PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 660–664 (1975)

An Improved Method of Fixation For Immunofluorescent Detection of SV40 T-Antigen in Infected Human Fibroblasts (38605)

MICHAEL M. KAPLAN,[1] DONALD J. GIARD,[1] WILLIAM A. BLATTNER,[2] ANTHONY S. LUBINIECKI,[1] AND JOSEPH F. FRAUMENI, JR.[2]

Increased susceptibility of fibroblasts to SV40-induced transformation has been demonstrated in certain groups at high risk for cancer (1, 7, 10, 11), and may serve as a means to identify cancer-prone patients (6). SV40 T-antigen is an easily quantified, viral specific marker that correlates with cell transformation (2, 3, 8). The nuclear immunofluorescent assay for measuring T-antigen required that cultured cells be grown on glass cover slips when acetone fixation was used. Employing a fixative which does not dissolve plastic would allow the use of a more simple method which required less time to execute. To remedy this problem, four fixatives, three temperatures and two culture substrates were tested and an improved method was developed.

Cell Culture. Human fibroblasts were grown from forearm skin biopsies of clinically normal individuals and persons with genetic predisposition (e.g., Fanconi's anemia and Down's syndrome) or strong familial susceptibility to cancer (4).

Cells were grown at 37° in an atmosphere of 10% CO_2 in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (Colorado Serum Co.), and penicillin and streptomycin. Cultures were maintained in 65 × 15 ml plastic cell culture petri dishes (Falcon Plastics) and subcultured using 0.1% trypsin in phosphate buffered saline (PBS). Tests were performed between the fifth and tenth passage before onset of the decline in growth rate characteristic of human fibroblast cultures (5).

Virus. The SV40 virus was a small plaque variant with a titer of 3×10^8 plaque forming units (PFU) per ml, propagated and assayed on African green monkey kidney cells (9).

Infection. Cell suspensions were transferred into 60 × 15 mm petri dishes; glass cover slips were used for acetone, but not for other fixatives. At 30–50% confluency (usually after 24 hr) cell sheets were washed twice with DMEM, infected with 0.5 ml of SV40 virus (multiplicity of infection approximately 100 PFU/cell) and rotated every 15 min for 3 hr in a CO_2 incubator. The inoculum was removed and cells washed once with DMEM. Cultures were then incubated under CO_2 in DMEM plus SV40 antisera for 3 days.

Fixation. Acetone was compared to absolute ethanol, methanol, and formalin at three temperatures: room temperature, 4° and −20°. For the standard method (2), cover slips were rinsed twice in PBS (pH 7.2), fixed in acetone solution for 15 min and, following distilled water rinse, placed on a dry gauze prior to staining.

For the other fixatives, cell cultures in petri dishes were washed with cooled PBS (except for room temperature assay) and maintained at 4° for 1 min. Five ml of fixative were then added to the petri dishes and incubated for 15 min at all three temperatures, the fixative removed, and the dishes air dryed at 4° for 5 min. Acetone fixation was not performed on plastic petri dishes since this solvent crazed the plastic.

Staining. Hamster anti-SV40 T-antigen, rhodamine, and fluorescein-conjugated rab-

This investigation was supported by contract NIH-CP-43216, National Cancer Institutes of Health, Bethesda, MD.

We would like to thank Dr. Thomas J. Mason for advice with the statistical analysis, and Yvette Preston, Nicolette Triantafellu and Mark Gunnell for skilled technical assistance.

[1] Meloy Laboratories, Inc., 6715 Electronic Drive, Springfield, VA 22151.

[2] Epidemiology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014.

TABLE I. COMPARISON OF CELL MORPHOLOGY AND SV40 T-ANTIGEN NUCLEAR FLUORESCENCE FOR VARIOUS METHODS OF FIXATION TESTED.[a]

Temperature of Fixation	Acetone		Absol. ethanol		Methanol		Formalin	
	Cellular morph.[b]	Nuclear fluor.[c]	Cellular morph.	Nuclear fluor.	Cellular morph.	Nuclear fluor.	Cellular morph.	Nuclear fluor.
Room Temperature	+	+	++	++	+	+	++	0
4°	+	+	++	++	+	+	++	0
−20° adjusted to −60°	+	++	+++	+++	++	++	ND[d]	ND

[a] Results reflect observations on two skin fibroblast strains assayed in duplicate.

