Measurement of T and B Cell Turnover with Bromodeoxyuridine

BrdU INCORPORATION TO DETERMINE T AND B CELL TURNOVER

The rate of turnover of T and B cells can be measured in terms of the time between two mitoses or between mitosis and death. This can be accomplished in vivo by administering DNA precursors and measuring the rate at which cells become labeled with these precursors. 5-bromo-2′-deoxyuridine (BrdU) is a thymidine analog that is incorporated into the DNA of cells during the S phase of the cell cycle and can be subsequently detected with a specific monoclonal antibody. In the Basic Protocol, BrdU is given to experimental animals either continuously or as a pulse, depending on the goal of the experiment. Lymphoid cells are then harvested and stained in vitro with an anti-BrdU antibody and labeled cells detected by flow cytometry. By combining cell surface staining for multiple lymphocyte markers with anti-BrdU staining, it is possible to determine the lifespan of phenotypically defined subsets. The Alternate Protocol is a variation of the Basic Protocol that has been further optimized for use with a potentially wide range of antibody-fluorochrome combinations. This approach may be useful in situations where the Basic Protocol proves incompatible with particular antibody-fluorochrome conjugates or other downstream staining procedures.

CAUTION: BrdU is potentially mutagenic and can be absorbed through skin or by inhalation. Therefore, when handling BrdU, wear suitable protective clothing, including gloves and eye and face protection.

Materials

- Experimental animals
- 0.8 mg/ml BrdU (Sigma) in water (for oral administration) or 4 mg/ml BrdU in PBS (for injection)
- PBS, pH 7.4 (APPENDIX 2A)
- 0.15 M NaCl, ice cold
- 95% ethanol, ice cold
- Paraformaldehyde fixative (see recipe)
- DNase I solution (see recipe)
- Anti-BrdU-FITC (Becton Dickinson Immunocytometry)
- 15 × 75-mm round-bottom polystyrene tubes
- Flow cytometer capable of 3- or 4-color analysis

Additional reagents and equipment for removal of mouse lymphoid organs (UNIT 1.9), preparation of mononuclear cell suspensions (UNIT 3.1), cell surface staining of lymphocytes for flow cytometry (UNIT 5.3), and flow cytometry (UNIT 5.4)

Treat animals with BrdU

1. Administer BrdU to animals in drinking water (dissolve in sterile drinking water at 0.8 mg/ml) or by i.v. or i.p. injection (dissolve in PBS to 4 mg/ml, then inject 0.2 ml—0.8 mg—per mouse as described in UNIT 1.6).

Because BrdU is light sensitive, it is necessary to prepare fresh BrdU-containing drinking water daily. Injection may be used in conjunction with administration in drinking water when the exact start time of BrdU treatment is critical. Injecting BrdU either i.m. or i.p. is preferable when a short pulse of BrdU labeling is desired.
Prepare cells and stain for surface markers

2. Collect lymphoid organs and make single-cell suspensions (UNITS 1.9 & 3.1). Prepare cells for immunofluorescence (UNIT 5.3).

3. Stain cells for surface markers using appropriate antibodies and fluorochromes (see Commentary for detailed description).

   Because the anti-BrdU antibody recommended in this protocol is FITC-conjugated, it is essential not to use FITC-labeled reagents for surface staining. If propidium iodide is to be used to distinguish live from dead cells (see UNIT 5.3), cells should subsequently be washed extensively (four to five times) to remove all extracellular propidium iodide before fixation and permeabilization (see steps 6 to 8). The fluorochromes used for staining will depend on the capabilities of the flow cytometer used for analysis but may include phycoerythrin, allophycocyanin, RED613, RED670, Texas red, CyChrome, and Tricolor, among others.

   If cell surface staining has been carried out in microtiter plates, cells should now be transferred to 15 × 75-mm round-bottom tubes for staining.

4. Wash cells by adding 1 ml PBS, then centrifuging 6 min at 300 × g, 4°C.

5. Pour off all supernatant and resuspend cells in 0.5 ml ice-cold 0.15 M NaCl.

Fix cells

6. While gently vortexing cells, add 1.2 ml ice-cold 95% ethanol dropwise. Incubate cells 30 min on ice.

   Gentle vortexing of the cells and slow addition of ethanol help prevent cell clumping.

