Supporting Information

Affordable uniform isotope labeling with $^2$H, $^{13}$C and $^{15}$N in insect cells

Agnieszka Sitarska, Lukasz Skora, Julia Klopp, Susan Roest, César Fernández, Binesh Shrestha and Alvar D. Gossert

Supplementary Figure 1: Pictorial summary of protocol for uniform labeling in Sf9 insect cells.
Protocol for uniform isotope labeling in Sf9 or Sf21 insect cells.

Media

- Amino acid-free basal media: Custom SF-4 (BioConcept) or Sf-900 III (Gibco/Life Technologies) medium without individual amino acids, glucose and yeast extract/yeastolate
  - Custom Sf-900 III media from can be ordered through a web-based custom media configurator.
    (Last accessed February 23rd, 2015)
  - Custom SF-4 media can be ordered by email: info@bioconcept.ch
- Standard SF-4 (BioConcept) or Sf-900 III (Gibco/Life Technologies) medium (depending on the cell line being used) for preparation of the starting culture.

Reagents

- ISOGRO® with desired isotope labeling pattern (Sigma-Aldrich)
- Tryptophan with desired isotope labeling pattern (Cambridge Isotope Laboratories or Sigma-Aldrich)
- L-cycloserine (Sigma-Aldrich, Cat. No. C1159)
- d-Glucose or u-13C d-glucose (Cambridge Isotope Laboratories or Sigma-Aldrich)
- 15NH4Cl (Cambridge Isotope Laboratories or Sigma-Aldrich)

Consumables

- Sterile culturing bottles with vented caps for 250 mL and 1 L culture volumes
- Sterile conical 500 mL centrifuge bottles (Corning, Cat. No. 431123)
- Sterile filtering devices with 0.22 µm pores (Millipore, Cat. No. SCGUP11RE)
Recipe for 1 L of medium for uniform isotope labeling in insect cells

1 L Amino acid-free basal medium

10 g ISOGRO® algal amino acid extract with desired labeling pattern

10 g D-Glucose (or 5 g of 13C-glucose)

2 mL Tryptophan stock solution (10 mg/mL) with the desired labeling pattern

833 µL 15NH₄Cl (300 mg/mL)

1 mL L-cycloserine (5 mg/mL)

Dissolve ISOGRO powder and glucose in basal medium by stirring with a magnetic bar for 15 min at room temperature. If needed, sonify medium to fully dissolve any solids. Add remaining stock solutions and stir for another minute. Check pH and osmolarity by measuring a 5 ml aliquot offline. If needed adjust with NaOH or HCl to pH=6.2±0.2 and osmolarity with NaCl to 320 mOsm/kg. Sterile-filter the medium through 0.22 µm pores into a sterile bottle and store closed at 4°C.

---

1 See materials above for ordering information.

2 In order to improve 13C labeling ratio form 75% to 80%, i.e. for backbone assignment experiments, 13C-labeled glucose can be used. It is incorporated primarily into Ala but also Glu, Gln, Asp, Asn by insect cell metabolism. Only 5 g of glucose can be used in this case. For SF-4 based media, generally only 5 g of glucose are used.

3 The tryptophan stock solution should always be stored protected from light at 4°C. The stock solution is at the limit of solubility, it should be prepared at room temperature.

4 For 13C labeling and 2H labeling the use of L-cycloserine is encouraged when non-labeled glucose is used. It will ensure full 13C and 2H labeling of alanine as it inhibits the enzyme (alanine transaminase) producing alanine from (unlabeled) pyruvate derived from glucose. For 2H-labeling it will additionally reduce the amount of deuterated α-ketoglutarate produced in this reaction, leading to less inhibition of the citric acid cycle and therefore improve cell viability.

5 The medium is designed to match these values, in practice we never had to adjust them.
Protocol for 1 L of insect cell culture

Day -2 (Friday) – Preparation of starter culture

0.1 Prepare a starter culture (Sf9 or Sf21 cells in SF-4 medium or in Sf-900 III) by passaging to a density of 0.7–1.0 x 10^6 cells/mL in 250 mL of working volume.

0.2 Incubate at 27°C, 220 rpm shaking speed, 25 mm shaking diameter.

Shaking speed should be adjusted to the properties of the incubator - shaking diameter mainly, as well as the size of a culturing vessel (the smaller shaking diameter, the higher shaking speed. The bigger size of a culturing vessel, the lower shaking speed). Cell suspension should be stirred efficiently in order to provide a proper aeration as well as the intake of nutrients from the medium. Cells must not aggregate on the bottom of the vessel.

Day 1 (Monday) - Infection

1.1 9:00 AM: Dilute starter cell culture (density around 8.0 x 10^6 cells/mL) with fresh full medium to a final volume of 1 L and approximately 1.5 x 10^6 cells/mL in a culturing bottle with vented cap.