[b] Criteria in judging morphology of cells: (+++) no apparent change in cell morphology; (++) noticeable change in cell morphology; (+) considerable change in cell morphology.

[c] Criteria in judging nuclear fluorescence: (+++) very sharp confluent nuclear fluorescence, with large nonfluorescing nucleoli; (++) observable nuclear fluorescence with distinguishable nonfluorescing nucleoli; (++) observable nuclear fluorescence with distinguishable nonfluorescing nucleoli; (+) dull nuclear fluorescence with no distinguishable nucleoli; (0) no nuclear fluorescence.

[d] ND Not Done (samples freeze at this temperature with cell detachment).

bit anti-hamster gamma globulin was obtained from Dr. Roger Wilsnak, Huntingdon Research Center, Baltimore, MD, as part of the Special Virus Cancer Program. Cells fixed on cover slips or petri plates were exposed to a few drops of hamster SV40 T-antibody (diluted 1:10 in PBS) and placed on a rocker platform in a CO_2 incubator for 30 min. Plates were then rinsed three times in PBS, and 0.5 ml of an equal mixture of rhodamine (diluted 1:30 in PBS) and fluorescein conjugated rabbit antihamster globulin were added. Plates were then incubated for an additional 30 min on a rocker platform, and rinsed three times with PBS. Cover slips were mounted, and plates and cover slips were employed and at times stored at −70°. Freezing and storage of fixed, stained petri dish cultures did not affect fluorescence for up to 6 mo.

Slides were examined at 250 × magnification with a Leitz Fluorescent microscope. Ten fields of cells were counted to determine the average number of cells per field. Enough cells were then viewed to give a total of 3000 cells (except in the cases where cell density did not permit) and the number of nuclei with positive fluorescence was recorded. When uninfected fibroblast cultures were stained by the above method, only nonspecific rhodamine-type fluorescence was observed.

Results. During the course of initial experiments, fixation at −60° gave the best nuclear fluorescence for methanol, ethanol, and acetone. However, cellular detachment remained a problem until the fixatives were precooled to −20° prior to addition to cell cultures. Subsequently, culture dishes were transferred to a −60° freezer for 15 min. This temperature modification was incorporated into the procedure in later experiments presented here.

As shown in Table I, the effect of the various fixation methods on cellular morphology and nuclear fluorescence was compared to room temperature, 4°, and −20° adjusted to −60°. Absolute ethanol gave the best nuclear fluorescence (Fig. 1), permitting brilliant and sharply contrasting T-antigen positive nuclei with crisp nonfluorescing nucleoli typical of SV40 T-antigen positive cells. Cellular morphology was well preserved, particularly with the low temperature modification. Fixation using methanol was associated with inferior nuclear resolution and greater disruption of cellular morphology. Acetone, although acceptable, was less desirable; cell nuclei assumed a rough appearance leading to hazy nuclear fluorescence which obscured differences between true and false positives. Formalin was unacceptable as a fixative, since it permitted no nuclear fluorescence. In general, the degree of cell detachment was

