EQUIPMENT AND PROCEDURES FOR MOUNTING MILLIPORE FILTERS AND COUNTING ASBESTOS FIBRES BY PHASE CONTRAST MICROSCOPY

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Consumer Protection and Environmental Health Service
Environmental Control Administration

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F O R E W O R D

To determine concentrations of asbestos dust in air for comparison with established hygiene limits, a simple reproducible method is required. A simple and inexpensive sampling method uses disposable (reloadable if desired) plastic membrane filter holders, pre-loaded with membrane filters. These membrane filters may be rendered transparent and the asbestos fibers counted with a phase-contrast microscope at 430X (or 400X). This method has been used by the Asbestosis Research Council in Great Britian and the U. S. