Pulse Chase of Suspension Cells

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[Abstract] Pulse-chase method is a powerful technique used to follow the dynamics of proteins over a period of time. The expression level, processing, transport, secretion or half-life of proteins can be tracked by metabolically labeling the cells, such as with radiolabeled amino acids (pulse step). This protocol describes the condition used to study the folding and disulfide bond formation of immunoglobulin in suspension cells. With some minor modifications, this protocol can be adapted to study the degradation rate or the secretion of target proteins.

Materials and Reagents

A. Pulse chase
1. Cells growing in suspension
2. HBSS (Life Technologies, Invitrogen™, catalog number: 14175-095)
3. RPMI without methionine and cysteine (Sigma-Aldrich, catalog number: R7513)
4. Dialyzed FBS (Life Technologies, Invitrogen™, catalog number: 26400044)
5. *N*-Ethylmaleimide (NEM) (Sigma-Aldrich, catalog number: R3876)
6. Cyclohexamide (CHX) (Sigma-Aldrich, catalog number: C7698)
7. Express ^{35}S protein labelling mix (Perkin Elmer, catalog number: NEG072014MC)
8. Methionine (Sigma-Aldrich, catalog number: M5308)
9. Cysteine (Sigma-Aldrich, catalog number: C7352)
10. Labeling medium (see Recipes)
11. Chase medium (see Recipes)
12. 2x stop buffer (see Recipes)

B. Cell lysis and immunoprecipitation
1. Antibody against protein of interest
2. Protein A/G beads (Thermo Fisher Scientific, catalog number: 20422)
3. Complete Protease Inhibitor Tablets (Roche Diagnostics, catalog number: 11836145001)
4. Lysis buffer (see Recipes)

C. SDS-PAGE
1. 4-12% Bis-Tris protein gel (Life Technologies, Invitrogen™)
2. MOPS running buffer (Life Technologies, Invitrogen™, catalog number: NP0001)
3. Amplify solution (GE Healthcare, catalog number: NAMP100)
4. Gel drying solution (Life Technologies, Invitrogen™, catalog number: LC4025)
5. Gel fixing solution (see Recipes)

Equipment

1. Incubator
2. Eppendorf tube
3. 26-gauge needle
4. 1 ml syringe
5. Heat block
6. Gel dryer
7. Phosphor imaging screen

Procedure

Cells are pulse-labeled and chased in a single tube and an aliquot of cells is removed from this tube for each time point of the chase.
After determining the numbers of chase time points (x), prepare enough cells for the experiment (x + 1, 2 x 10^6 per sample). See Note 1.

A. Pulse chase
1. Wash cells (2 x 10^6 per sample) with 2 ml of HBSS.
2. Pellet cells at 500 *x g* for 3 min at room temperature. Resuspend cells in 2 ml/sample of pre-warmed labeling medium. Mix gently.
3. Incubate the cells for 20 min at 37 °C incubator.
4. Pellet cells at 500 *x g* for 3 min at room temperature and resuspend cells in 100 µl/sample of pre-warmed labeling medium. Keep the cells at 37 °C, either in a water bath or an incubator during the labeling and chase periods.
5. Pulse for 2 min at 37 °C with [^{35}S] methionine (100 µCi/ml) (see Note 2).
6. Add 400 µl/sample of chase medium. Pipet up and down gently to ensure proper mixing.
7. Immediately take out 500 µl for the 0 min sample. Transfer to an eppendorf tube filled with 500 µl of 2x ice cold stop buffer (see Note 3).
8. Spin cells at 500 *x g* for 2 min at 4 °C and freeze pellet.

9. Repeat steps 7-8 for every time point.
10. Proceed to cell lysis or keep the pellet frozen in -80 °C.

B. Cell lysis and immunoprecipitation
   1. Add 1ml cold lysis buffer to each cell pellet.
   2. Apply mechanical shearing force to the cell lysate by passing it through a 26-gauge needle attached to a 1 ml syringe (repeat 5-10 times for thorough lysis). Incubate on ice for 15 min. Spin at 16,000 *x g* for 15 min at 4 °C to clarify the lysate. Transfer the clarified cell extracts to a new tube.
   3. Add antibody against protein of interest to equal volume of cell extracts and rotate overnight at 4 °C (see Note 4).
   4. Add 30 µl of Protein A/G beads and incubate for another 2 h at 4 °C.
   5. Wash the immunoprecipitates twice with 1 ml of lysis buffer.
   6. Add 50 µl of sample buffer without reducing agents (such as DTT or 2-mercaptoenthanol). See Note 5.
   7. Heat the samples 65 °C for 5 min. Centrifuge briefly before proceeding to non-reducing SDS-PAGE.

