Dynamics of Chromosome Spreading

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Consistency of optimum chromosome spreading during harvest of cytogenetic specimens remains a major concern. We have tested the idea that a precise control of the drying rate (the time with which metaphase cells dry), as fixed cell suspension is placed on a slide or an in situ culture in last fixation, may be the answer. Amniocyte and lymphocyte cultures were allowed to dry at defined combinations of relative humidity (RH) and temperature (T) in a modified Thermotron environmental control unit. We were able to demonstrate, based on 2,250 amniocytes and 1,650 lymphocytes, that the metaphase area after drying was a function of RH and T for both in situ and non-in situ culture systems. As the RH and T increase, the metaphase area increases until a threshold is reached. Also, as RH increases, the slide drying time increases. Data obtained using a response surface regression, proportional hazards regression analysis and slide drying time studies are consistent with our model of chromosome spreading. Optimum metaphase areas can be achieved at various combinations of RH and T. We propose that the use of an environmental control unit is a practical way of achieving optimum chromosome spreading routinely and in a highly consistent manner. © 1996 Wiley-Liss, Inc.

KEY WORDS: chromosome spreading, optimum cytogenetic harvesting, environmental control, chromosome harvest chamber

INTRODUCTION

During the past few decades there have been numerous advancements in cytogenetic methodology. Some of these landmark developments include the use of hypotonic solution, phytohemagglutinin-stimulated blood cultures, in situ culture methods, introduction of high resolution and robotic harvesting. While these developments have greatly improved the quality of cytogenetic techniques, consistency in the optimum spreading of chromosomes remains a major problem. We have developed a model for the dynamics of chromosome spreading for both in situ and non-in situ cultures. The basic premise of this model is that chromosome spreading characteristics are dependent upon the speed with which the chromosomes dry on the coverslip or microscope slide. As the fixative evaporates, the surface tension of the fixative on the metaphase cell makes the cell thinner from top to bottom and wider from side to side. The cell continues to get thinner and wider as the fixative evaporates. As the cell widens and reaches its widest diameter potential and then dries (Fig. 1b), it results in a suitable metaphase spread. If the drying rate is too fast, the cell dries before it has a chance to reach the optimum diameter. This results in a tight, compact metaphase where many chromosomes are overlapped (Fig. 1a). On the other hand, if the drying rate is too slow, the cell gets wider as above. The suitable cell diameter potential is reached; however, the cell does not dry. At this point, the cell suffers two usual fates: (1) the membrane breaks and chromosomes spill out. Brownian movement and currents in the fixative solution move the chromosomes, then the cell dries resulting in a broken/scattered metaphase (Fig. 1c), or (2) the cell membrane does not break and the currents in the fixative solution move the metaphase cells. The chromosomes within the cell roll and clump together as they move. The metaphase dries, resulting in a tight "rolled" metaphase (Fig. 1d). Therefore, metaphases that dry too fast are most often tight. Metaphases that dry at the optimum rate are spread with few overlaps and metaphases that dry too long are characterized by both broken and tight "rolled" metaphase spreads. This phenomenon has been observed by us during the past several years. Video images (Fig. 2) of an amniocyte are shown at different stages of the drying process.

By simply controlling the factors which influence metaphase cells drying rate, one can influence the quality of chromosome spreading. We demonstrate that controlled optimum chromosome spreading is a function of relative humidity, temperature and drying time.

MATERIALS AND METHODS

In situ amniocyte cultures from 5 different chromosomally normal subjects were trypsinized and passed to 22 × 22 mm coverslips in 25-mm Petri dishes. Each am-
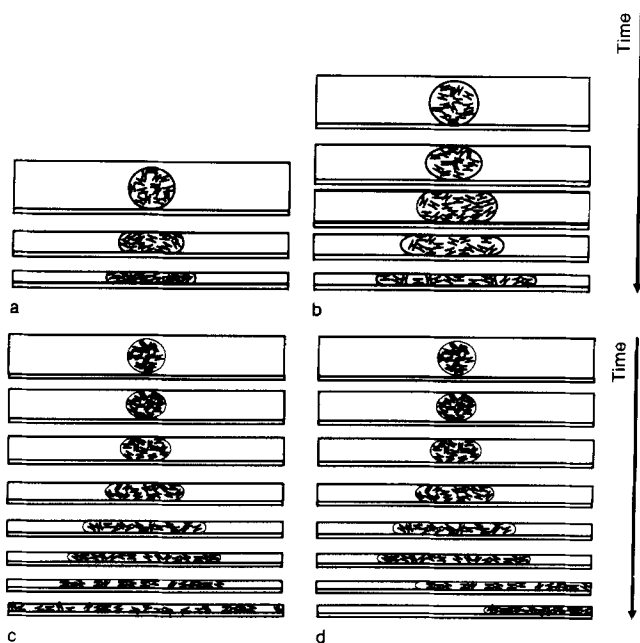
Fig. 1.   Metaphase spread as a function of the duration of drying time. **a:** Metaphases that dry too fast are often tight with many overlapping chromosomes. **b:** Metaphases that dry at the optimum rate (top view in Fig. 2) have few overlaps and are not broken. **c:** Metaphases that dry too slowly are characterized by both broken metaphases and (**d**) by tight "rolled" metaphases. This figure illustrates a side view where the top of the fixative solution is represented by a single line and the slide surface is represented by a double line.

