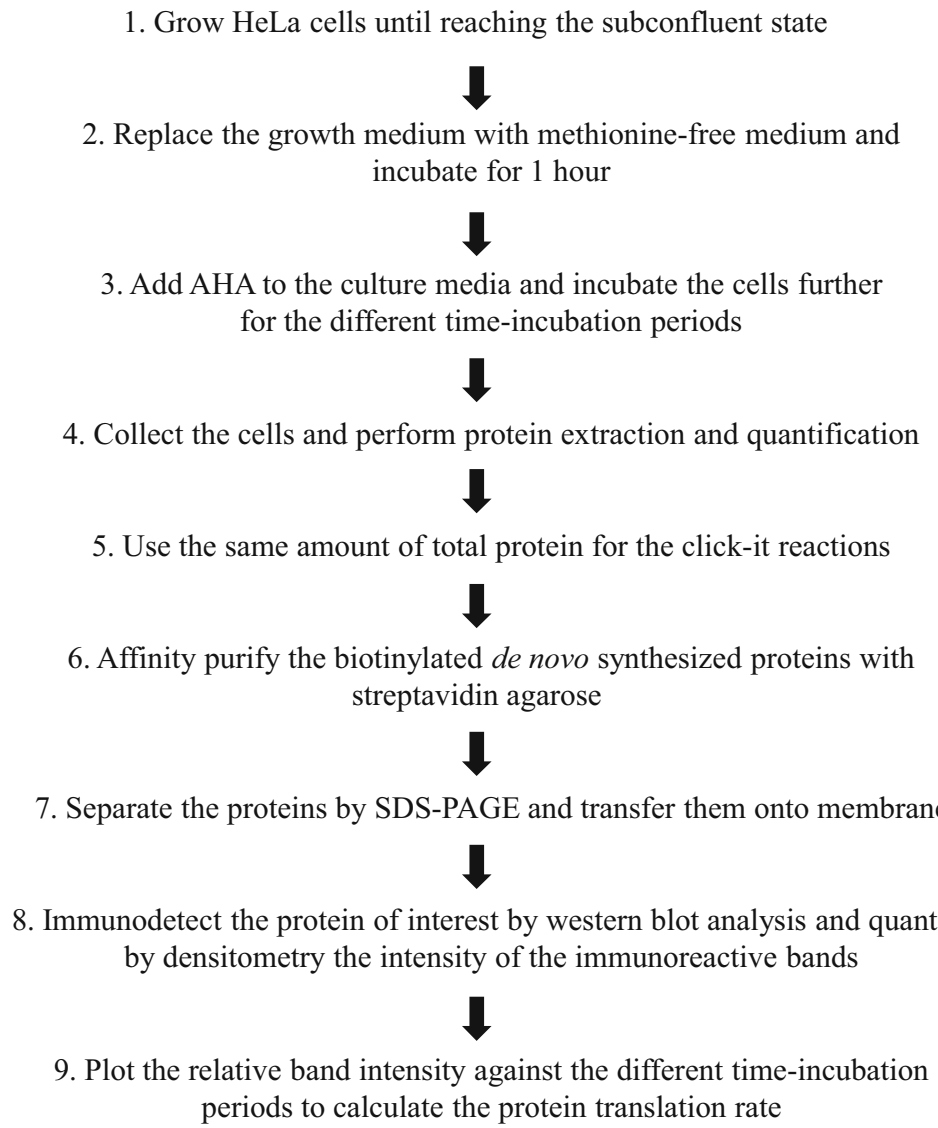

Fig. 1 Summary of the methods used for quantitation of protein translation rate in vivo with bioorthogonal Click-chemistry

1. Seed 3×10^5 exponentially growing HeLa cells in 2 mL of DMEM/F12 in each well. After 24 h, replace medium with fresh DMEM/F12 and incubate at 37 °C and 5% CO_2 for 24 h until reaching the subconfluent state (1.5×10^6 cells/cm^2). Each sample/culture of subconfluent HeLa cell monolayers contributes sufficient tissue to extract at least 100 μg of total protein to prepare the click-it reactions.
2. Once at the subconfluent state, wash cells twice with 1 mL of PBS.
3. Remove PBS and add 1 mL of DMEM-Met prewarmed at 37 °C. Incubate the cultures for 1 h at 37 °C.