7. Wash cells by adding 2 ml PBS, then centrifuging 6 min at 450 × g, 4°C.

   Because ethanol-fixed cells do not form a tight pellet, it is necessary to centrifuge at a higher speed during all washing steps from this point onward to minimize cell loss.

8. Pour off supernatant. Resuspend cells in 1 ml paraformaldehyde fixative. Incubate 30 min at room temperature.

9. Centrifuge cells 6 min at 450 × g, 4°C.

Stain to detect BrdU and analyze results

10. Pour off supernatant. Resuspend cells in 1 ml DNase I solution. Incubate 10 min at room temperature.

11. Repeat step 7.

12. Pour off supernatant. Resuspend cells and add 10 µl anti-BrdU-FITC. Incubate 30 min at room temperature.

13. Repeat step 7.

14. Resuspend cells in 500 µl PBS. Analyze by flow cytometry (UNIT 5.4).

   Samples may be analyzed immediately or stored up to 1 week protected from light at 4°C before being analyzed.

ALTERNATE PROTOCOL

SIMULTANEOUS MEASUREMENT OF T CELL TURNOVER USING HI-DIMENSIONAL FLOW CYTOMETRY

This procedure is a variation of the Basic Protocol that has been further optimized for use with a potentially wide range of antibody-fluorochrome combinations. This approach may be useful in situations where the Basic Protocol proves incompatible with particular antibody-fluorochrome conjugates or other downstream staining procedures. This protocol has been used extensively in flow cytometric analyses involving the simultaneous measurement of >11 fluorescence parameters (hi-dimensional) including a wide array
of surface markers and, in some cases, transcription factors. This protocol has also been successfully used in more typical flow cytometric strategies involving 3 or 4 colors.

Materials

- Experimental animals
- Staining buffer (see recipe)
- Fixing buffer (see recipe)
- Permeabilization buffer (see recipe)
- DNase solution (see recipe), prewarmed to 37°C
- Fluorochrome-conjugated anti-BrdU antibody (BD Biosciences)
- 12 × 75–mm round-bottom polystyrene tubes
- Water bath, prewarmed to 37°C
- Flow cytometer capable of multi-color analysis

Additional reagents and equipment required for BrdU treatment (Basic Protocol), the removal of mouse lymphoid tissues (UNIT 1.9), preparation of single cell suspensions (UNIT 3.1), cell-surface staining for flow cytometry (UNIT 5.3), and flow cytometry (UNIT 5.4)

Label cells in vivo with BrdU

1. Treat animals with BrdU as described in step 1 of the Basic Protocol.

Prepare cells for surface marker staining

2. Harvest tissues, dissociate into single-cell suspensions, and prepare resulting suspension for cell surface staining in 12 × 75–mm round-bottom tubes (UNITS 1.9, 3.1, & 5.3).

3. Perform cell surface staining using appropriate antibodies and fluorochromes.

   IMPORTANT NOTE: The volumes suggested from this point forward assume the use of 12 × 75-mm tubes. If tubes of different sizes are employed, volumes will need to be adjusted accordingly.

   As suggested in the Basic Protocol, be certain that the other staining reagents used for surface staining are not conjugated with the same fluorochrome as the anti-BrdU antibody.

4. Wash cells by adding 2 ml of staining buffer and then centrifuging for 6 min at 300 × g, 4°C.

5. Carefully suction off supernatant and vortex the cell pellet making sure that it is thoroughly resuspended.

Fix and permeabilize surface-stained cells

6. Add 1 ml of fixing buffer and incubate in the 37°C water bath for 5 min.

7. Repeat steps 4 and 5.

8. Add 1 ml of permeabilization buffer and incubate on ice for 1 hour.

9. Repeat steps 4 and 5.

DNase treat fixed, permeablized cells

10. Add 1 ml of DNase solution (prewarmed to 37°C) to each tube and incubate at 37°C for 10 min.

11. Repeat steps 4 and 5.

Detect BrdU incorporation with specific antibodies

12. Add 20 µl/tube of fluorochrome-conjugated anti-BrdU antibody and incubate 40 min at room temperature in the dark.
13. Repeat steps 4 and 5 and analyze by flow cytometry or proceed with intracellular staining to detect intracellular proteins.

**REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see APPENDIX 5.*

**DNase solution**

Make stock solution of 1000 Kunitz U/ml deoxyribonuclease I (from bovine pancreas; Sigma) in phenol red–free RPMI 1640 (APPENDIX 2A) and store at –80°C for up to 6 months. Make a fresh solution before each experiment by diluting the stock solution to 50 U/ml in phenol red–free RPMI 1640.

**DNase I solution**

Dissolve deoxyribonuclease I (from bovine pancreas; Sigma) to 50 Kunitz U/ml in 4.2 mM MgCl2/0.15 M NaCl, pH 5. Make solution fresh before use or make in concentrated form (e.g., 500 U/ml) and store up to 2 months at –20°C.

**Fixing buffer**

In 30 ml of staining buffer (see recipe), dilute the contents of one ampule (10 ml) of 16% paraformaldehyde solution (Electron Microscopy Sciences, no. 15710) and 4 µl Tween 20. Final concentration of paraformaldehyde and Tween 20 should be 4% (w/v) and 0.01% (v/v), respectively. Store covered or in a dark place at room temperature for up to 6 months.

**Paraformaldehyde solution**

Dissolve 1% (w/v) paraformaldehyde and 0.01% (v/v) Tween 20 in PBS. Stir at low heat (70°C) to dissolve paraformaldehyde. Store up to 6 months at room temperature. Filter through a 0.2-µm before use.

**Permeabilization buffer**

Dissolve 1 ml of Triton X-100, 4 ml of 0.5 M EDTA (Biosource International), and 5 g of BSA in 1000 ml of PBS (APPENDIX 2A). Sterilize by vacuum filtration through a 0.2µm pore size filter unit. Final concentrations should be 0.5% (w/v) for BSA, 2 mM for EDTA, and 0.1 % (v/v) for Triton-X 100. Store up to 3 months at 4°C.

**Staining buffer**

Dissolve 5 g of bovine serum albumin (BSA) and 4 ml of 0.5 M EDTA (Biosource International) in 1000 ml of PBS (APPENDIX 2A). Sterilize by vacuum filtration through a 0.2-µm pore size filter unit (Millipore). Final concentration should be 0.5% (w/v) for BSA and 2 mM for EDTA. Store up to 3 months at 4°C.

**COMMENTARY**

**Background Information**

The turnover of T and B cells has been a topic of study for many years. Lymphocytes comprise dynamic populations, and direct in vivo measurement of cell lifespan is clearly crucial to understanding the normal functioning of the immune system. In practice, cell lifespan is measured by infusing DNA precursors and determining either the rate at which cell populations incorporate these labels (i.e., arise from precursors) or the rate at which labeled cell populations disappear (through death or division) following cessation of treatment. [3H]thymidine has been used extensively in studies of in vivo cell turnover. However, there are certain limitations to this approach. First, because 3H-labeled cells must be detected by autoradiography, cells of a given phenotype must be extensively purified prior to enumeration of labeled cells. Second, counting
large numbers of $^3$H-labeled cells under a microscope is a labor-intensive, time-consuming process. Third, thymidine released from dying cells can be efficiently reutilized in vivo (Feinendegen, 1967); this is a particular problem in studies of cell kinetics in the thymus, where extensive cell death followed by thymidine release and reuptake occurs continuously (Penit and Papiernik, 1986).

The feasibility of giving BrdU in the drinking water was first demonstrated by Wynford-Thomas and Williams (1986). BrdU offers several advantages over $[^3$H]thymidine in studying cell turnover. The half-life of free BrdU in the bloodstream is very short: the majority is cleared by 1 hr after injection (Kriss and Revesz, 1962). Because BrdU incorporated into DNA can be detected with a monoclonal antibody, phenotypically identifiable cell populations can be analyzed for BrdU labeling rapidly and quantitatively by flow cytometry with no requirement for prior cell purification. Furthermore, although BrdU administration yields efficient labeling of cycling cells in vivo (Van Furth and Van Zwet, 1988; Westermann et al., 1989; Schittek et al., 1991), BrdU released from dying cells is very poorly reutilized, probably because of its low affinity for thymidylate kinase (Feinendegen et al., 1973; Forster and Rajewsky, 1990).

The first anti-BrdU antibody useful for flow cytometry was reported in 1982 (Gratzner, 1982), and several others have subsequently been produced. The first protocols for BrdU staining placed severe limitations on the fluorochromes that could be used for cell surface staining. Thus, staining of BrdU incorporated into cellular DNA involves technical procedures not required for staining of typical cell surface molecules. As mentioned earlier, cells must be permeabilized to allow access of the anti-BrdU antibody to the DNA. Also, because anti-BrdU antibodies are able to bind BrdU in single- but not double-stranded DNA, detection requires pretreatment of the cells to generate single-stranded DNA. This can be accomplished by exposing permeabilized cells to denaturing conditions such as acid or base (Gratzner, 1982; Houck and Loken, 1985). Though effective in generating single-stranded DNA, this treatment is harsh and destroys phycoerythrin and allophycocyanin (Houck and Loken, 1985). Therefore, when using acid or base treatment for BrdU staining, surface staining with first-generation BrdU-specific antibodies essentially restricts measurements to a single color (usually Texas red). Moreover, analysis of the turnover of defined cell populations can only be achieved following purification of such cells.

The development of the current protocol, which allows staining of multiple cell surface markers in combination with BrdU, owes its origin to a chance contamination of an anti-BrdU antibody–producing hybridoma. It was found that an antibody previously reported to bind BrdU in double-stranded DNA lost its ability to do so when the antibody was purified from culture supernatant (Gonchoroff et al., 1986). In fact, the hybridoma producing this antibody was found to be mycoplasma-contaminated and supernatant from these cells contained DNase activity in addition to antibody. The key finding was that addition of DNase to the purified antibody reconstituted its ability to bind to cell-incorporated BrdU (without any other denaturation steps). Thus, DNase treatment of permeabilized cells also generates single-stranded DNA. Significantly, this treatment does not require harsh acid or base conditions and therefore does not destroy cell surface labeling.

Following this observation, methods for BrdU staining were reported that utilized DNase (Carayon and Bord, 1992) or a combination of endonucleases and exonuclease (Dolbeare and Gray, 1988). The protocol described here was originally published in 1994 (Tough and Sprent, 1994) and was modified from a method reported by Carayon and Bord in 1992 (Carayon and Bord, 1992).

Lymphocytes are not a static, homogeneous population of cells; rather, T and B cells progress through numerous phenotypically and functionally distinct stages, both during their differentiation from immature precursors and following their activation during an immune response. BrdU administration in combination with flow cytometric analysis of cell surface molecules can therefore be used to decipher the sequence of phenotypic changes that occur during development or activation, or can be used to measure the turnover time (lifespan) of cell populations.

The mode and duration of BrdU administration will depend on the purpose of the experiment. Because BrdU is not reutilized, “pulse-chase” studies can be used to follow the sequence and timing of phenotypic changes among post-cycling lymphocytes. Both early lymphocyte precursors and activated mature cells are cycling; these cells will become labeled with BrdU during a brief pulse period.
Phenotypic changes in these BrdU-labeled cells can then be followed during the chase period. Such a strategy has been used to determine the sequence of phenotypic changes that occur during thymocyte development (Lucas et al., 1994). In this study, mice were given two BrdU injections 4 hr apart and the BrdU-labeled thymocytes followed over the next 10 days.

Continuous BrdU labeling can be used to measure the turnover time of phenotypically identifiable lymphocyte populations. Thus, the time taken to label 100% of the cells in a population is the time necessary for complete replacement of that population from precursors. This technique may be used to determine the lifespan of lymphocytes at any stage of development for which phenotypic markers are available.

The type of information that can be obtained from BrdU labeling experiments is exemplified in the authors’ recent study on mature T cells in mice (Tough and Sprent, 1994). In this study, both continuous BrdU labeling and pulse-chase experiments were employed to examine the turnover of naive- and memory-phenotype T cells.