niocyte culture was treated with ethidium bromide (10 μg/ml) for 90 minutes and colcemid (100 ng/ml) for 45 minutes and harvested with a Tecan 505 robotic harvester according to our standard method [Spurbeck et al., 1988; Spurbeck, 1991] using 0.8% sodium citrate hypotonic and methanol and glacial acetic acid (2:1) fixative, except the cultures were trypsinized prior to harvest and coverslips in the last fixative were transferred to the environmental chamber. Seventy-two hour PHA-stimulated blood cultures, also from 5 chromosomally normal subjects, were harvested by standard method, using 0.075 M KCl for 15 minutes, colcemid at 25

ng/mL and ethidium bromide at 5 μg/mL for 2 hours. Two drops (12.5 μL/drop) of cell suspension were dropped onto different areas on a dry microscope slide 1 cm from the surface, inside the Thermotron environmental chamber. Coverslips and slides were dried at three different temperatures of 20°, 25° and 30°C. For each of the three temperatures, 13 relative humidity (RH) settings at 25%, 26%, 27%, 28%, 30%, 32%, 33%, 35%, 38%, 45%, 55%, and 65% were used for amniocyte specimens and 5 RH settings at 35%, 38%, 45%, 55% and 65% were used for lymphocyte specimens. The barometric pressure was obtained from the Rochester Weather Bureau at the start of each harvest (about 12:00 noon). Amniocytes were harvested when all the cultures reached similar average densities of about 10 cells per 300 microns^2 (range of 4 to 18). Blood cultures were dropped at an approximate average density of 15 cells per 300 microns^2 (range 8 to 23). Cell densities were calculated from one subject by counting the number of cells in a 300/300-micron square five times in each of the above temperature and humidity settings for both specimen types. The metaphases were viewed by phase contrast microscopy at 250×.

The chromosome spreading was tested by a Thermotron environmental control chamber (SM5.5C). The equipment was purchased from Thermotron (Holland, MI) and modified by us for ease of access to the chamber and control of air flow. Relative humidity and temperature readings were calibrated by Thermotron personnel. The drying speed was determined by recording the time it took for 3 to 5 amniocyte coverslips or lymphocyte microscope slides to completely dry at 30%, 35%, 45%, 55% and 65% RH and 20°, 25° or 30°C temperature (T) settings from 5 amniocyte and 5 lymphocyte samples.

Statistical Analysis

Thirty consecutive diploid (tetraploid cells were not used) metaphases were scored at defined RH and T settings (Tables I and II). The area of each metaphase (Fig. 3) was approximated as an ellipse using the values of the long (x) and short (y) axes:
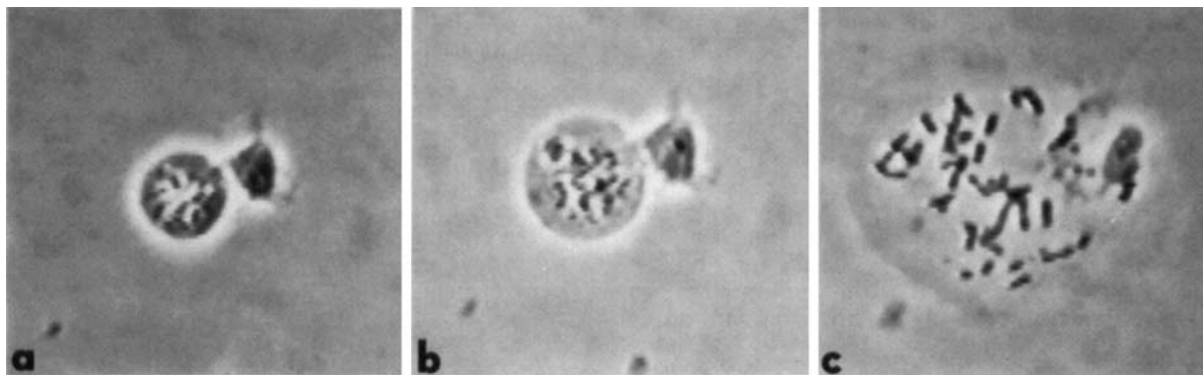
$$Area = \pi * \frac{x}{2} * \frac{y}{2}$$

Fig. 2.   Videotape images of the same amniocyte at early (**a**), intermediate (**b**), and near optimum (**c**) stages during the drying process.

TABLE I. Summary* of the Distribution of Observed Areas: Amniocytes

	Temperature (°C)		
Relative humidity (%)	20	25	30
25	—	—	982 (884) 0/90
26	—	977 (942) 0/30	—
27	—	799 (805) 0/60	1,715 (1,610) 0/30
28	—	—	941 (942) 0/30
29	—	1,052 (942) 0/30	—
30	1,096 (1,100) 0/30	—	—
32	820 (766) 0/30	—	—
33	—	1,397 (1,247) 0/30	—
35	1,367 (1,257) 0/180	1,583 (1,414) 0/150	1,879 (1,728) 3/150
38	2,275 (2,140) 2/60	—	—
45	2,097 (1,964) 3/150	1,825 (1,885) 13/150	1,854 (1,767) 16/150
55	2,145 (1,964) 11/150	1,692 (1,571) 21/150	1,542 (1,443) 22/150
65	1,743 (1,757) 26/150	1,845 (1,728) 26/150	1,478 (1,571) 38/150

* Mean area, μ^2 (median) and the number (/total) broken or scattered; mean based on unbroken scattered.

If a metaphase clearly had chromosomes missing, it was not scored. A total of 2,250 amniocytes and 1,650 lymphocytes were scored for determination of the metaphase area. If a metaphase was broken but appeared to have 46 chromosomes, it was scored as broken/scattered, and the area considered as a "censored" value. That is, it could have had an area bigger than the computed one based on the observed values of x and y.

