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A Microcomputer program for calculating cell population doubling time in vitro and in vivo

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Abstract Determination of the doubling time for a population of cells can involve tedious calculations. We have developed computer software for MS-DOS microcomputers to expedite the analysis of tumor cell growth in vitro and in vivo. This program, DOUBLE-TIME, assists in the collection of cell numbers into a database and calculates the doubling time for a population of cells from the plot of cell growth over time. For experiments where tumor mass is measured in vivo, the software collects measurements of tumor size, calculates tumor volume (mass), generates growth curves for tumor volume change over time, and determines the doubling time of the tumor and the mean for multiple tumors. DOUBLE-TIME plots both total and viable cell numbers over time, calculates standard error of the doubling time, and the doubling time for a selected portion of a growth curve. This software also automates the cell counting process with a software-generated cell counter that allows cell counts to be tallied directly into the computer via a mouse.

Key words Cell growth kinetics · Nude mice · Microcomputer · Computer software

Introduction

The time required for a cell population to double (i.e. doubling time) represents, in a single numerical value, the growth rate of that population. Commonly evaluated during the log phase of growth as an intrinsic property of a cell line, a population's doubling time can be calculated by performing cell counts at intervals over a period of time [12, 23]. Correlations can then be established in relation to potential growth-altering events which are exogenous (i.e. changes in fetal calf serum, culture medium, temperature, growth factors, treatment with drugs [8, 11, 12, 14, 15, 41]) or endogenous (i.e. activation of oncogenes, deactivation of tumor suppressor genes [13, 18, 40, 43]).

Methods used to tabulate the total number of cells in such experiments include counting with a hemocytometer or an electronic counter (i.e. Coulter counter), and measuring wet or dry cell weight, cell volume, total DNA content, or total protein content [12, 16, 20, 23, 28, 29, 32, 34, 46]. Hemocytometer-assisted counts are utilized in many laboratories because of their relatively low cost, the user's ability to count cells in clumps, and the ability to differentiate between viable and dead cells [12]. The standard graphic interpretation of the data collected can then be represented by semilogarithmic "growth" curves from which a population's doubling time can be determined [12, 23].

If measurements of tumor size are collected, it is also possible to determine the doubling time for the tumor mass in vivo [24, 31, 44, 47]. In such experiments, including those in which subcutaneous tumors are grown in athymic (nude) mice, the intrinsic growth rate of the tumor cells in vivo and the effects of chemotherapeutic agents [4, 6, 9, 10, 26, 27], hormones [3, 25, 39], growth factors [33], biological response modifiers [1, 45], monoclonal antibodies [19, 38], or radiation [17, 21, 37] can be assessed.

The collection and manipulation of cell counts or tumor measurements can be time consuming [23], as can be the inherent calculations (i.e. cell concentration, percent viability, tumor volume, doubling time, etc.). We have developed a computer program, DOUBLE-TIME, designed to collect and organize cell counts and perform the calculations necessary to determine the doubling time for either in vitro cell cultures or for

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tumor mass in vivo. This program also includes various time-saving functions relevant to the counting of cells with a hemocytometer, including a computer-generated version of a laboratory counter which tabulates cell counts from a hemocytometer directly into the computer with a Microsoft-compatible mouse.

Materials and methods

Hardware

The program was developed on an IBM PS/2 Model 60 and can be run on any MS-DOS system equipped with either a VGA or EGA color monitor. DOUBLE-TIME supports the following printers: HP Laser jet + and HP Laser Jet Series II and III; Epson MX-80, MX-100, FX-80, LX-80, and FX-100; and Okidata Microline 292, 293, and 294 Plus. The cell-counting portion of the program will support any Microsoft-compatible mouse.

Software

DOUBLE-TIME was written using Turbo PASCAL ver 5.0 (Borland International, Scotts Valley, Calif.). HALO'88 (Media Cybernetics, Silver Springs, Md.) which is a library of graphic subroutines for software design, was used to generate graphs and data input screens as well as to configure the various hardware specifics (i.e. printer, color card, etc.). Routines to manipulate data files were written using TOPAZ (Software Science Inc., Brisbane, Calif.) which is a set of software tools that allow dBASE files to be managed within Turbo PASCAL. DOUBLE-TIME is an executable file which can be run independently of other software programs but requires the HALO'88 device drivers (these are supplied with DOUBLE-TIME).