1.2 Incubate for 8 h under the same conditions as for the starter culture.

1.3 Meanwhile, prepare the expression medium for isotope labeling according to the recipe above.

1.4 5:00 PM: Take the pre-incubated 1 L cell culture and infect it with the respective baculovirus. The infection ratio (MOI) depends on the potency of a particular viral construct, which should be evaluated experimentally beforehand.

Infection has to be performed in the full culturing medium. Ammonium chloride, which is a component of the labeling medium, inhibits the viral entry and renders viral infection inefficient.

1.5 Incubate the infected culture overnight (16 h) under the same parameters.

Protein over-expression induced by the polyhedrin promoter starts 24–48 hours post-infection. Therefore, 16 h of incubation post-infection in full medium, does not significantly compromise the incorporation of isotopic label. It greatly increases protein yields and ensures reproducibility by allowing efficient viral entry.
Day 2 (Tuesday) – Medium Change

2.1 9:00 AM: Pre-warm the labeling medium to 27°C in a water bath (for at least 15 min).

If washing of the cells for increased isotope incorporation is planned, also pre-warm basal medium or PBS to 27°C. The amount needed is about one fourth of the volume of the expression culture.

2.2 Check cell culture parameters (cell count, cell diameter and viability) of the infected culture before centrifuging.

2.3 Transfer the cell suspension into four 500 mL – centrifuge bottles and spin them for 5 min at 300 x g and 27°C.

When 500 mL centrifuge bottles are only half filled, the cell suspension spin lasts shorter and requires lower centrifuging force. The cell pellet, while still not truly compact, is yet more stable while handling.

2.4 Carefully decant supernatant and discard it.

2.5 Resuspend the cell pellet in 50 mL per centrifuge bottle of warm (27°C) basal medium or PBS in order to wash away remaining unlabeled medium. Centrifuge again for 5 min at 300 x g and 27°C.

This step improves the labeling ratio by about 5%, depending how efficiently the culture medium can be removed from the pellet in the previous step. Cell viability and likewise the productivity of the culture may however be compromised by this additional handling. This step may therefore be omitted.

2.6 Carefully decant supernatant and discard it.

2.7 Resuspend the cells immediately in pre-warmed labeling medium (27°C).

2.8 Check cell count, cell diameter and viability of the infected culture after these manipulations.

2.9 Incubate for additional 72 h or until viability drops below 40%.
Day 3–5 (Wednesday – Friday) – Monitoring of culture

3.1 Regularly monitor cell culture parameters (cell count, cell diameter and viability)

Viability drop and increasing cell diameter over time indicates an efficient viral infection. For cytosolic proteins, viability should not fall below 30-40%, because subcellular compartmentalization in dying cells will be weakened, leading to elevated proteolysis in the cytosol. The cell count should also not drop significantly, because lysed cells will release protein into the supernatant. Therefore, a decrease in culture viability compromises protein yields. For optimal results, expression should be continued for 56–80 h from the medium change point, which is 72–96 h post-infection. The infection ratio with the particular baculovirus should be adjusted accordingly.

Day 5 (Friday) – Harvest:

5.1 5:00 PM (or when viability drops below 40%): Harvest the cells by centrifugation for 15 min at 1500 x g and 4°C.

5.2 Freeze the cell pellet at -80°C or process supernatant if the target protein is secreted.
Supplementary Figure 2: Example cell culture data for expression of uniformly $^{2}$H,$^{13}$C,$^{15}$N-labeled Abl. During the entire cell culture period, cell count still keeps increasing (top chart). Due to the activity of the baculovirus, cell diameter increases in the initial phase of infection (bottom). After protein production is initiated, cell viability (middle) and average diameter drop. The virus amount employed, was optimized in previous small scale experiments in order that cell viability drops below 80% between 72 and 96 h post infection. Cells were harvested as soon as viability was below 40–50%. $^{2}$H,$^{13}$C,$^{15}$N labeling as shown here can be considered a worst case scenario in terms of cell growth, since deuterated amino acids affect cell metabolism slightly negatively. Data were obtained on a Vi-CELL cell viability analyzer (Beckmann Coulter).
Supplementary Figure 3: Mass spectrometry data of uniformly $^2$H, $^{13}$C, $^{15}$N-labeled Abl kinase. Mass spectra of unlabeled (green) and triply labeled (magenta) Abl kinase are shown. Note the approximately 20-fold broader signal of the labeled molecule, due to inhomogeneous label incorporation. Data were obtained on an Acquity ultra-high pressure liquid chromatography station coupled to a Xevo G2-S QTof mass spectrometer with electrospray ionization (Waters). In order to obtain a clear signal, maximum entropy processing using data point spacing of 7 Da had to be applied for the labeled molecule as opposed to 0.4 Da in the unlabeled case.