

Micromethod for Assaying Reverse Transcriptase of Human T-Cell Lymphotropic Virus Type III/Lymphadenopathy-Associated Virus

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A micromethod for assaying the reverse transcriptase enzyme of human T-cell lymphotropic virus type III/lymphadenopathy-associated virus in cocultures of clinical specimens for viral isolation was developed and compared with the macromethod in use. Ultracentrifuged, pelleted, and solubilized viral culture supernatants were transferred into either tubes (macromethod) or microtiter plates (micromethod) and incubated with tritiated enzyme substrate. Trichloroacetic acid-precipitated DNA was collected on individual filter papers with a Millipore filtration manifold (macromethod) or on filter sheets using a semiautomated cell harvester (micromethod). Filters were then placed in scintillation fluid and counted on a beta scintillation counter. Results of the micromethod significantly correlated to those of the macromethod, with a linear relationship between the two. The cutoffs for positivity based on the mean + 2 standard deviations for a set of known negative specimens ($n = 19$) was 4,973 cpm for the micromethod compared with 5,336 for the macromethod. The intrarun and interr run variations were comparable for both methods. There was a 67% increase in the maximal daily number of specimens which could be run (100 versus 60) as well as a reduction in reagent use. In summary, the micromethod utilizing a semiautomated cell harvester is comparable to the existing macromethod in accuracy and is an improvement due to savings in time and reagents.

The identification of a human retrovirus as the etiologic agent of the acquired immunodeficiency syndrome has caused a surge of interest in this class of agents. Many new laboratories are involved in acquired immunodeficiency syndrome research, and this often entails the culture of clinical specimens from patients with the disease or associated conditions, the culture of specimens from animals infected with the agent or related agents, and the study of interactions between the agent and cell lines. Cultures of the virus most often consist of the cocultivation of potentially infected cells or secretions and phytohemagglutinin-stimulated lymphoblasts or continuous, virus-receptive cell lines. These cultures are usually monitored for virus replication by assaying for the presence of the viral enzyme reverse transcriptase in supernatants from the cultures. The procedure is quite laborious, which limits the number of assays that can be done in 1 day.

To increase the daily assay capacity, we adapted the standard method currently used in our laboratory for measuring reverse transcriptase activity (a modification of earlier methods [1, 2, 4]) so that a portion of the assay is done in microtiter plates, which are then harvested by using semiautomated equipment. A comparison of the standard method (the macromethod) and the semiautomated method (the micromethod) is the subject of this report.

MATERIALS AND METHODS

Reagents. The virus solubilization buffer consists of 0.5% Triton X-100 (a detergent; Bio-Rad Laboratories, Rockville Centre, N.Y.) in 0.8 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride (an antiprotease, anti-RNase; Sigma Chemical Co., St. Louis, Mo.), 20% (vol/vol) glycerol (Fisher Scientific Co., Inc., Norcross, Ga.), and 50 mM Tris hydrochloride

(pH 7.8). The reaction mixture consists of 52 mM Tris (pH 7.8), 10 mM MgCl₂, 5 mM dithiothreitol (a reducing agent to prevent oxidation of the enzyme; Bethesda Research Laboratories, Inc., Gaithersburg, Md.), 5 µg of poly(rA)-oligo(dT) (the template primer; Pharmacia Biochemicals, Inc., Piscataway, N.J.) per ml, 83 µg of dATP (to prevent hydrolysis of TTP; Pharmacia) per ml, and 52 µCi of [³H]TTP (10 to 20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml. tRNA (GIBCO Life Technologies, Inc., Chagrin Falls, Ohio), derived from bakers' yeast, is used as a carrier for the precipitated DNA at a concentration of 2.5 mg/ml in 10 mM Tris-0.1 M NaCl-1 mM EDTA (Fisher). Trichloroacetic acid (TCA; J. T. Baker Chemical Co., Phillipsburg, N.J.) solutions, 5% and 10% containing 0.02% sodium pyrophosphate (to prevent adsorption of [³H]TTP, Fisher), were used to precipitate DNA.

Samples (3 ml) of supernatant from retroviral cultures were collected and placed into 15-ml centrifuge tubes. These were centrifuged at 300 to 600 × *g* for 10 min to remove cellular debris. A 2-ml sample of the supernatant was collected and placed into a 10-ml polycarbonate ultracentrifugation tube (Sybron/Nalgene, Rochester, N.Y.). These supernatants could be frozen at -70°C and stored in these tubes until assayed. The samples were centrifuged at 100,000 × *g* for 30 min, and the supernatants were discarded. The tubes were inverted onto absorbent paper and allowed to drain before an absorbent swab was used to carefully remove any remaining liquid without disturbing the pellet. Solubilization buffer (100 µl) was added to each tube to solubilize the virus and release the reverse transcriptase located in the viral core. The tubes were vortexed to disrupt the pellet and incubated at 4°C for 10 min.