Calculations

The doubling time calculation is based on a semilogarithmic plot (log base 2) of a population's growth over time. A population's doubling time is the period of time required for the population size to increase by one unit in a semi-$\log_2$ graph and is represented by the inverse slope of the regression line [23]. The program also calculates a confidence interval for the doubling time from the standard error of the inverse slope. The program calculates this interval at three limits of confidence: 95%, 90%, and 80% [5, 36].

The calculations performed during the automated cell counter portion of the program include calculations of the cell concentration (total and viable) per milliliter, the total cell numbers in a sample, the percentage difference between the top and bottom hemocytometer squares (in order to check for uneven loading), and the percentage viability.

Cell culture

Sample files were generated from actual data taken from human neuroblastoma and primitive neuroectodermal (PNET) cell lines. These cell lines, SK-N-MC and LA-N-5, were obtained from their originators [2, 35]. Cells were maintained in vitro at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 with 10% fetal calf serum (FCS). Both cell lines were tested by culture and DNA staining and found to be free of mycoplasma [22].

Cells were harvested from tissue culture flasks with Pucks saline A plus 10 m*M* HEPES and 1 m*M* EDTA (Pucks-EDTA) [30]. Cells were then pipetted into uniform suspension in Pucks-EDTA, centrifuged, resuspended in RPMI-1640 plus 10% FCS, and 100 µl of cells in suspension was mixed with 900 µl 0.06% trypan blue in phosphate-buffered saline without Ca^{++} or Mg^{++} (PBS). Viable and total cell numbers were counted using a hemocytometer [12].

Tumor growth in athymic (nude) mice

Cultured neuroblastoma cells were removed from tissue culture flasks using Pucks-EDTA and then washed and resuspended in PBS. Cell clumps were dissociated and cell numbers and viability determined as above Balb/c nu/nu mice were injected subcutaneously with 10^8 cells through 22-gauge needles between the scapulae [42]. Mice were housed in a filter top caging system and all food, bedding, and water were autoclaved. Day O of the experiment was the first day a palpable tumor mass was identified and all subsequent tumor measurements were made weekly.

Tumor volume was calculated from three dimensions taken with vernier calipers–length (longest dimension), width (perpendicular to the length and parallel to the animal), and height (perpendicular to the length and width). The formula used to calculate tumor volume, $L \times W \times H \times \pi/6$, has been established as best representing the actual mass of a tumor and is therefore the most accurate method for assessing tumor doubling time [42]. Those investigators who choose to measure only two dimensions can enter the width into the program a second time as a substitute for the height measurement. For multilobed tumors, total tumor volume is most accurately determined by taking measurements of individual lobes and calculating their respective tumor volumes [42]. The doubling time for tumor volume over time is calculated as described above for cell counts.

Program description

DOUBLE-TIME was written with several objectives, the main one being to develop a program that would calculate the doubling time for a population of cells in vitro or a tumor mass in vivo. In addition, we wanted to develop a program that could be used in a laboratory to simplify the process of data collection and to organize cell counts into easily arranged databases.

DOUBLE-TIME organizes each experiment by storing data in individual dBASE files. For every file created, a description of the experiment (passage numbers, number of replicates, start date and time) is stored as a single record in a separate "directory" file. In the experiment shown in Table 1, the following information was saved in a directory file: SK-N-MC (passage 3) as the cell line, starting data of 07/25/91 at 3:30 p.m., and three replicates per entry. By storing the experimental parameters in a directory file, DOUBLE-TIME is able to recall and display descriptive information for each experiment. The program also provides various functions that allow for the manipulation of the dBASE files, including the ability to copy and delete files as well as the ability to move files to other disks.

After a file has been created or loaded, the cell count file can be updated or graphed. The program allows for two methods of updating a cell count – either by

Table 1 Sample printout from DOUBLE-TIME for in vitro cell counts

Experiment Label: Example cell count
Cell Line: SK-N-MC
Start Date:07/25/91
Start Time: 15:30
Comments:
Passage: 3
Filename: Test