First, continuous BrdU labeling was used to measure the turnover of T cell populations defined by their expression of cell surface molecules associated with T cell memory (CD44, CD45RB, and L-selectin). This was done in adult thymectomized mice to exclude the contribution T cells newly generated in the thymus; turnover was therefore restricted to the peripheral lymphoid organs. Mice were given BrdU in their drinking water and sacrificed at various times up to 5 weeks after the start of BrdU treatment. Cell suspensions were made from lymph nodes and spleen, and the cells stained for CD4 or CD8 versus the above memory markers and BrdU. The key finding was that there was only very low labeling of naive-phenotype (L-selectin<sup>lo</sup>, CD45RB<sup>hi</sup>, CD44<sup>lo</sup>) T cells; >80% of these cells remained BrdU<sup>−</sup> after 5 weeks of continuous labeling. In contrast, L-selectin<sup>lo</sup>, CD45RB<sup>lo</sup>, CD44<sup>hi</sup> memory-phenotype T cells displayed a more rapid turnover, with 40% to 80% (depending on the marker concerned) becoming BrdU<sup>+</sup> over the same time period. Continuous BrdU labeling thus demonstrated a large difference in the turnover rate of naive- versus memory-phenotype T cells in the peripheral lymphoid organs.

Second, a chance observation made it possible to assess the rate at which naive T cells are released from the thymus. This became feasible when it was found that dividing lymphocytes incorporated less BrdU on a per-cell basis than did peripheral T cells. Thus, when thymectomized mice were given BrdU in their drinking water for 9 days, BrdU-labeled T cells in lymph node and spleen were uniformly BrdU<sup>hi</sup>. However, in similarly treated euthymic animals, BrdU-labeled T cells in the periphery included both BrdU<sup>lo</sup> and BrdU<sup>hi</sup> cells. In these latter mice, the intensity of BrdU staining amongst the peripheral BrdU<sup>lo</sup> cells corresponded to that of the BrdU-labeled single positive (mature) thymocytes. Therefore, the BrdU<sup>lo</sup> cells in the peripheral lymphoid organs represented cells that had recently emigrated from the thymus. By examining the BrdU<sup>lo</sup> cells present in the periphery of euthymic mice it was thus possible to define the phenotype of naive, recent thymic emigrants. In addition, by enumerating total BrdU<sup>lo</sup> T cells in spleen plus pooled lymph nodes over time during continuous BrdU labeling, it was possible to determine the rate of thymic output of newly generated T cells.

Third, pulse-chase BrdU-labeling studies were done with thymectomized mice to follow the fate of T cells which had divided in the periphery. Mice were given BrdU in their drinking water for 9 days and then given normal drinking water for up to 71 days. After the chase period, a considerable proportion of BrdU-labeled cells remained; these cells constituted a mixture of naive- and memory-phenotype cells. There was evidence of dilution of label (i.e., cell division) during the chase period, but some cells remained BrdU<sup>hi</sup> for at least 71 days. Thus, the phenotype and fate of post-division lymphocytes can be followed over long periods of time using BrdU pulse-chase strategies.

The ability to discriminate BrdU incorporation by individual cells present among populations of total lymphocytes using flow cytometry has greatly facilitated our understanding of lymphocyte turnover in a variety of different contexts. The advent of flow cytometers capable of hi-dimensional analysis (i.e., simultaneous measurement of >12 fluorescence parameters) now allows for the identification and characterization of novel lymphocyte subsets in a manner that was not previously possible. In the authors’ experience, protocols suitable for 3- or 4-color analyses may not necessarily be compatible with hi-dimensional staining procedures due to the sensitivity of some tandem fluorochromes to the particular treatments employed. The Alternate Protocol described in this unit was developed to
better facilitate such hi-dimensional analyses. With just a few relatively minor variations to the Basic Protocol, simultaneous measurement of up to 12 different fluorescence parameters including BrdU incorporation is routinely accomplished.

Critical Parameters

Two significant aspects of the Basic Protocol (in which it differs from the original Carayon and Bord procedure; see Background Information) is the use of two short fixation steps (30 min in ethanol followed by 30 min in paraformaldehyde) rather than overnight fixation in paraformaldehyde. In addition, the DNase digestion step has been shortened, and is carried out at room temperature rather than at 37°C. With these modifications, it is possible to stain and analyze cells in a single day.

The particular anti-BrdU antibody used for detection is important. The Becton Dickinson antibody suggested in the Basic Protocol works well (clone B44; Gratzner, 1982), whereas at least one other anti-BrdU antibody that has been tested did not work in this assay. This may be related to the affinity of the antibody for BrdU, as descriptions of other protocols utilizing enzyme disruption of DNA have reported a requirement for a high-affinity antibody (Gonchoroff et al., 1986; Dolbeare and Gray, 1988).