Macromethod. A 20-µl solubilized sample was added to triplicate 12- by 75-mm plastic Falcon tubes (Becton Dickinson Labware, Oxnard, Calif.) containing 180 µl of the reaction mixture. The first and last triplicate tubes were

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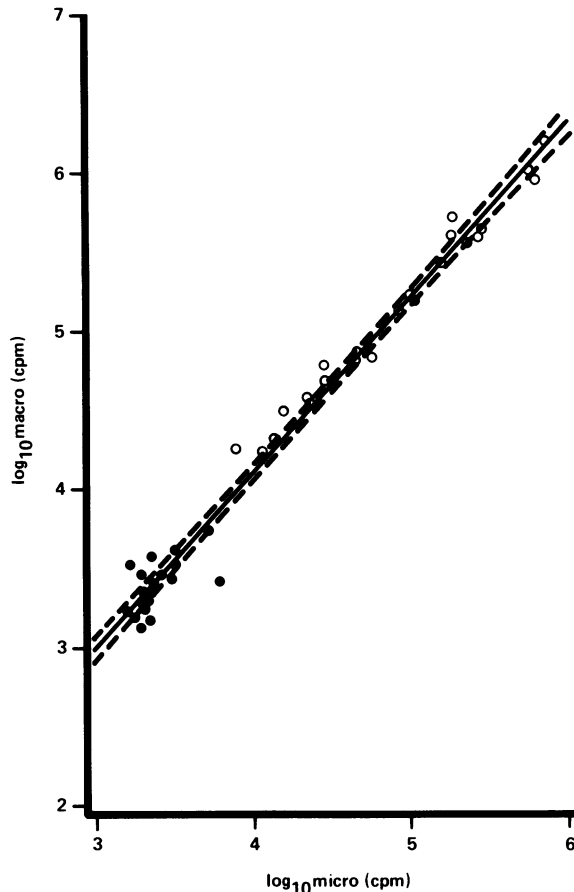


FIG. 1. Plot of logarithmically transformed results of reverse transcriptase assay, micromethod versus macromethod, with regression line (—) and 95% confidence limits (---). Symbols: ●, negatives ($n = 19$); ○, positives ($n = 23$).

labeled as controls, and 20 μ l of virus solubilization buffer was added to each. The tubes were covered and incubated at 37°C in an incubator for 2 h. After incubation, 10 μ l of tRNA buffer was added to each tube to cause the strands of DNA to clump together. Cold 10% TCA (3 ml) was added to each tube to precipitate the DNA. The precipitated DNA was then collected on 27-mm-diameter glass fiber filters (grade 25; Schleicher & Schuell Co., Keene, N.H.) in a Millipore sampling manifold (Millipore Corp., Bedford, Mass.). This was done by allowing each tube to drain into a separate well of the apparatus. Each tube was then washed twice with 5% TCA, which was poured onto the filters, and the filters were washed with an additional 4 volumes of TCA followed by 1 volume of 70% ethanol. The filters were then removed from the apparatus and dried. Each filter was placed into a glass scintillation vial, and 8 ml of scintillation fluid (Scintilene; Fisher) was added.

Micromethod. In the micromethod, the 20- μ l samples of solubilized virus were placed into the wells of 96-well flat-bottomed microtiter plates (Costar, Cambridge, Mass.) containing 180 μ l of the reaction mixture. Solubilization buffer controls were set up in the first and last three wells. The plates were covered and incubated at 37°C for 2 h. Then 10 μ l of tRNA buffer was added to each well, followed by 100 μ l of cold 10% TCA. After precipitation, the plates were harvested with a semiautomatic cell harvester (Skatron, Inc., Sterling, Va.) (the harvester must be designed to be

used with TCA). A glass fiber filter sheet (grade 25; Schleicher & Schuell) was prewet with 70% ethanol for 2 s, followed by automatic wash cycles that consist of 5% TCA followed by 70% ethanol, each for 10 s. This process was repeated three times for an empty 12-well row (to clear the machine) before harvesting the sample rows. The glass fiber filter sheet was dried, the disks containing the precipitable DNA were removed and placed into scintillation vials, and 2 ml of scintillation fluid was added to each vial.

The vials from both the macro- and micromethods were counted for 15 s in a beta scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.), and the results are expressed as counts per minute.

RESULTS

The micromethod was compared with the macromethod in three experiments. In the first, both methods were used on a series of known human T-cell lymphotropic virus type III/lymphadenopathy-associated virus negative ($n = 19$) and known virus-positive ($n = 23$) culture supernatants. To examine the relationship between the micro- and macromethods, linear regression analysis with the unweighted least squares method was used (3, 5). Graphic analysis of the regression residuals suggested that transformation provides a better fit to the data. The data were logarithmically transformed, and a significantly linear relationship was shown between the results by the two methods ($r^2 = 0.98$, $P < 0.0001$). The resulting regression equation was \log_{10} macromethod (cpm) = $-0.290 + [1.096 \times \log_{10}$ micromethod (cpm)] (Fig. 1), where cpm is counts per minute.