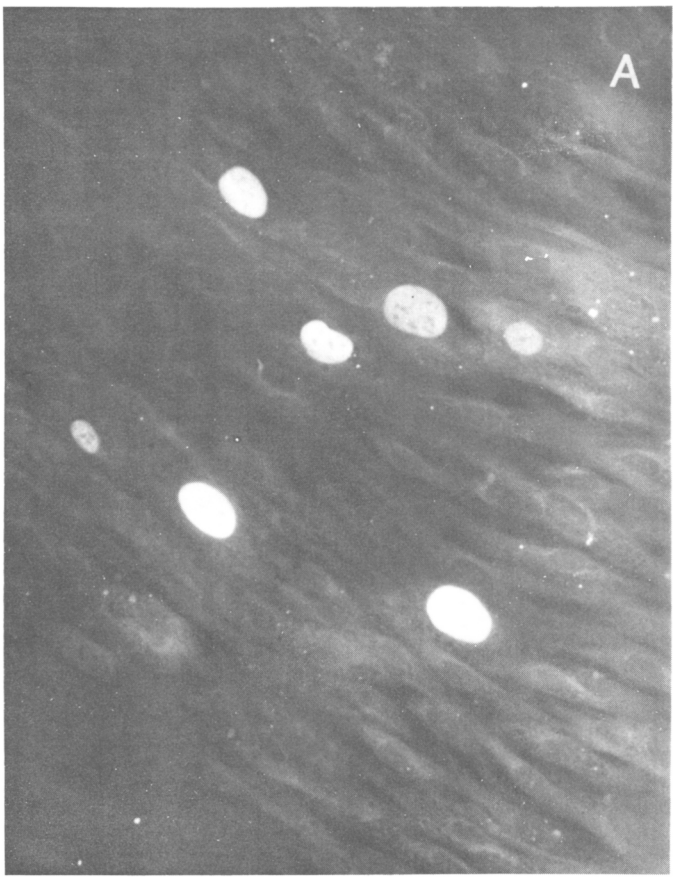

FIG. 1A SV40 T-antigen positive skin fibroblasts (fifth passage) 3 days after infection. Note fluorescing nuclei with various levels of T-antigen expression against a background of antigen-negative, non-fluorescing cells. Mag. 250×. B. SV40 T-antigen positive skin fibroblasts (fifth passage) 3 days after infection. Note two brightly fluorescing nuclei containing nonfluorescent nucleoli. Mag. 450×.

similar to the level of alteration in cell morphology.

Table II demonstrates that the employment of the ethanol method did not significantly alter the cell density, compared with the older acetone fixation method. The former technique consistently showed slightly smaller variability in several replicate experiments employing normal and cancer-prone cell lines.

To test whether the method of fixation causes quantitative variation in T-antigen production, skin biopsies from a total of 45 persons (normal individuals and persons genetically prone to cancer) were cultured and assayed using both acetone and absolute ethanol fixation. As summarized in Table III, there were no significant differences (paired *t* test) in T-antigen production between the ethanol and acetone methods.

Ethanol fixation of petri dish cultures also resulted in considerable savings of time compared with acetone-fixation. The former method dispersed with the lengthy manipulation of cover slips, one distilled water rinse, and cover slip mounting procedures employed in the latter method.

Conclusion. The assay for SV40 T-antigen shows promise as a means of identifying persons at high risk of cancer (6). To clarify its epidemiologic significance, the test will require application to various groups of