Date	Cells Viable (10^6)	Cells Total (10^6)	%Viability	Average ± SD	Log_2Avg
07/25/91					
Day #: 0.00				0.165 + / − 0.000	− 2.599
Rep 1	0.165	0.165	100.00		
Rep 2	0.165	0.165	100.00		
Rep 3	0.165	0.165	100.00		
07/26/91					
Day #: 1.02				0.221 + / − 0.078	− 2.175
Rep 1	0.277	0.277	100.00		
Rep 2	0.166	0.166	100.00		
Rep 3	0.000	0.000	NA		
07/29/91					
Day #: 3.83				1.356 + / − 0.158	0.439
Rep 1	1.028	1.244	82.64		
Rep 2	0.000	0.000	NA		
Rep 3	1.284	1.468	87.47		
07/30/91					
Day #: 4.97				6.133 + / − 0.371	2.167
Rep 1	3.540	6.100	58.03		
Rep 2	3.760	5.780	65.05		
Rep 3	4.090	6.520	62.73		
07/31/91					
Day #: 5.85				10.483 + / − 1.276	3.390
Rep 1	9.690	11.460	84.55		
Rep 2	8.900	10.950	81.28		
Rep 3	6.850	9.040	75.77		

manually entering numerical values into the program (using a keyboard) or by performing a cell count via the computer-controlled cell counter with a Microsoft-compatible mouse. The counts tallied can then be saved directly to an existing file and/or printed.

Manual data entry is both quick and easy. The "update" screen displays horizontally each cell count entry in a grid. For in vitro experiments, the user inputs the date of the count and the values determined from any two of the three cell counting possibilities – number of cells viable, dead, or total. DOUBLE-TIME then calculates the sequential number of days (or hours) for the entry relative to the starting day or hour, as well as percent viable, average, and standard deviation of the replicates. For example, in the last entry of the SK-N-MC experiment (Table 1), DOUBLE-TIME calculated the average number of cells for the three replicates to be 10.48×10^6 cells with a standard deviation of 1.27×10^6. The log base 2 of the average was 3.39 and that value was plotted in the semilogarithmic growth curve as point #5 (Fig. 1A).

For in vivo experiments, DOUBLE-TIME calculates a tumor's volume for a given length, width, and height; calculation of tumor volume for multilobed tumors is based on the dimensions of individual lobes [42]. The sequential number of days and log base 2 of the total volume for each entry are also calculated (Table 2). The program allows for the analysis of numerous mice per experiment, and calculates individual tumor doubling time for each mouse as well as an average doubling time for all mice.

The computer-controlled cell counter simulates counting with a laboratory counter. The right portion of the "counter" screen displays both the top and bottom grids of a hemocytometer as seen in Fig. 2. The user counts by moving the cross-hair with the mouse to the desired hemocytometer square (representing the square currently being counted under the microscope) and pressing the left and right buttons to tally the cells. All the necessary calculations are performed, adjusting for the total volume and the dilution factor of the sample. These calculations include the number of viable, dead, and total cells, the percentage difference between top and bottom grids (to ensure even loading), the average number of cells counted per square, the cell concentration (viable and total), and the percentage

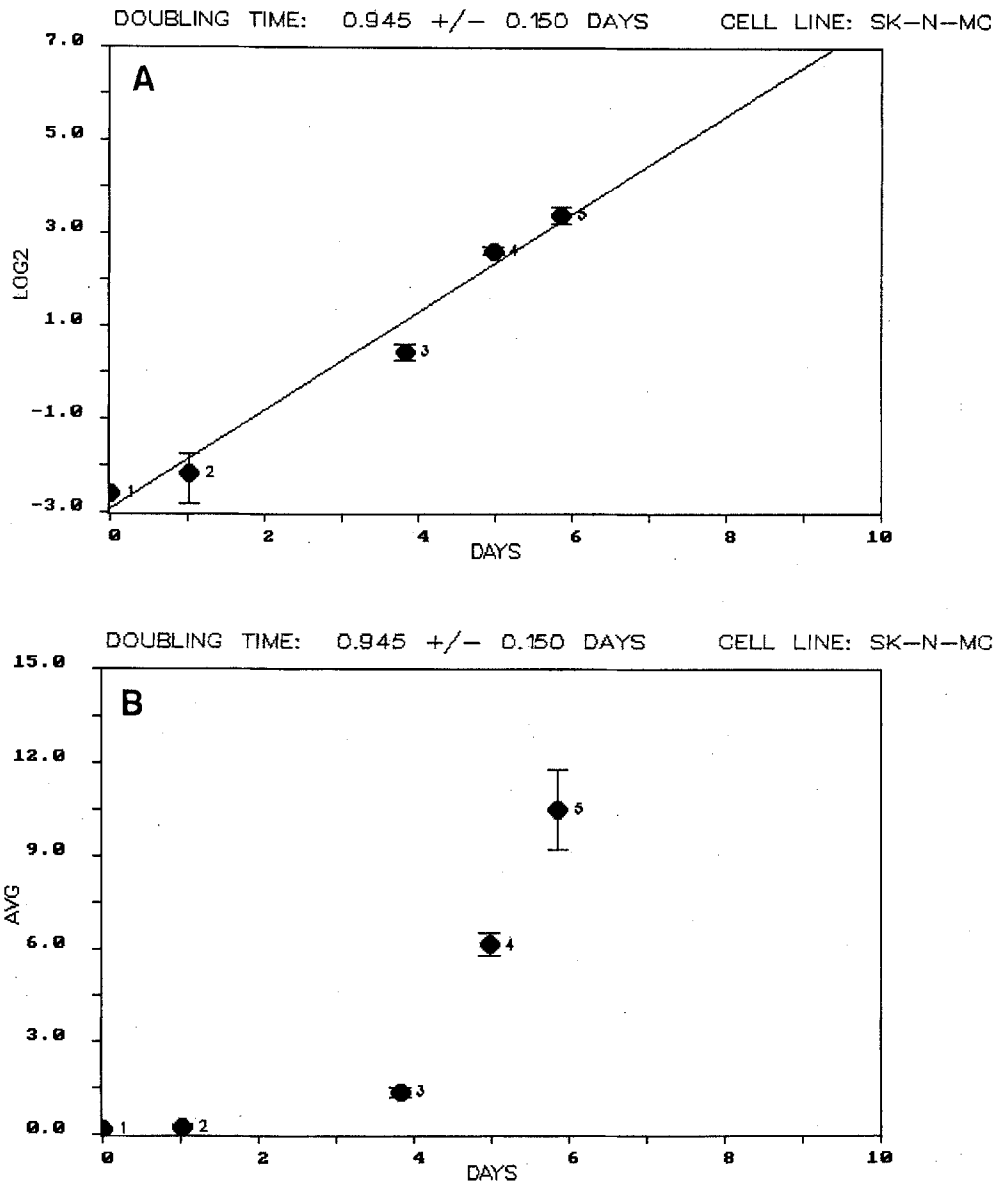

Fig. 1A Sample semilogarithmic (log base 2) growth curve generated by DOUBLE-TIME for an in vitro experiment. The graph represents the growth of the total number of SK-N-MC cells over time. The confidence interval of the doubling time was calculated with a 90% confidence limit on the inverse slope. **B** Linear (non-logarithmic) growth curve of the cell counts used in **A**

viability. The user can save a count to an existing file and/or print a count on either paper or labels (for pasting into a laboratory notebook).

DOUBLE-TIME generates a graphic representation of a cell count by plotting cell growth over time. The program can produce two formats: a plot of the average number of cells (total or viable) log base 2 versus time as seen in Fig. 1A or a plot of the average number of cells (total or viable) versus time (Fig. 1B). As shown in Fig. 3A, similar plots can be generated for tumor volume over time. The program calculates the doubling time from log base 2 plots of cell number or tumor volume. For example, using data from a nude mouse xenograft, the doubling time of the tumor's volume was calculated to be 9.33 days +/− 0.64 days with 95% confidence (Fig. 3A). For in vivo experiments, the graph and doubling time for each mouse is displayed and the average doubling time for all mice is calculated.

The program allows the user to select the confidence interval to be used in calculating the doubling time (80, 90, or 95%). The user may also select which cell count parameter to represent in the graph and calculations: number of viable cells or total number of cells. Data points are numbered on all graphs to allow excluding points from analysis or determining the doubling time for a selected area of a growth curve. Both of these operations involve simply entering the numbered

Table 2 Sample tumor volume doubling time printout from DOUBLE-TIME

Experiment Label: Xenograft of LA-N-5 in nude mouse
Injection Date: 03/20/90
First Measurement: 03/09/90
Mouse #: 1
Filename:

Date	Length (mm)	Width (mm)	Height (mm)	Volume (mm^3)	Log_2V
03/20/90 Day #: 0 Lobe: 1	6.600	7.200	2.500	62.202	5.959
04/02/90 Day #: 13 Lobe: 1	11.700	12.000	9.100	668.951	9.386
04/09/90 Day #: 20 Lobe: 1	12.800	13.000	8.200	714.419	9.481
04/16/90 Day #: 27 Lobe: 1	14.900	15.100	9.900	1166.230	10.188
04/23/90 Day #: 34 Lobe: 1	15.900	15.500	11.200	1445.216	10.497
04/30/90 Day #: 41 Lobe: 1	18.800	19.000	14.900	2786.657	11.444
05/07/90 Day #: 48 Lobe: 1	19.600	19.500	15.500	3101.760	11.599

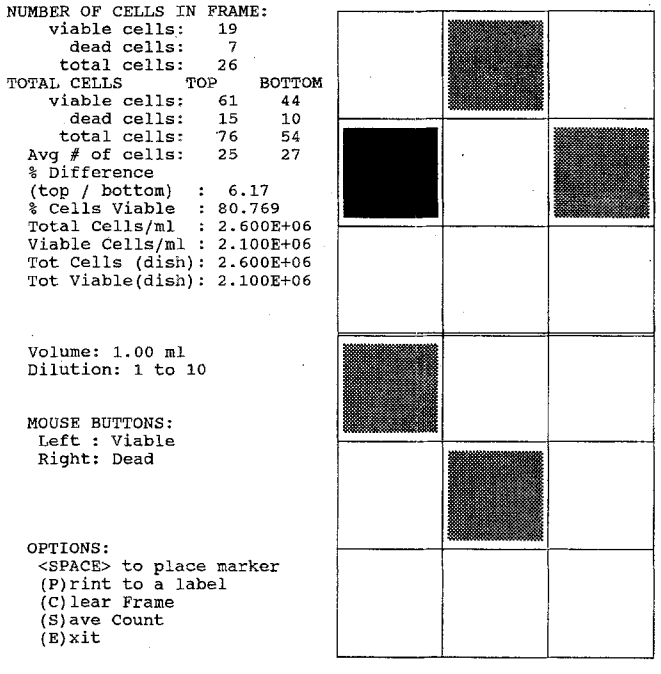

Fig. 2 Actual DOUBLE-TIME screen. Cell counts can be entered via the computer mouse. The right portion of the figure represents the top and bottom grids of a Neubaur hemocytometer. The dimensions of each of the 18 squares are 0.1 mm × 0.1 mm with a depth of 0.1 mm. To the left are the functions and calculations performed during a cell count. The solid dark box represents the square where cells are currently being counted while the other shaded boxes mark the locations of squares where counts have been already performed

data points to be excluded. The effect of excluding non-representative data on a doubling time calculation is shown in Fig. 3.

Discussion

Evaluation of the doubling time in vitro or in vivo is necessary for a variety of biological and therapeutic studies [1, 3, 4, 6, 8–15, 17–19, 21, 24–27, 31, 33, 37–41, 43–45, 47]. Although the calculation is not necessarily a complex one, it often proves to be labor intensive. Consequently, we have developed a program that calculates the doubling time for a population of cells and eliminates much of the excess data manipulation previously necessary.

In addition to calculating a population's doubling time, one of our goals in designing DOUBLE-TIME was to streamline the cell counting process, which can involve first counting cells manually with a laboratory cell counter, then entering the results into notebooks, and eventually inputing the data into a computer graphing program. With DOUBLE-TIME, counts can be tallied directly into the computer so that the data do not have to be re-entered or translated. Thus, the program allows for instantaneous calculation of the data and provides the organization needed for long-term experiments. Another program which functions as a computer cell counter using the computer keyboard

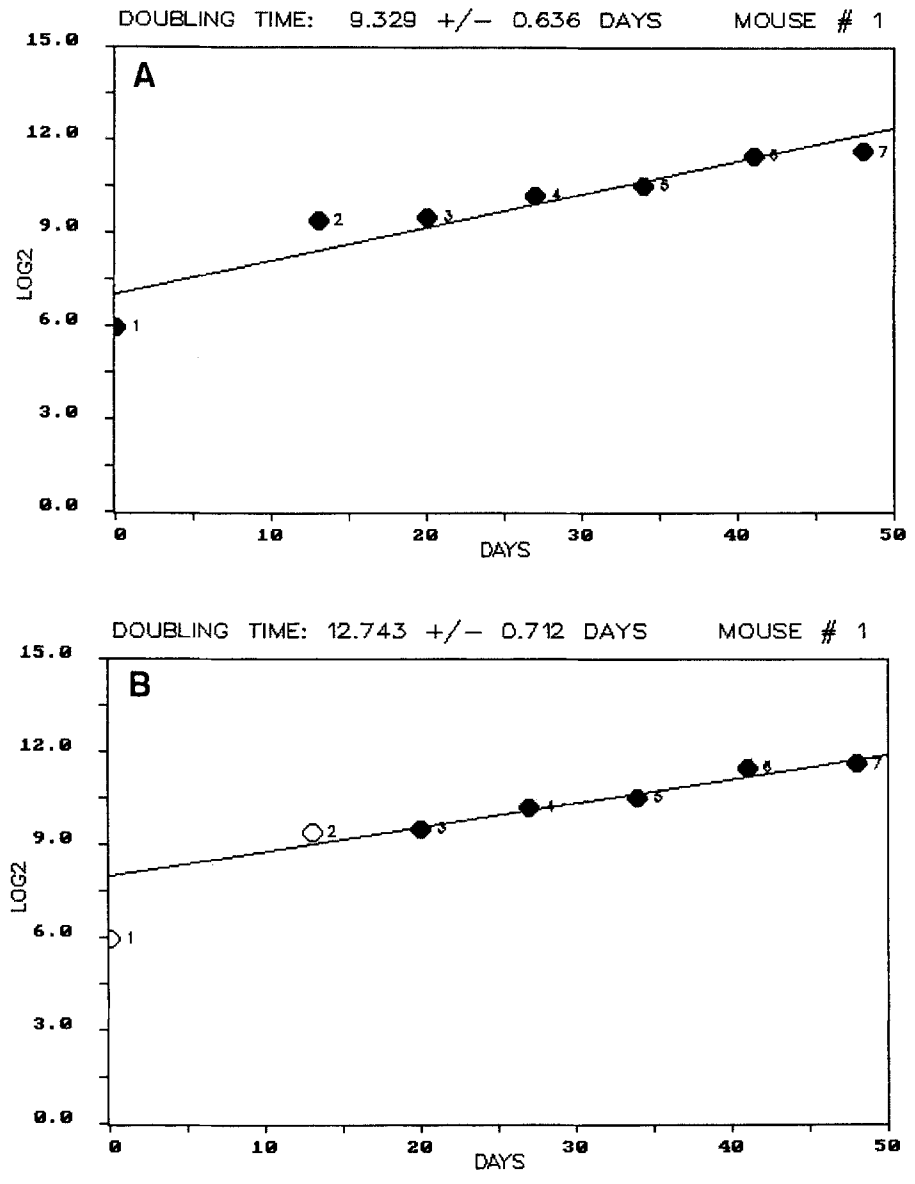

Fig. 3A Sample semilogarithmic (log base 2) growth curve generated by DOUBLE-TIME for an in vivo experiment. The graph displays tumor volume growth over time for a xenograft of the neuroblastoma cell line LA-N-5. The confidence interval of the doubling time was calculated with 95% confidence. **B** Graph of data from **A** for which two points (1 and 2) have been ignored from the calculation of the tumor's doubling time in order to better represent steady-state tumor growth; note that the calculated doubling time has changed from 9.3 to 12.7 days

has recently been described [7]. However, it lacks any of the doubling time calculation or data management features of our software, and we find the mouse to be easier than a keyboard for tallying cells when working at a microscope.

DOUBLE-TIME provides a single, flexible program that can be used to generate growth curves for experiments conducted either in vivo or in vitro. For in vitro experiments, the program allows for up to six replicates per entry. These replicates may be counted either manually or through a computer mouse and may be entered either daily or hourly depending on the type of experiment being performed. For in vivo experiments, DOUBLE-TIME generates growth curves for tumor volume measurements over time from a single mouse or from several mice. These data management features and the graphic displays of this program simplify the task of collecting and analyzing growth curve data. In addition, the ability to easily determine the doubling time for selected areas of a growth curve is useful in quantitating biphasic growth data (such as an increase in growth rate during or after drug treatment).

This program can be run on any IBM compatible MS-DOS computer and should prove to be a beneficial laboratory accessory. DOUBLE-TIME is available free of charge by sending a self-addressed stamped

envelope and a 3.5 inch MS-DOS formatted floppy diskette to the corresponding author.

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