The effects of T and RH on metaphase area were analyzed two ways. For just the "uncensored" values (i.e., not broken or scattered), a second-order response surface regression analysis with linear, quadratic, and cross-product terms [Box et al., 1978] for T and RH was used to model the logarithm of metaphase areas. Secondly, using all values, including "censored" values, a proportional hazards regression analysis [Kalbfleisch and Prentice, 1980] incorporating T, RH, and squared- and cross-product terms was used to assess these effects when the broken/scattered data were also included.

RESULTS

Because of space restrictions, it is not possible to provide all the raw data from 3,900 metaphases. However, a summary of the mean metaphase area, median metaphase area, total number of metaphases scored (both censored and uncensored) at each RH and T setting for amniocytes and lymphocytes are presented in Tables I and II. Metaphase areas increase with in-

crease in RH for each T setting until a threshold is reached. As the RH increases at each T setting, the proportion of broken/scattered metaphases also increases. For example, at a setting of 30°C, the number of broken/scattered metaphases for amniocytes were 0/90, 3/150, 16/150, 22/150 and 38/150 at 25, 35, 45, 55 and 65 RH, respectively (Table I).

The response surface regression analysis for amniocytes indicated that the metaphase area increased with increasing RH up to about 55% RH and then decreased (Fig. 4). Increasing T tended to increase the metaphase area at lower RH, but a significant $(P < .05)$ quadratic effect and interaction with RH resulted in an opposite effect at higher RH (above roughly 50%). The plateau followed by a drop in the metaphase area starting at 55% RH most likely is due to both the occurrence of a larger number of broken metaphases not scored and a greater number of less well spread "rolled" metaphases.

Including the broken/scattered areas as censored values in the proportional hazards regression analysis (Fig. 5) indicated similar effects of T and RH. Both these factors were significant, as was the interaction term in this model for amniocytes. A summary of the predicted results for metaphase area as a function of T (settings 20°, 25°, 30°C) and RH (24% to 66%) for amniocytes is given in Table III. The overall pattern of increased metaphase size with increasing RH was observed for all three T settings. However, the effect of increasing RH differed over the range of T settings. The

TABLE II. Summary* of the Distribution of Observed Areas: Lymphocytes

	Temperature (°C)		
Relative humidity (%)	20	25	30
35	—	—	1,880 (1,728) 2
38	—	1,357 (1,247) 0	—
45	1,897 (1,728) 0	1,697 (1,571) 1	2,120 (2,081) 4
55	2,440 (2,199) 3	1,988 (1,885) 2	2,042 (1,905) 13
65	2,345 (1,964) 0	2,146 (1,964) 8	2,069 (2,003) 22

* Mean area, μ^2 (median) and the number (out of 150) broken or scattered; mean based on unbroken/scattered.
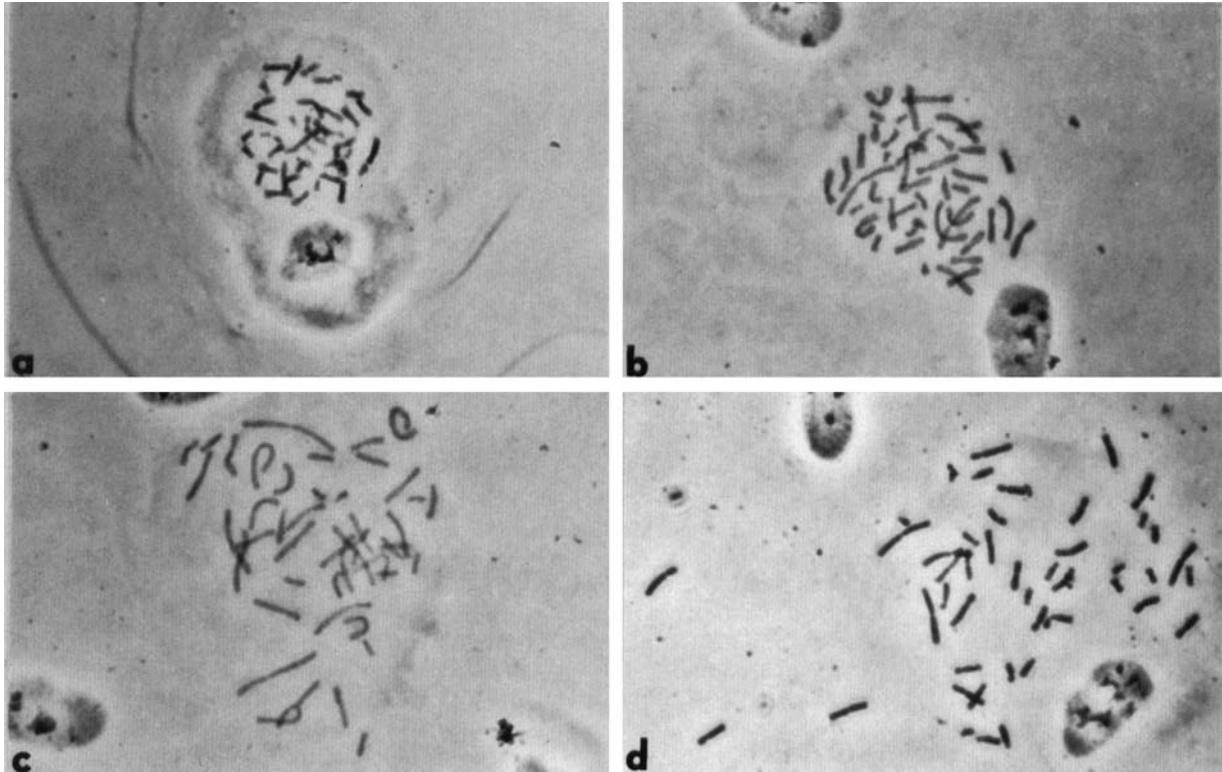
Fig. 3.   Four different metaphase areas from amniocytes: **a**) 1,100 μ^2, **b**) 2,120 μ^2, **c**) 4,946 μ^2, **d**) 8,537 μ^2 considered broken/scattered (censored).