4. Add 1 μL of 50 mM AHA (50 μM final concentration) to each growth culture media and incubate for different time periods. In this case example the time-incubation periods are 0, 1, 2, 4, 6, and 8 h (*see* **Note 8**).
5. After each time-incubation period, wash three times with 1 mL of cold PBS.
6. After the last wash, add 1 mL of cold PBS once again and harvest the cells with a cell scraper.
7. Collect the cell suspensions in 15 mL tubes and put them on ice.
8. Centrifuge the samples at 7000 × *g* for 5 min at 4 °C and discard supernatant. Store the samples at −80 °C before use.

3.2 Total Protein Extraction and Quantification

1. Add 60 μL of 1× PEB to the cell pellets and mix by using the vortex. Incubate the lysates for 30 min on ice vortexing every 5 min.
2. Centrifuge the lysates at 12,000 × *g* for 15 min at 4 °C, and collect individually the supernatants, which are the total protein extracts, into new 1.5 mL tubes and keep them on ice.

   The quantification of the total proteins in each sample (Bradford assay) is depicted in the next steps. First, a calibration curve representing the absorbance at 595 nm (A_{595nm}) versus the concentration of protein (mg/mL) is needed. The calibration curve is made by measuring the A_{595nm} of different BSA concentrations.
3. Use a BSA stock solution (2 mg/mL) to make different dilutions by mixing with cold PBS. Maintain the serial dilutions on ice:
   (a) Prepare 1.4 mg/mL BSA by mixing 70 μL of 2 mg/mL BSA with 30 μL of PBS. Vortex.
   (b) Prepare 1 mg/mL BSA by mixing 71.4 μL of 1.4 mg/mL BSA with 28.4 μL of PBS. Vortex.
   (c) Prepare 0.50 mg/mL BSA by mixing 50 μL of 1 mg/mL BSA with 50 μL of PBS. Vortex.
   (d) Prepare 0.25 mg/mL BSA by mixing 50 μL of 0.50 mg/mL BSA with 50 μL of PBS. Vortex.
   (e) Prepare solution 0 mg/mL BSA with 100 μL of PBS.
4. Dilute the protein extract five times by mixing 3 μL of each protein extract with 12 μL of cold PBS. Vortex and keep on ice.
5. Mix 5 μL of each BSA or protein extract dilutions with 200 μL of Bradford reagent. Do it in duplicate.
6. Pippete the mixes separately in a 96-well microtiter plate and measure the A_{595nm} with the microplate reader.

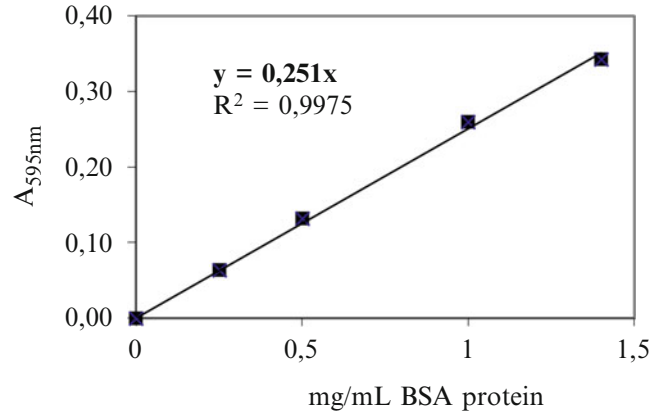

Fig. 2 Standard calibration curve for protein quantification from data obtained with the Bradford assay. The resulting linear regression equation is shown in bold. The *y* means the A_{595nm} while the *x* refers to the protein concentration

7. Represent the obtained standard calibration curve. Figure 2 represents the calibration curve and the linear regression equation obtained for this case example.
8. After measuring the A_{595nm} for every sample, calculate the protein concentration in the extracts by using the linear regression equation obtained from the standard calibration curve. Table 1 shows the protein concentration of the extracts for this case example.

