Benchmarks

Simultaneous Preparation of RNA and Nuclei for Northern Blot and Flow Cytometric Analysis


Several methods have been developed to quantify RNA synthesis during the progression of the cell cycle. The rate of RNA synthesis can be detected during different stages of the cell cycle by staining cells with agents that intercalate with nucleic acids. For example, following staining of mammalian cells with acridin orange, the green and red fluorescence that correlates with DNA and RNA content, respectively, can be analyzed by flow cytometry (5). Increase in RNA content during the progression of cells through the cell cycle can be measured after staining with acridin orange (1). RNA synthesis resulting from the stimulation of quiescent cells with various growth factors has also been demonstrated by labeling cells with bromo-uridine and using the anti-bromo-deoxyuridine antibody (3). These methods allow measurement of the overall RNA content in cells; however, they do not allow the measurement of the levels of specific mRNAs throughout the cell cycle. Current methods to quantify specific mRNAs generally require the preparation of a large number of cells (5–10 × 10^6 cells) to carry out flow cytometric analyses and to isolate RNA for Northern blot analysis or solution hybridization. In this report, we describe a method of simultaneously preparing RNA and nuclei for Northern blot and flow cytometric analyses, respectively. The minimum number of nuclei required to obtain flow cytometric data and the effect of conserving nuclei in methanol for several days are also presented.

Confluent cultures of Chinese hamster ovary (CHO) cells were maintained for 5–7 days in serum-free F12 medium (Sigma Chemical, St. Louis, MO, USA); this condition yielded an enriched population in the G_0/G_1 phase of the cell cycle. The cells were stimulated to enter the cell cycle by subculturing at a density of 20,000 cells/cm^2 in F12-medium containing 20% serum. Our preliminary studies had shown that the progression through the cell cycle could be observed at 0, 7, 17, 20, 23 and 25 h after subculturing. Therefore, cells were harvested at these time points and

Figure 1. Comparison of flow cytometric measurements of CHO cells fixed in methanol and isolated nuclei. After enriching CHO cells in G_0/G_1, cells were harvested at 0, 7, 17, 20, 23 and 25 h during their progression into the cell cycle. A part of the cells (1 × 10^6 cells) was fixed in ice-cold 70% methanol, and the rest (4 × 10^6 cells) was stored in -80°C for preparation of nuclei. Samples were treated with RNase A and propidium iodide for 2 h at 4°C before flow cytometric analysis.
Benchmarks

Table 1. Percentage of Cells That Were in G₀/G₁, S and G₂ at the Indicated Time Points

<table>
<thead>
<tr>
<th>Time in Hours</th>
<th>% G₀/G₁ Cells</th>
<th>% G₀/G₁ Nuclei</th>
<th>% S Cells</th>
<th>% S Nuclei</th>
<th>% G₂ Cells</th>
<th>% G₂ Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>57</td>
<td>66</td>
<td>23</td>
<td>24</td>
<td>20</td>
<td>10</td>
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<td>42</td>
<td>56</td>
<td>26</td>
<td>20</td>
<td>32</td>
<td>24</td>
</tr>
</tbody>
</table>

These percentages were obtained after subculturin serum-starved CHO cells into F12 medium containing 20% serum. Flow cytometric analysis of methanol-fixed cells and isolated nuclei are compared.

Benchmarked methanol-fixed cells were prepared for flow cytometric analysis by washing once in 5 mL DPBS and incubating in 0.05% Triton X-100 in DPBS for 20 min on ice. Cells were washed again in DPBS and suspended in 2 mL DPBS containing RNase A and propidium iodide at the same concentrations as described for the nuclei. For direct comparison, both isolated nuclei and methanol-fixed cells were prepared for flow cytometric analysis at the same time.

Samples were analyzed using a FACStar™ Plus flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The intercalated propidium iodide was excited with an argon laser at 488 nm. The emitted light was collected through a 575/26 band-pass filter. The fluorescent signal was gated to include only events containing propidium iodide.

The DNA tracings for the isolated nuclei and the methanol-fixed cells showed the progression of the CHO cells through the cell cycle (Figure 1). The percentage of cells in G₁, S and G₂ was quantified using the MacCycle™ software (Phoenix Flow Systems, San Diego, CA, USA) as described in the manufacturer’s manual. The percentage of cells in G₁, S and G₂ at the different time points for both isolated nuclei and

Figure 2. Northern blot analysis of RNA isolated from CHO cells. Confluent CHO cell populations were enriched in G₀/G₁ and harvested at 0, 7, 17, 23 and 25 h during their progression through the cell cycle. Isolated RNA (20 µg/lane) was fractionated on a 1% denaturing agarose gel and blotted onto a Nytran membrane (Amersham, Arlington Heights, IL, USA). The blot was then hybridized to 32P-labeledcyclin D1 and GAPDH cDNA probes.