largest median metaphase area (about 1,850 μ^2) was reached around 50% RH for 25°C, while the metaphase area reached the largest median value (about 2,000 μ^2) at 55% RH for the 20°C and 30°C temperature setting.

The response surface regression model for lymphocytes is summarized in Figure 6 and the proportional hazards model (including the broken/scattered censored values) is summarized in Figure 7. Both types of regression analyses indicated similar results to those

for amniocytes; an overall increase in the metaphase area was reached at about 55% RH over the three temperature settings. In contrast to the results for amniocytes, there was not a marked downward trend after the highest thresholds were achieved for the proportional hazards regression analysis, even at 65% RH, though a plateau was reached between 55% to 65% RH. A summary of predicted results for metaphase area in lymphocytes as a function of T (20°, 22°, 24°, 26°, 28°, and 30°C) and RH (24% to 66%) is presented in Table IV. As the RH increases, the numbers of broken and scattered scorable (censored) metaphases also increase. For example, in lymphocytes at 30°C the number of scorable broken and scattered were 2, 14, 13 and 22; for 35%, 45%, 55% and 65% RH, respectively (Table II).

The drying time, expressed as mean ± S.E. (standard error) in seconds is represented in Figure 8 for amniocytes and Figure 9 for lymphocytes. For amniocytes and lymphocytes, the drying time tended to increase as RH increased. These data are consistent with the model that as the drying time increases, the metaphase area also increases until a threshold is reached.

During these experiments, the barometric pressure was noted. However, no effect of barometric pressure on metaphase area was detected for either amniocytes or lymphocytes. The range of barometric pressure (in inches of mercury corrected at sea level) for amniocytes was 29.74 to 30.44, and for lymphocytes it was 29.79 to 30.03.
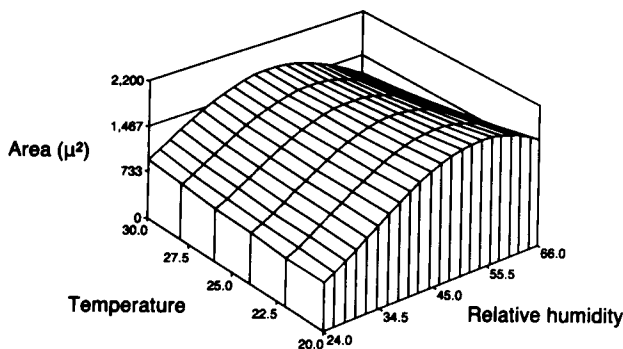

Fig. 4.   Predicted mean metaphase area for amniocytes as a function of temperature (°C) and relative humidity (%) based on a least squares response surface regression analysis using only unbroken/scattered cells.

TABLE III. Predicted Median Metaphase Areas (micron^2) for Amniocytes as a Function
of T and RH

Relative humidity (%)	Temperature (°C)					
	20	22	24	26	28	30
24	857	857	857	884	977	1,008
26	884	942	977	1,008	1,008	1,080
28	1,008	1,008	1,008	1,008	1,104	1,172
30	1,034	1,034	1,104	1,172	1,172	1,268
32	1,172	1,172	1,172	1,172	1,268	1,365
34	1,172	1,172	1,268	1,308	1,404	1,404
36	1,325	1,325	1,365	1,404	1,404	1,500
38	1,404	1,404	1,404	1,404	1,482	1,644
40	1,461	1,461	1,461	1,515	1,627	1,691
42	1,551	1,551	1,551	1,644	1,691	1,770
44	1,691	1,691	1,691	1,691	1,770	1,785
46	1,770	1,734	1,734	1,770	1,770	1,929
48	1,770	1,770	1,770	1,770	1,839	1,945
50	1,858	1,770	1,770	1,785	1,929	2,017
52	1,929	1,858	1,821	1,858	1,929	2,017
54	1,978	1,929	1,858	1,929	1,929	2,017
56	2,107	1,929	1,910	1,929	1,929	2,017
58	2,017	1,929	1,858	1,858	1,929	1,978
60	1,995	1,929	1,804	1,785	1,839	1,929
62	1,945	1,858	1,770	1,770	1,770	1,839
64	1,929	1,770	1,770	1,770	1,770	1,770
66	1,821	1,770	1,734	1,691	1,691	1,734