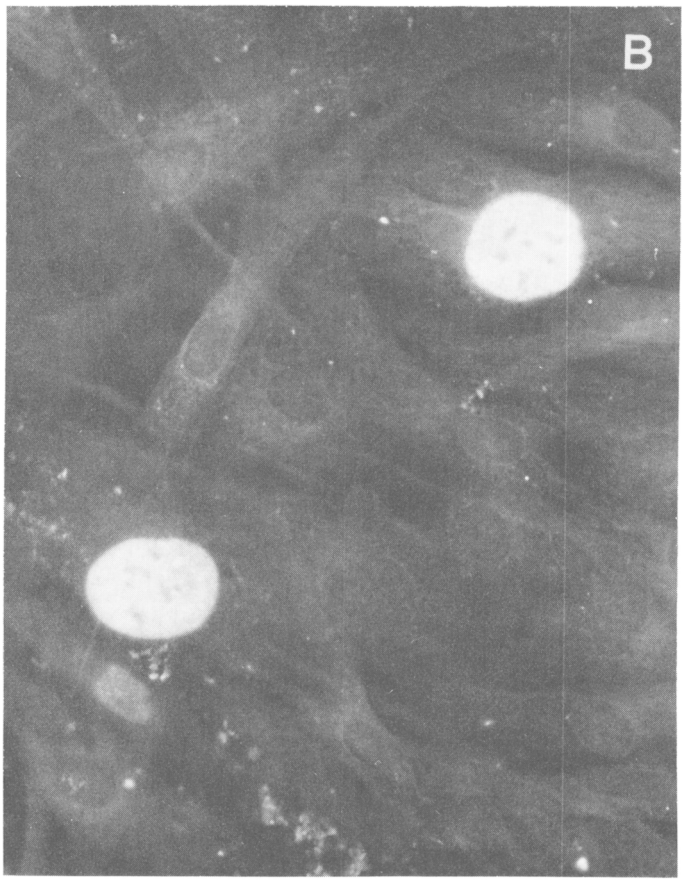

FIG. 1B

TABLE II. EFFECT OF ETHANOL OR ACETONE FIXATION ON CELL DENSITY IN FIBROBLAST CULTURES.

Cell density	Normals		Cancer prone	
	Acetone	Ethanol	Acetone	Ethanol
Cell density[a]	2500.64 (53)	2942.73 (51)	2344.77 (35)	2826.31 (38)
Standard deviation range	496–4900	1000–4800	1000–3990	1400–6000

[a] Mean number of cells per 10 microscope fields. Number in parentheses indicates the number of cultures examined.

individuals with cancer and predisposing conditions. In efforts to improve the efficiency of the assay for expanded testing, we found that ethanol is superior to other fixatives. It is not destructive to plastic and allows the entire assay to be performed in petri dishes without cover slips. It is more convenient, results in considerable saving of time, and has at least equal reproducibility. Furthermore, a modification of the temperature during the period of fixation leads to superior nuclear fluorescence with little cell detachment. The new fixation method does not alter the sensitivity of the T-antigen assay in discriminating between normal and susceptible cell lines.

TABLE III. EFFECT OF ACETONE OR ETHANOL FIXATION ON MEAN % T-ANTIGEN POSITIVE NUCLEI IN FIBROBLAST CULTURES INFECTED WITH SV40 VIRUS.

Fixation	Number of samples	Mean % Positive nuclei (range)	Alcohol–acetone paired *t*-statistic (*P* value)
		Normal population	
Ethanol	24	0.90 ± 0.61 (0.1–1.9)	0.21[a] (0.8)
Acetone	24	0.86 ± 0.57 (0.0–2.3)	
		Cancer-prone population	
Ethanol	21	3.37 ± 2.20 (0.5–9.3)	−1.38 (0.15)
Acetone	21	3.98 ± 3.40 (0.7–16.2)	

[a] In both normal and cancer prone populations, analysis by paired *t* test showed no statistically significant differences in T-antigen values between the two methods of fixation.

Summary. Improved fixation for immunofluorescence staining in the SV40 T-antigen test has been developed utilizing ethanol in place of acetone. The modified method improves resolution, results in smaller variability of cell numbers, and reduces the time of assay, while maintaining its sensitivity in discriminating between normal and cancer-prone groups.

1. Aaronson, S. A., and Lytle, O. D., Nature (London) **228,** 359 (1970).
2. Aaronson, S. A., and Todaro, G. J., Virology **36,** 254 (1968).
3. Black, P. H., Rowe, W. P., Turner, H. C., and Huebner, R. J., Proc. Nat. Acad. Sci. U.S.A. **50,** 1148 (1963).
4. Fraumeni, J. F., Jr., *in* "Cancer Medicine" (J. F. Holland and E. Frei, eds.) Lea and Febiger, Philadelphia (1973).
5. Hayflick, L., Exp. Cell Res. **37,** 614 (1965).
6. Miller, R. W., and Todaro, G. J., Lancet **1,** 81 (1969).
7. Mukerjee, D., Bowen, J. M., Trujillo, J. M., and Cork, A., Cancer Res. **32,** 1518 (1972).
8. Pope, J. J., and Rowe, W. P., J. Exp. Med. **120,** 124 (1964).
9. Takemoto, K. K., Todaro, G. J., and Hable, K., Virology **35,** 1 (1968).
10. Todaro, G. J., Nat. Cancer Inst. Monogr. No. **29,** 271 (1968).
11. Todaro, G. J., and Martin, G. M., Proc. Soc. Exp. Biol. Med. **124,** 1233 (1967).

Received October 21, 1974. P.S.E.B.M. 1975, Vol. 148.