Table 1

Protein concentration of the different samples/time-incubation periods with AHA are determined by using the linear regression equation obtained with the calibration curve

Samples/time-incubation periods with AHA (hours)	A_{595nm}	mg/mL of protein in the extract
0	0.103	2.06
1	**0.102**	**2.03**
2	0.103	2.05
4	0.137	2.73
6	0.115	2.29
8	0.132	2.63

The limiting concentration sample is highlighted in bold (*see* **Note 9**)

3.3 Performing the Click-It Reactions

The same amount of total protein has to be used to prepare the click-it reactions for every time-incubation sample (*see* **Note 9**).

1. For each click-it reaction mix 10 μL of 4 mM biotin-PEG4-alkyne with 90 μL of Click-iT® reaction buffer (Component A) in a 1.5 mL tube.
2. Add up to 200 μg of total protein extract labeled with AHA in a maximum volume of 50 μL (*see* **Note 10**).
3. Bring the volume to 160 μL with ultrapure 18 MΩ water and vortex for 5 s.
4. Add 10 μL of 40 mM $CuSO_4$ (Component C) and vortex for 5 s.
5. Add 10 μL of Click-iT® reaction buffer additive 1 (Component C) and vortex for 5 s. Wait for 2–3 min, but not longer than 5 min before **step 7**.
6. Add 20 μL of Click-iT® reaction buffer additive 2 (Component D) and vortex for 5 s. This mixture turns color into bright orange.
7. Incubate the reactions for 20 min in the end-over-end rotator.

3.4 Purification of Biotinylated "De Novo" Synthesized Proteins

1. To clean up the excess of free biotin-PEG4-alkyne, use the PD pipette MiniTrap G-25 (GE Healthcare) columns. Remove the top and bottom caps and transfer the columns into 15 mL tubes (*see* **Note 11**).
2. To equilibrate the columns add 2 mL of 1× EB per column and allow the EB to enter the packed bed completely. Discard the flow-through and repeat this step twice.
3. Centrifuge the columns at 1000 × *g* for 2 min.
4. Place the columns into new 15 mL tubes and add separately the samples (0.2 mL) into the columns in the middle of the packed bed.
5. Elute and collect the samples by centrifugation at 1000 × *g* for 2 min (*see* **Note 12**).
6. To purify the biotinylated proteins from the total extract protein background, use a column of Streptavidin-Agarose (Sigma-Aldrich). First, place the 10 μm pore filter into the 1 mL Mobicol classic column until well adjusted. Place the columns into 2 mL tubes.
7. Add 50 μL of Streptavidin-Agarose slurry per column and wash twice with 400 μL of PBS centrifuging at 1800 × *g* for 2 min. Discard the flow-through and place the columns into new 2 mL tubes.
8. Put a bottom cap into the column, add the samples to each Streptavidin-Agarose column and incubate the columns in the end-over-end rotator for 1 h.

9. Remove the bottom cap, put the columns into new 1.5 mL tubes, centrifuge at 1800 × *g* for 2 min and collect the unbound sample to streptavidin protein extract. Store it at −20 °C as a control to check binding to the agarose matrix if necessary.
10. Place the columns into new 2 mL tubes, wash the Streptavidin-Agarose beads with 400 μL of 1× WB and centrifuge at 1800 × *g* for 2 min. Repeat this step three times.
11. Place the columns into new 1.5 mL tubes and add 50 μL of 2× LB. Incubate at 95 °C for 10 min in the Thermoblock (*see* **Note 13**).
12. Elute and collect completely the biotinylated proteins by centrifugation at 1800 × *g* for 2 min. Store the samples at −20 °C until use.