DISCUSSION

The breakthroughs in cytogenetic techniques have been of landmark proportions in the last few decades. Introduction of the hypotonic treatment in the 1950s [Hsu, 1952], air drying techniques [Rothfels and Sim-minovitch, 1958], use of PHA to stimulate lymphocyte culture in the 1960s [Nowell et al., 1960a,b], the ability to induce high resolution banding [Yunis, 1976], development of in situ culture methods [Peakman et al., 1979; Spurbeck et al., 1988] and robotic harvesting [Spurbeck et al., 1988] during the 1970s and 1980s. In spite of these remarkable advancements in cytogenetic techniques, consistency of optimum spreading of chromosomes remains a major problem.
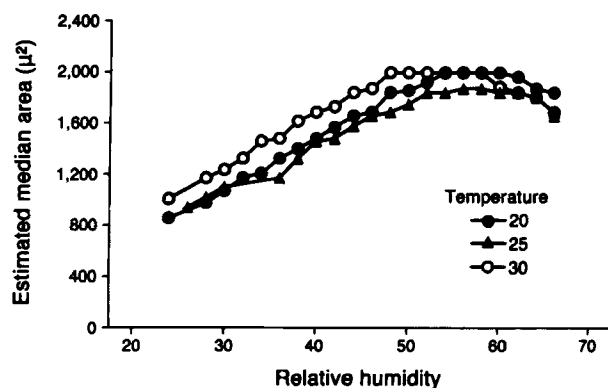

Fig. 5. Estimated median metaphase area for amniocytes as a function of temperature (°C) and relative humidity (%) based on proportional hazards regression analysis.

It has been our belief that the dynamics of chromosome spreading, both for in situ and non-in situ cultures, are dependent at the rate (time) with which chromosomes dry on a glass surface after the final fixation step. If the metaphase dries too fast, it often results in overlapped chromosomes and prominent cytoplasm. On the other hand, if the drying rate is too slow, both metaphases with scattered chromosomes and metaphases with tightly spread chromosomes are encountered.

To control the drying time of the cell suspension on a glass surface in a predictable manner, we used an environmental chamber to test in situ and non-in situ procedures at defined T and RH. The use of response surface regression models, proportional hazards models and slide drying timing experiments indicates that optimum spreading can be achieved for defined T and RH settings.

The metaphase area increased until a threshold was reached for in situ cultured amniocytes for each of the three T settings (20°, 25° and 30°C) as RH increased from 27% to 65%. The drop in the metaphase area for the population mean beyond the threshold was most likely indicative of rejection of nonscorable scattered or broken metaphases and increased numbers of less well-spread "rolled" metaphases. Although we did not record the nonscorable scattered metaphases, these broken metaphases were mostly encountered at the highest RH setting of 65%. The maximum median metaphase areas appeared to be 1,800 to 2,000 μ^2 which could be achieved at all three temperature settings at 55% RH (Fig. 6 and Table III).

TABLE IV. Predicted Median Metaphase Areas (microns^2) for Lymphocytes as a Function of T and RH

Relative humidity (%)	Temperature (°C)					
	20	22	24	26	28	30
24	1,005	1,005	1,005	1,005	1,080	1,242
26	1,055	1,005	1,005	1,055	1,172	1,316
28	1,139	1,080	1,080	1,139	1,242	1,422
30	1,172	1,172	1,172	1,172	1,268	1,480
32	1,242	1,172	1,172	1,242	1,422	1,532
34	1,291	1,242	1,242	1,268	1,422	1,595
36	1,422	1,291	1,268	1,422	1,480	1,694
38	1,480	1,422	1,422	1,422	1,532	1,718
40	1,532	1,450	1,422	1,480	1,571	1,782
42	1,571	1,496	1,480	1,532	1,661	1,887
44	1,679	1,548	1,532	1,548	1,694	1,908
46	1,694	1,635	1,548	1,661	1,741	1,965
48	1,761	1,679	1,661	1,694	1,761	2,085
50	1,824	1,694	1,694	1,694	1,806	2,085
52	1,887	1,741	1,694	1,741	1,887	2,125
54	1,944	1,782	1,741	1,761	1,908	2,171
56	1,965	1,824	1,761	1,782	1,908	2,217
58	2,085	1,887	1,782	1,824	1,965	2,242
60	2,085	1,887	1,806	1,845	1,965	2,242
62	2,105	1,908	1,824	1,887	1,965	2,242
64	2,148	1,926	1,887	1,887	1,965	2,242
66	2,171	1,944	1,887	1,887	1,965	2,242

The metaphase area also experienced an increase for non-in situ lymphocyte cultures for each of the three T settings (20°, 25° and 30°C) as RH increased from 27% to 65%. However, unlike the results for amniocytes, the proportional hazards regression analysis indicated no clear downward trend in metaphase area beyond the threshold for maximum values. This may be due to the relatively smaller number of scattered or broken metaphases that were rejected. The largest median metaphase areas of 1,800 to 2,150 μ^2 occurred at about 55% to 60% RH for T settings of 25°C and 30°C and was about 2,200 μ^2 at (65% RH and 20°C (Fig. 7 and Table IV). Estimated median metaphase areas can be predicted both for in situ and non-in situ systems, at a

given T (20° to 30°C) and RH (25% to 65%; Tables III and IV). These data are consistent with the model of chromosome spreading proposed.