The geometric means and standard deviations for the human T-cell lymphotropic virus type III/lymphadenopathy-associated virus-negative group were calculated. The geometric mean plus 2 standard deviations for this group was used to determine the cutoff point for positivity for each method. For the micromethod, this value was 4,973 cpm (geometric mean, 2,451 cpm; \log_{10} standard deviation, 0.307) and for the macromethod, it was 5,336 cpm (geometric mean, 2,511 cpm; \log_{10} standard deviation, 0.329). Although the reverse transcriptase activity with the micromethod was lower than that with the macromethod, this difference, although greater at higher reverse transcriptase values, was less than 7% of the value with the macromethod at the cutoff point for positivity.

The second experiment was a comparison of the intrarun variation of each method. This was done by setting up 10 sets of four viral dilutions (A through D) covering a range of possible positive values. These 10 sets were then assayed by both methods on a single day.

Interrun variation was measured in the third experiment. Additional sets of four viral dilutions, as in the second experiment, were prepared. These were assayed, one set per day, for 8 days, by both methods.

The means and standard deviations were calculated for each method by dilution in these experiments and used to determine the coefficients of variation (Table 1). The mean coefficient of variation over the range of dilutions for the micromethod was 19.7% compared with 18.8% for the macromethod for the second experiment and 21.1% compared with 20.8% for the third experiment. This indicates that the methods are comparable in both their intrarun and interrun variations and that there is not a major increase in interrun variation over intrarun variation.

We also compared the amount of time required for an average number of assays by both methods and the amounts

TABLE 1. Comparison of intrarun and interrune results of the reverse transcriptase assay methods

Comparison (n)	Viral dilution	Micromethod			Macromethod		
		Mean (cpm)	SD (cpm)	Coefficient of variation (%)	Mean (cpm)	SD (cpm)	Coefficient of variation (%)
Intrarun (10)	A	559,073	69,657	12.5	845,061	128,256	15.2
	B	224,308	24,618	11.0	282,869	30,381	10.7
	C	39,263	12,531	31.9	50,612	16,961	33.5
	D	9,197	2,145	23.3	13,039	2,077	15.9
Interrun (8)	A	388,328	73,906	19.0	702,930	101,460	14.4
	B	157,789	42,542	27.0	230,255	68,717	29.8
	C	40,749	7,452	18.3	55,463	7,372	13.3
	D	6,227	1,243	20.0	9,022	2,332	25.8

of reagents involved. For an average daily run of 45 specimens set up in triplicate, both methods would require about 4 h for supernatant preparation before virus solubilization. This preparation can be done the day before the assay if the virus pellets are stored at -70°C until assay. Virus solubilization requires 10 to 15 min. Tube labeling in the macromethod requires about 15 min, compared with 2 min for plate labeling. The transfer of the solubilized virus samples to the tubes or plates requires about 30 min, and incubation with the reaction mixture takes 2 h. The addition of tRNA followed by 10% TCA takes about 8 min. The major difference in the two methods is in the harvesting step. The macromethod requires 75 min, whereas the micromethod requires only 15 min. Filter drying, transfer to the scintillation vials, adding of scintillation fluid, and counting require about 30 min. Overall there is a time saving of about 1 h at this level of sample load. The maximal number of specimens that could be assayed in a day by an experienced person by the macromethod is 60 as compared with 100 by the micromethod.

Reagent use was also substantially less for most reagents in the micromethod. TCA usage was considerably less; 13.5 ml of 10% TCA and 650 ml of 5% TCA were used in the micromethod, compared with 405 and 2,400 ml, respectively, for the macromethod. The use of scintillation fluid was also less; 270 ml was used in the micromethod, compared with 1,080 ml in the macromethod. This saving was due to the small size of the filter disks in the micromethod. Ethanol use increased in the micromethod from 50 to 650 ml. We have shown that this could be reduced to 120 ml without any change in assay results by reducing the ethanol wash cycle to 2 s.

DISCUSSION

The results of a micromethod for measuring the reverse transcriptase of human T-cell lymphotropic virus type III/lymphadenopathy-associated virus utilizing a semiautomated cell harvester highly correlate with the results of the existing macromethod. Intrarun variation and interrune variation for the two methods are comparable. There is a significant reduction over the macromethod in both the time required to run the assay and in reagent utilization. Although the reverse transcriptases of retroviruses vary in substrate and ion requirements and reaction kinetics and assays for their measurement differ in these areas, the assays have in common a requirement for harvesting the derived DNA. The modification of the harvesting procedure described here should therefore be useful in assays for the reverse transcriptases of other retroviruses as well.

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