3.5 Separation of the Purified Biotinylated Proteins in a SDS-PAGE

1. Place the previously polymerized 10–14 % SDS-PAGE gel and the Mini Cell Buffer Dam (Bio-Rad) facing each other into the Mini-Protean® Tetra handcast systems (Bio-Rad). Fill completely the inner space between plates with 1× RB. Check the wells to be entirely filled with 1× RB. Fill the outside space until the line marked for 2 gels in the buffer tank.
2. Pippete 25 μL of each sample (the order of the pipetting in this case example is 0, 1, 2, 4, 6, and 8 h of time-incubation periods with AHA) into the well. Pipette in the right or in the left corner side of the gel, the prestained MW markers (*see* **Note 14**).
3. Run the gel electrophoresis until the dye has reached the bottom of the gel.
4. Disassemble the plates and place the gel in a cuvette with 1× TB.

3.6 Protein Transfer from SDS-PAGE Gel to Membrane

1. Activate the PVDF membrane by rinsing in methanol for 1 min and next place the membrane in a cuvette with 1× TB.
2. Prepare the transfer in wet conditions with 1× TB by using the Mini Trans-Blot® cell system (Bio-Rad) and two pieces of Extra Thick Blot Paper (Bio-Rad).
3. Fill totally the tank with 1× TB and place the magnetic stir bar inside.
4. Place the system on a magnetic stirrer during the transfer.
5. Transfer the proteins from the SDS-PAGE gel to the membrane at 2–3 V/cm overnight.

3.7 Protein Immunodetection by Western Analysis

1. Disassemble the transfer system and place the PVDF membrane in a cuvette with 1× TBS.
2. Discard the 1× TBS and incubate to block the membrane with abundant 1× BB for 1 h in the rocking platform (*see* **Note 15**).

3. Discard the 1× BB and incubate the membrane with the primary antibody dissolved in 1× BB for 1 h in the rocking platform (*see* **Note 15**).
4. Discard the solution, and wash with abundant 1× BB by incubating for 10 min in the rocking platform. Repeat this step three times.
5. Discard the 1× BB and incubate the membrane with the secondary antibody dissolved in 1× BB for 1 h in the rocking platform (*see* **Note 15**).
6. Discard the solution, and wash with abundant 1× BB by incubating for 10 min in the rocking platform. Repeat this step three times.
7. Prepare the chemiluminescence detection mix. To prepare this, mix one volume of solution I with one volume of solution II (*see* **Note 16**).
8. Place face up the wet membrane on a Saran Wrap piece and pipette the chemiluminescence detection mix on the membrane. Cover the membrane with another piece of Saran Wrap to spread completely the detection reagent mix on the membrane and incubate for 1–5 min.
9. Unwrap the membrane and discard the excess of detection mix contacting the corner of the membrane.
10. Place face down the membrane on a new Saran wrap piece (*see* **Note 17**) and cover with another one.
11. Place face up the wrapped membrane into the cassette.
12. In the darkness, expose the X-ray film with the membrane (*see* **Note 18**).
13. Develop the X-ray film by using the X-ray film processor. The Fig. 3a shows the autoradiography results obtained in this case example.

3.8 Estimation of Protein Synthesis Rate

1. Scan the autoradiography with the image scanner.
2. Quantify by densitometry the intensity of the bands by using the ImageJ software.
3. Calculate the relative band intensity as the ratio with respect to the reference value (*see* **Note 19**).
4. Plot the relative band intensity against the time-incubation periods.
5. The slope of the curve reflects the translation rate of the protein of interest. Figure 3b shows the case example of GAPDH protein analysis (*see* **Note 18**).