It is difficult to define the optimum metaphase area. The optimum metaphase area will be different for a 400 band stage compared to a 850 band stage metaphase. However, if one defines optimum as a rounded metaphase with the fewest number of overlapping chromosomes without being broken or scattered, for a 550 band stage metaphase, one may estimate that between 2,500 μ^2 to 4,500 μ^2 may represent an optimum metaphase area range, although a metaphase area of 2,100 μ^2 (~550 band stage) seen in Figure 3 is very acceptable. The fitted model indicated that for amniocytes
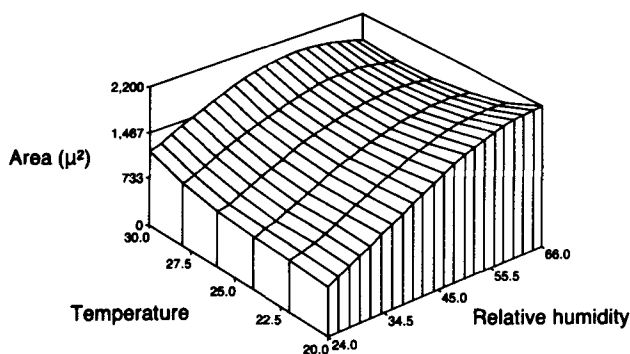

Fig. 6. Predicted mean metaphase area for lymphocytes as a function of temperature (°C) and relative humidity (%) based on a least squares response surface regression analysis using only unbroken/scattered cells.
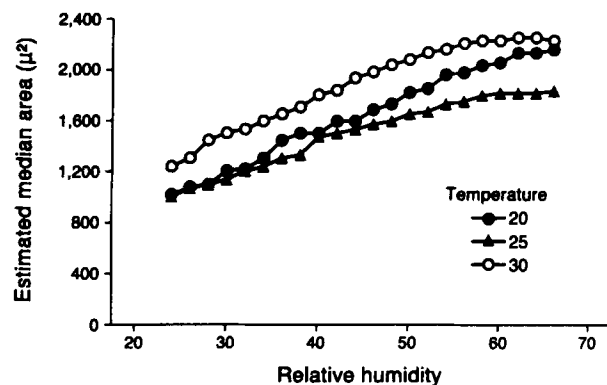

Fig. 7. Estimated median metaphase area as a function of temperature (°C) and relative humidity (%) for lymphocytes based on proportional hazards regression analysis.
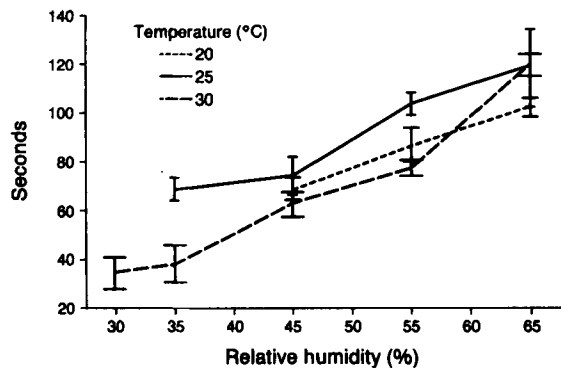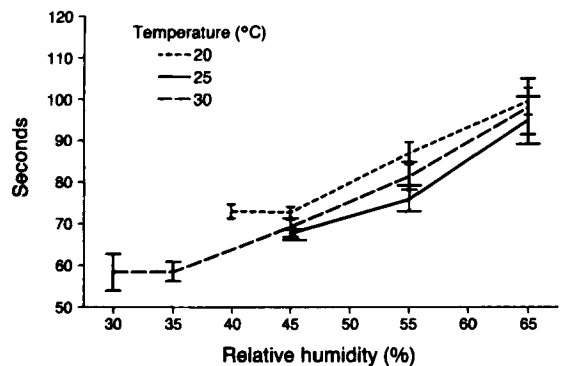
Fig. 8. Association of temperature (°C) and relative humidity (%) with slide drying time (mean ± S.E.) for amniocytes.

55% RH and 20°C, and for lymphocytes 60% RH and 20°C would provide 30% or more metaphases with a metaphase area between 2,500 to 4,500 μ^2. In practice, however, we usually prefer the use of 50% RH and 25°C for both amniocyte and lymphocyte cultures. At the lower RH setting, there are less broken/scattered metaphases and, therefore, less "floater" or "nomadic intruder" chromosomes to deal with. We therefore pre-

Fig. 9. Association of temperature (°C) and relative humidity (%) with slide drying time (mean ± S.E.) for lymphocytes.

fer metaphases to be a little tighter and less broken/scattered. It is possible for 75% to 90% of metaphases, in a given specimen, to be suitable for analysis by adjusting RH and T.

It should be noted that these findings were obtained by specific slide drying methods (air-dried with average cell density of 10 cells per 300 μ^2 for amniocytes and 15 cells per 300 μ^2 for lymphocytes). Other drying techniques such as flaming, blowing on slides or using different cell densities have not been tested by us.

It seems that the optimum metaphase areas can be achieved for in situ and non-in situ culture systems at defined RH and T settings. The use of an environmental control chamber can help increase the probability of obtaining a large number of metaphase suitable for chromosome analysis.

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A Note From Thermotron

The preceding abstract presents a summary of Mayo Clinic's research on "The Dynamics of Chromosome Spreading." The results of Mayo's research demonstrate that the use of an environmentally controlled drying chamber optimizes chromosome spreading.

Thermotron, in cooperation with Mayo Clinic Cytogenetic Laboratory, has developed a Temperature - Humidity Chamber that accurately and consistently controls these vital drying conditions.

Thermotron proudly displayed the new CDS-5 Cytogenetic Drying Chamber at the Annual Meeting of the Association of Cytogenetic Technologists in San Antonio, TX. Designed for ease of use in the Cytogenetic Laboratory environment, the CDS-5 features a full visibility window and comfortable arm ports to promote efficient preparation of slides inside the chamber.