Some researchers have found toxicity associated with prolonged administration of BrdU to certain mouse strains (e.g., C57BL/6). In our experience, C57BL/6 mice can be given BrdU drinking water for up to 5 weeks with no toxicity. When BrdU water is given for longer than 5 weeks, toxicity may sometimes be observed. Because toxicity will stress the animal and therefore possibly alter lymphocyte turnover, mice showing overt signs of toxicity (e.g., sickness, hair loss) obviously should not be used. A simple test for more subtle evidence of toxicity is to check the thymus of the recipient animal; a smaller-than-normal thymus suggests toxicity.

Although similar in principle, the Alternate Protocol differs from the Basic Protocol in several respects. First, only one rapid (5 min) fixation step in paraformaldehyde at 37°C is called for compared to two separate steps, one in ethanol and the other in paraformaldehyde, outlined in the Basic Protocol. In addition, bovine serum albumin (BSA) is present in all of the solutions used here in the Alternate Protocol, which greatly diminishes the propensity for large cell aggregates to form. This is particularly an issue following the fixation step. The DNase solution used in the Alternate Protocol is also entirely different, consisting simply of DNase enzyme dissolved in phenol red–free RPMI 1640. RPMI 1640 is used because it contains significant amounts of magnesium ions, which are normally absent from standard PBS formulations. Magnesium ions appear to be a required co-factor for DNase enzymatic activity. Phenol red is avoided as it may increase the auto-fluorescence of some cell types which could lead to problems with compensation of flow cytometry data during acquisition.

Anticipated Results

This assay is sensitive and highly reproducible. Using the Basic Protocol, BrdU staining can be incorporated into 3- and 4-color analyses of lymphocyte populations. A useful positive control to test the efficiency of both in vivo labeling and in vitro staining is to examine a population known to turn over rapidly (e.g., thymus lymphocytes, of which the vast majority label in 4 to 5 days; see Fig. 4.7.1). In addition to distinguishing BrdU+ from BrdU− cells, it is possible to differentiate cells based on the amount of BrdU that they have incorporated. This latter parameter may reflect the environment in which the cell divided (e.g., local free thymidine will compete with BrdU for incorporation into DNA) and/or the number of times that a cell has divided.

This protocol has been successfully used in procedures where the detection of BrdU incorporating lymphocytes in conjunction with staining of 11 or more additional surface or intracellular proteins was required. It is recommended that whenever possible lymphocytes from animals not treated with BrdU be processed in parallel with experimental samples, rather than staining with nonspecific isotype antibodies, be used as a control to determine the relative specificity of the anti-BrdU staining.

Time Considerations

The time required to complete the assay will vary depending on the time needed to generate cell suspensions and the number of samples stained. After surface staining, the procedure can generally be completed in 2 to 3 hr. Subsequently, cells must be analyzed by flow cytometry; the length of time required for this step will also depend on the number of samples and method of analysis. Fixation times with ethanol and paraformaldehyde may be lengthened if necessary. After paraformaldehyde
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4.7.8

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**Figure 4.7.1** BrdU labeling of thymocytes. Thymocytes from adult C57BL/6 mice, either untreated or given BrdU in drinking water for 7 days, were stained for CD4, CD8, and BrdU. CD4 vs. CD8 staining of total thymocytes from (A) an untreated and (B) a BrdU-treated mouse is illustrated. Histograms show BrdU staining of CD4+CD8+ thymocytes (boxed area in A and B) for (C) the untreated and (D) the BrdU-treated mouse. >99% of CD4+CD8+ cells from the BrdU-treated mouse are BrdU+.

fixation, cells may be washed, resuspended in PBS, and left at 4°C overnight before the assay is continued the next day.

As in the Basic Protocol, the amount of time required to perform the Alternate Protocol will depend largely on the number of samples and amount of time required to make single-cell suspensions. Typically, from start to finish, the procedure takes 5 to 6 hr. However, this time may increase if large numbers of samples are processed in parallel. Following the fixation step, samples can be washed and resuspended in staining buffer and left at 4°C overnight and continued the following day.

**Literature Cited**


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