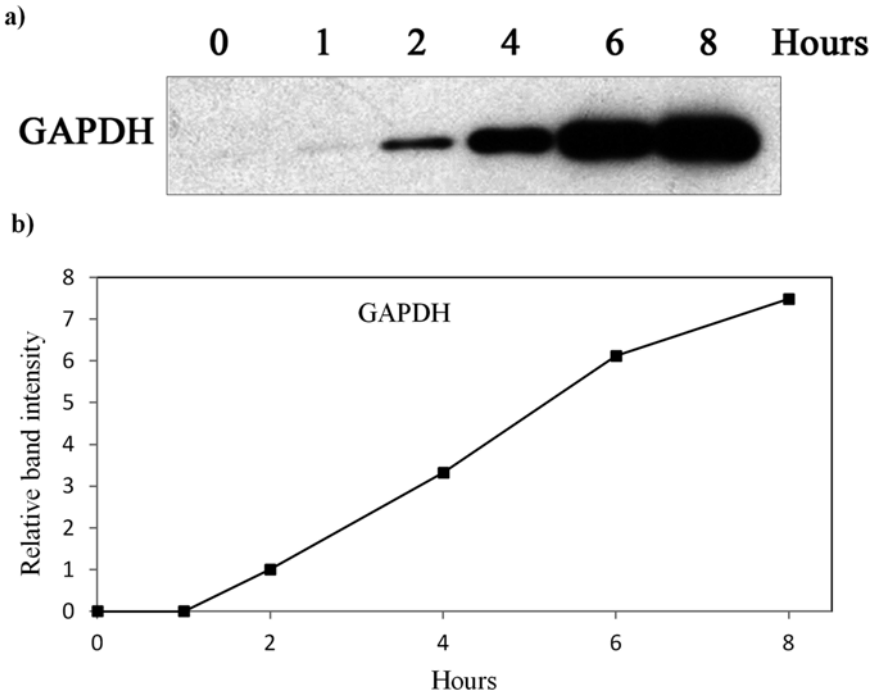

Fig. 3 Estimation of GAPDH protein synthesis rate can be calculated from the slope of the plot of the relative band intensity against the time-incubation periods. The immunodetected bands used are shown in (**a**). The relative values, depicted in (**b**), were obtained using as the reference value the scanned signal of the first immunodetectable band of *lane 2* (*see* **Note 18**)

4 Notes

1. In the case example described in this chapter, six AHA time-incubation periods are used. In the case of adherent cultures of HeLa cells, the growth area (9.5 cm^2/sample) provides suitable quantity of protein to perform the click-it assays.
2. To prepare the DMEM cell culture solutions, mix 50 mL of 100% FBS (Gibco®, Life Technologies) and 450 mL of the specific DMEM. Then add 5 mL of 10,000 U/mL Penicillin-Streptomycin (Gibco®, Life Technologies). Store at 4 °C.
3. It is useful to prepare the following stock solutions in order to facilitate the elaboration of all the buffers and reagents depicted in this chapter. Unless otherwise indicated all the solutions should be autoclaved for 20 min at 120 °C and 1 atm of pressure and stored at room temperature:
   (a) 1 M Tris–HCl pH 6.8, 7.6, 8.0, or 8.8 (500 mL): weigh 60.57 g of Tris base (MW = 121.14) and mix with 250 mL of water. Adjust the pH to 6.8, 7.6, 8.0, or 8.8 with HCl and bring the volume to 500 mL with water.

(b) 10% SDS (500 mL): weigh 50 g of SDS and dissolve it in water up to 500 mL.

(c) 10% APS (10 mL): weigh 1 g of APS and dissolve it in water up to 10 mL. Do not autoclave. Store at −20 °C.

(d) 5 M NaCl (500 mL): weigh 146.10 g of NaCl (MW = 58.44) and dissolve it in water up to 500 mL.

(e) 10% DOC (100 mL): weigh 10 g of DOC and dissolve it in water up to 100 mL. Do not autoclave.