THE DYNAMICS OF CHROMOSOME SPREADING. JL Spurbeck, AR Zinsmeister, KJ Meyer, SM Jalal. Cytogenetics Laboratory, Mayo Clinic, Rochester, Minnesota 55905

Consistency of optimum chromosome spreading during harvest of cytogenetic specimens remains a major concern. We have tested the idea that a precise control of the drying rate (the time with which metaphase cells dry), as fixed cell suspension is placed on a slide or an *in situ* culture in last fixation, may be the answer. Amniocyte and lymphocyte cultures were allowed to dry at defined combinations of relative humidity (RH) and temperature (T) in a modified Thermotron environmental control unit. We were able to demonstrate, based on 2,250 amniocytes and 1,650 lymphocytes, that the metaphase area after drying was a function of RH and T for both *in situ* and non-*in situ* culture systems. As the RH and T increase, the metaphase area increases until a threshold is reached. Also, as RH increases, the slide drying time increases. Data obtained using a response surface regression, proportional hazards regression analysis and slide drying time studies are consistent with our model of chromosome spreading. Optimum metaphase areas can be achieved at various combinations of RH and T. We propose that the use of an environmental control unit is a practical way of achieving optimum chromosome spreading routinely and in a highly consistent manner. Figures 1 to 4 illustrate some data obtained.
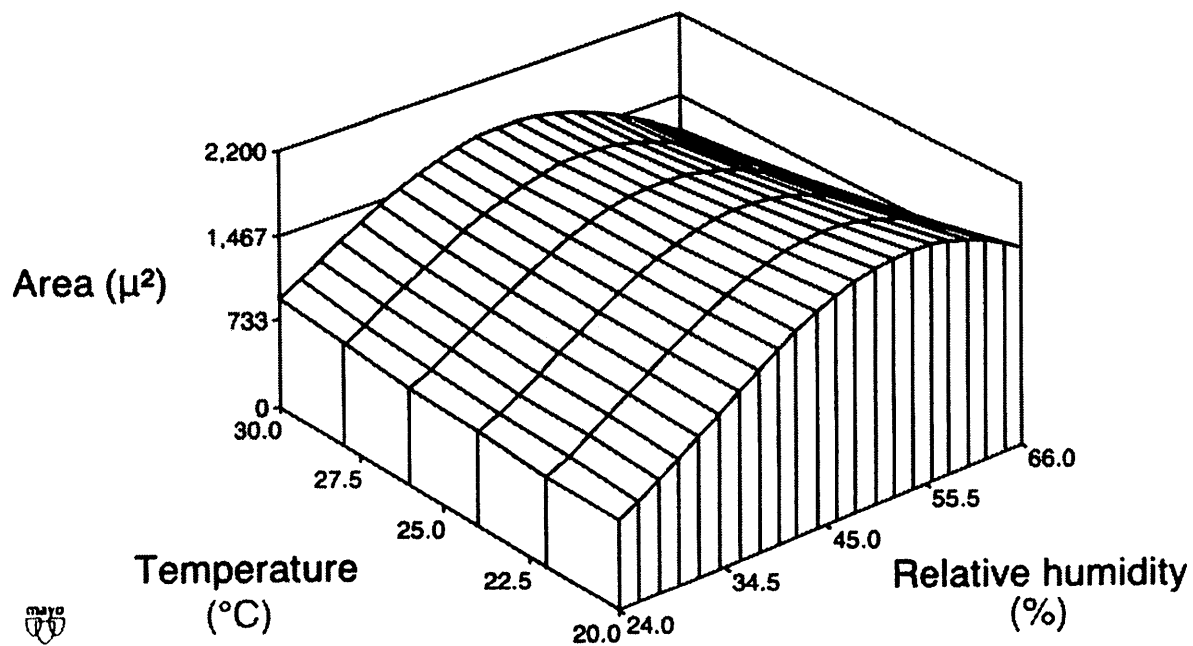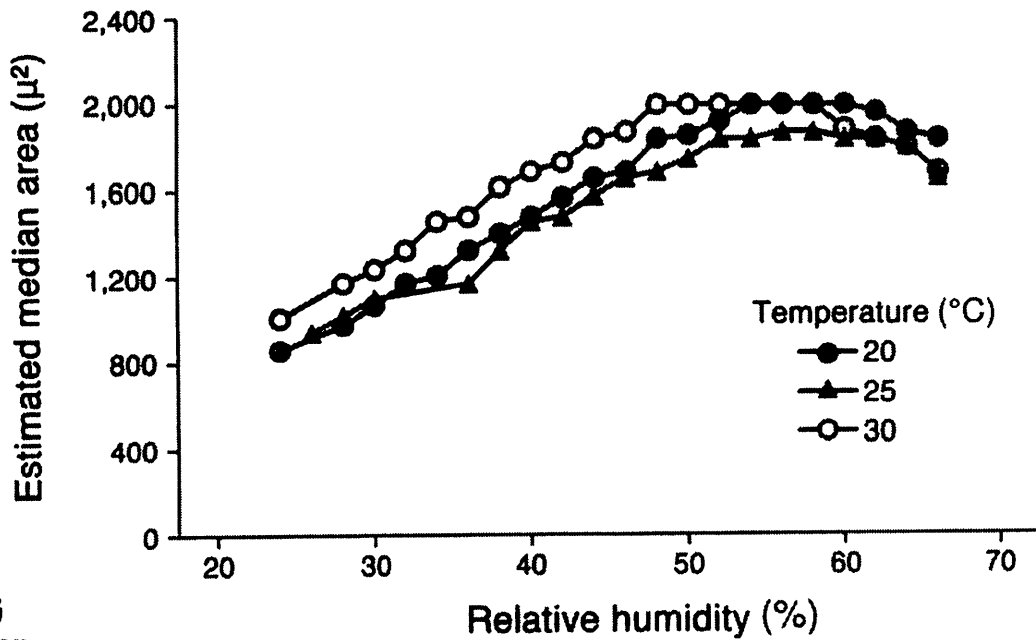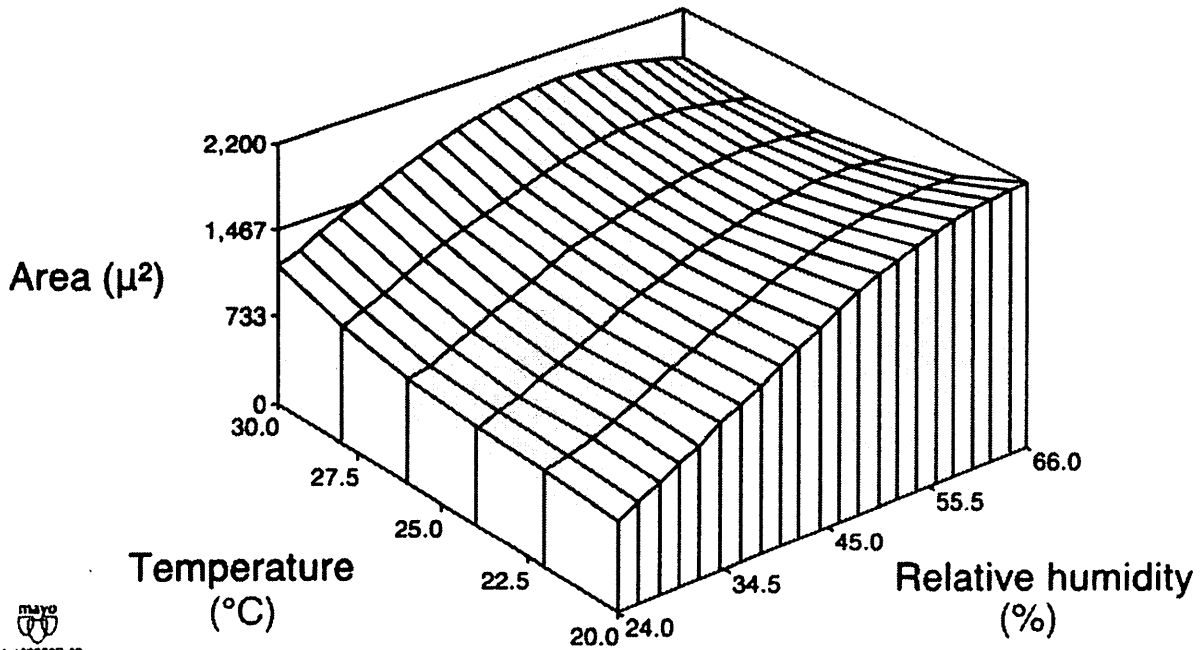
Figure 1