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methanol-fixed cells (Table 1) shows that cells progressed from G1 at 7 h, to 
S at 17 h and to G2 at 20–23 h.

RNA prepared from these samples was analyzed after Northern blotting as 
described previously (6). Hybridization to the glyceraldehyde-3-phosphate de-
hydrogenase (GAPDH) cDNA probe showed that similar amounts of RNA 
were fractionated on each lane (Figure 2). The expression of cyclin D1, known 
to be expressed only during the G2 (4), increased at 7 h and decreased as cells entered the S-phase at 
17 h (Figure 2).

In a separate set of experiments, 13 
× 10^6 cells were serially diluted to obtain cell suspensions ranging from 13 
× 10^5 cells to 5 × 10^4 cells/mL. The cells were lysed, and isolated nuclei were 
analyzed by flow cytometry. DNA measurements showed that at least 2 
× 10^5 cells were necessary to obtain reliable flow cytometric data, while 5 
× 10^4 cells did not yield enough counts to show the distribution of DNA content in 
the cell population (Figure 3). Although 2 × 10^5 cells may not yield enough RNA for a Northern blot analy-
sis, the isolated RNA is sufficient for cDNA synthesis using reverse transcriptase followed by polymerase chain reactions (PCR) to detect specific RNAs (6).

In these experiments, nuclei were used for flow cytometric analysis immediately after isolation from frozen 
cells. In an attempt to conserve isolated nuclei for several days before prepara-
tion for flow cytometric analysis, cells and isolated nuclei (10^6) were fixed in 
ice-cold methanol and stored at 4°C. Furthermore, an equivalent number of CHO cells were frozen at -80°C to obtain freshly isolated nuclei for comparison. After 7 days, cells and nuclei were washed in 5 mL DPBS and prepared for flow cytometry as described earlier. Comparable results were obtained for freshly isolated nuclei and methanol-
fixed cells. However, DNA traces of nuclei fixed in methanol did not show a 
distinct separation of the G1, S and G2 phases (Figure 4). Forward and side 
scatter measurements of nuclei fixed in methanol indicated that these nuclei 
changed in size and density compared to freshly isolated nuclei (data not 
shown) perhaps because the structure of the chromatin was partially de-
graded. Therefore, we concluded that methanol is not a suitable agent to con-
serve nuclei for analysis by flow cy-
tometry.

In summary, the simultaneous 
preparation of RNA and nuclei from 
cells can be useful in cases where large numbers of cells are not available, e.g., 
after isolation of primary cell isolates and cells that are difficult to propagate. 
Nuclei isolated from as few as 2 × 10^5 cells can routinely be used for analysis 
by flow cytometry and the cytosolic extract from the same cells for analysis of the 
mRNA. Because nuclei cannot be fixed in methanol, the flow cytometric 
analysis of nuclei must be carried out immediately after isolation from fresh 
or frozen cells.

REFERENCES
1 Darzyynkiewicz, Z. 1990. Differential staining of DNA and RNA in intact cells and isolated 

Press, San Diego.
2 Gaugh, N.M. 1988. Rapid and quantitative 

preparation of cytoplasmic RNA from small 
3 Jensen, P.O., J. Larsen, J. Christiansen and 
J.K. Larsen. 1993. Flow cytometric measure-
ment of RNA synthesis using bromouridine 
labeling and bromodeoxyuridine antibodies. 
Cytometry 14:455-458.
4 Sherr, C.J. 1994. G1 phase progression: cy-
5 Staiano-Coco, L., Z. Darzyynkiewicz and 
C.K. McMahon. 1989. Cultured human ker-
atinoocytes: discrimination of different cell cy-

cle compartments based upon measurement of 
nuclear RNA or total cellular RNA content. 
6 Tesfaigzi, J., P.S. Wright, V. Oreffo, G. An, 
R.Wu, H. Witschi and D.M. Carlson. 1993. A small proline-rich protein regulated by vita-

min A in tracheal epithelial cells is induced in 
9:434-440.

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Figure 3. Minimum number of cell nuclei for flow cytometric measurements. CHO cells (13 × 10^6) were serially diluted and lysed to prepare nuclei for flow cytometric analysis. A minimum number of cell nuclei (2 × 10^5) was necessary to obtain flow cytometric data.

Figure 4. Comparison of flow cytometric ana-
lysis of cells and nuclei fixed in methanol. Flow 
cytometric analyses of cells fixed in methanol 
(A), of freshly isolated nuclei (B) and of isolated 
nuclei fixed in methanol (C) are shown.