4. In this case example 1.0 mm integrated spacer plates were used to pour 10 mL of resolving gel and 5 mL of stacking gel mixes to prepare two SDS-PAGEs gels. Scale the volumes to perform more than two SDS-PAGEs gels or to use different spacer plates. Add the APS just before pipetting the liquid mixes inside the plates in order to start polymerization of the gels. Immediately pour the resolving gel mix (4.70 mL per gel) and cover with a layer of 2-butanol until polymerization is completed. Next, eliminate the 2-butanol by decantation and add the stacking gel mix (freshly prepared) up to the upper limit of the plates. Immediately place the comb to set the wells.
5. The correct percentage of acrylamide–bis-acrylamide depends on the molecular weight of the proteins of interest.
6. The acrylamide–bis-acrylamide and the TEMED solutions should be stored at 4 °C and −20 °C respectively.
7. For each gel to transfer, prepare a PVDF membrane section of 7 × 9 cm.
8. The time-incubation periods with AHA depend on the protein turnover under study. This should be checked empirically in order to optimize the technique. As an approximation to address protein turnover under standard growth conditions in HeLa cells, the tool PepTracker (http://www.peptracker.com/epd/search/) can be helpful.
9. The maximum quantity of protein used is restricted to the sample with less protein concentration. This limiting amount of total protein has to be equal for every sample. In the case example, the sample of 1 h of time-incubation period with AHA (2.03 mg/mL) is the limiting one.
10. In this case example, the maximum quantity of protein of the limiting sample, which has to be used for the click-it reactions, is 50 µL × 2.03 µg/µL = 101.5 µg of total protein. Next, calculate the volume for 101.5 µg of total protein to add of each sample/time-incubation period for the click-it reactions. For example, for the sample of 4 h of time-incubation period with AHA (2.73 mg/mL), add 37.18 µL of protein extract.
11. The use of 15 mL tube centrifuge adapters is recommended.
12. The sample will still have bright orange color.

13. The elution of the biotinylated proteins will start in this step. Take care to avoid losing the eluted proteins.
14. The prestained MW marker helps visualization of the SDS-PAGE progression and the transfer onto PVDF membrane.
15. The blocking or the antibody-incubation conditions depends on the antibody used. Check them empirically before use. In this case example, the PVDF membrane was blocked for 1 h with 1× BB. Then the membrane was first incubated for 1 h with the anti-GAPDH primary antibody diluted 1:5000 in 1× BB and, after washing, with the anti-mouse IgG-HRP secondary antibody diluted 1:50,000 in BB for 1 h.
16. 500 μL of the detection reagent mix is enough to cover a 7×9 cm PVDF membrane.
17. Try to avoid the appearance of bubbles between the plastic and the membrane.
18. The exposure time depends on the protein of interest or the antibody used. Optimize the exposure time empirically.
19. The reference value is the intensity data of the first detectable scanned band of the time-incubation periods. The slope of the curve reflects the protein translation rate and can be used to compare translation rates of any protein of interest under different conditions such growth conditions, pharmacological treatments, genetic alterations, etc.

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References

1. Man O, Pilpel Y (2007) Differential translation efficiency of orthologous genes is involved in phenotypic divergence of yeast species. Nat Genet 39(3):415–421. doi:10.1038/ng1967
2. Nissley DA, O'Brien EP (2014) Timing is everything: unifying codon translation rates and nascent proteome behavior. J Am Chem Soc 136(52):17892–17898. doi:10.1021/ja510082j
3. Boisvert F-M, Ahmad Y, Gierliński M, Charrière F, Lamont D, Scott M, Barton G, Lamond AI (2012) A quantitative spatial proteomics analysis of proteome turnover in human cells. Mol Cell Proteomics 11(3). doi: 10.1074/mcp.M111.011429
4. Best MD (2009) Click chemistry and bioorthogonal reactions: unprecedented selectivity in the labeling of biological molecules. Biochemistry 48(28):6571–6584. doi:10.1021/bi9007726
5. Breinbauer R, Köhn M (2003) Azide-alkyne coupling: a powerful reaction for bioconjugate chemistry. Chembiochem 4(11):1147–1149. doi:10.1002/cbic.200300705
6. Prescher JA, Bertozzi CR (2005) Chemistry in living systems. Nat Chem Biol 1(1):13–21. doi:10.1038/nchembio0605-13
7. Dieterich DC, Link AJ, Graumann J, Tirrell DA, Schuman EM (2006) Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT). Proc Natl Acad Sci 103(25):9482–9487. doi:10.1073/pnas.0601637103