C. SDS-PAGE
   1. Load 25 µl of the immunoprecipitates on SDS-polyacrylamide gel.
   2. Immerse the gel in fixing buffer for 15-30 min at room temperature.
   3. Immerse the gel in amplify solution for 15 min at room temperature.
   4. Immerse the gel in gel drying solution for another 15 min at room temperature.
   5. Dry the gel on filter paper on top of a gel dryer of choice.
   6. [^{35}S] methionine-labeled proteins can be visualized after exposure to a Phosphor imaging screen.

Representative data

1. The protocol described here was used to examine the maturation kinetics of pentameric IgM complexes during their passage through the secretory pathway. IgM assembly begins with the coupling of a heavy chain (H) and a light chain (L), resulting in a monomeric heavy and light chain intermediate (HL). A H2L2 unit is assembled and followed by large multimers of “H2L2”. At later points of the chase time, the signals for these IgM intermediates, especially the high molecular weight species, will decrease due to the successful assembly and secretion of mature IgM.

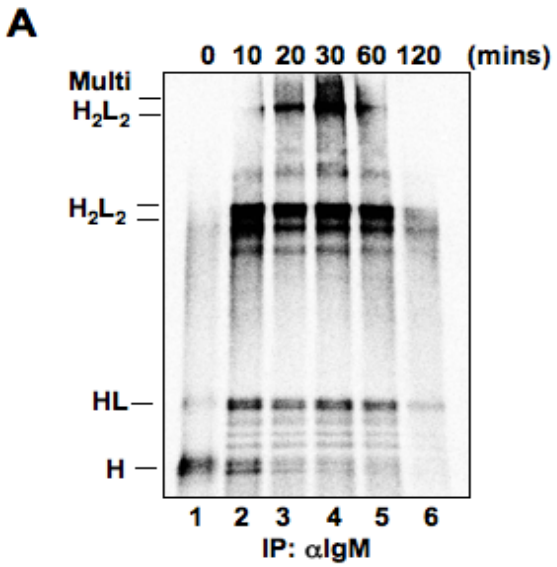

Figure 1. I.29 μ^+ mouse lymphoma cells were pulse labeled with ^{35}S-methoninie for 2 minutes and chased for the indicated times. The cell extracts were immunoprecipitated with αIgM and the immunoprecipitates were resolved by non-reducing SDS/PAGE. IgM assembly intermediates are indicated.

Notes

1. In order to ensure equal number of cells are used in each samples, it is necessary to determine the number of chase time points (x) and the volume of chase medium before starting the experiment. We normally prepare enough cells and medium for x (number of chase time points) plus 1 to account for any fluid loss during the experiment.
2. The incubation time with radioactive amino acids should be optimized depending on the target protein and the cells used. Subsequently, keeping the labeling step consistent, especially the number of cells and 'pulse' time with radioactive amino acids, will reduce variability in the end results.
3. Alternatively, the cells can be added directly to ice cold 2x lysis buffer and cell lysis can commence immediately.
4. The amount of antibody and cell extracts to be added and the incubation time for antibody-antigen binding should be determined and optimized according to each antibody and antigen. Incubation time can vary from 1 h to overnight at 4 °C.
5. Denaturing sample buffer with reducing agents can be used if disulfide bond formation is not being monitored.

6. Dialyzed FBS is used to prevent contamination of ‘cold’ methionine and cysteine. The amount added to the medium should be determined according to the cell line used.
7. Cyclohexamide (CHX) is added to reduce the level of newly translated protein. CHX as well as 'cold' methionine and cysteine should be added fresh to the medium.
8. NEM is an alkylating agent that covalently attaches to free SH-groups found on cysteines. It is added to prevent disulfide bonds from forming once the chase period has ended. In pulse-chase experiments that track the formation of disulfide bonds, NEM must be added to the stop and lysis buffers.

Recipes

1. Labeling medium
   RPMI lacking methionine or cysteine
   1% Penicilin and Streptomycin
   1% glutamine
   5-10% dialyzed FBS (see Note 6)
2. Chase medium (see Note 7)
   Labeling medium (see above) plus
   5 mM cysteine
   5 mM methionine
   1 mM CHX
3. 2x stop buffer
   HBSS with 40 mM NEM (see Note 8)
4. Lysis buffer
   50 mM Tris (pH 7.4)
   150 mM NaCl
   0.5% NP-40
   0.5% Na deoxycholate
   20 mM NEM
   Protease inhibitors
5. Gel fixing solution
   25% methanol
   15% acetic acid

Acknowledgments

The protocol presented here was adapted from van Anken *et al.* (2009). This work was partly supported by grants from the National Institutes of Health (CA082057, CA31363, CA115284, CA180779, AI105809, and AI073099); the Hastings Foundation; the Fletcher Jones Foundation and BaCaTec (J.U.J.). We also thank the Jung laboratory members for their support and discussions.

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