Predicted mean metaphase area for amniocytes as a function of temperature and relative humidity based on a least squares response surface regression analysis using only non-broken/scattered cells.
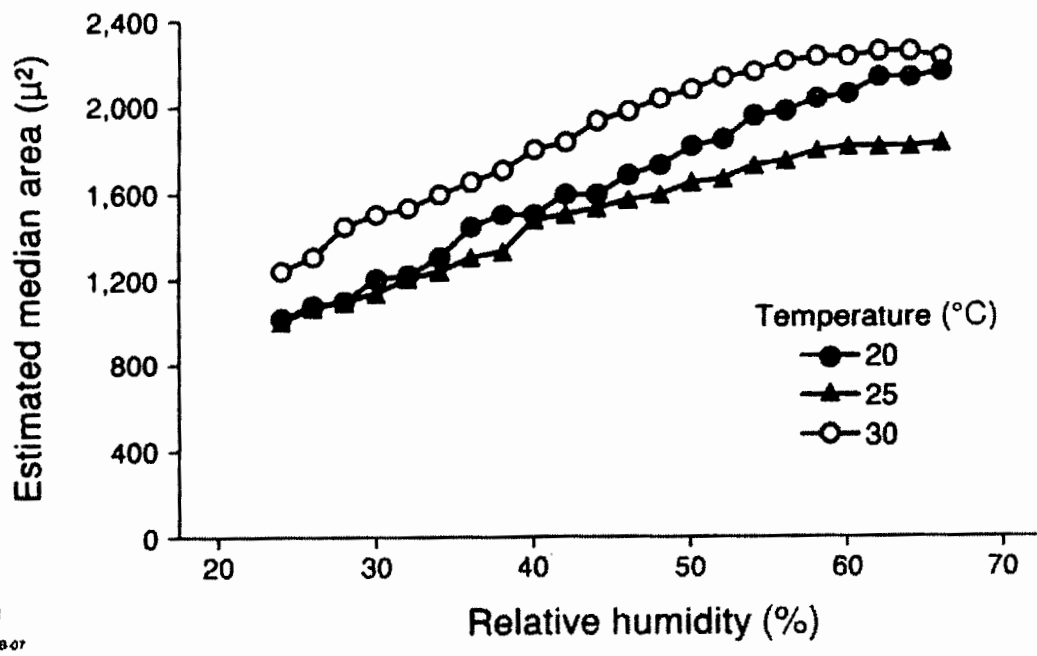
Figure 2
Estimated median metaphase area for amniocytes based on proportional hazards regression analysis.

Figure 3
Predicted mean metaphase area for lymphocytes as a function of temperature and relative humidity based on a least squares response surface regression analysis using only non-broken/scattered

Figure 4
Estimated median metaphase area for lymphocytes based on proportional hazards regression analysis.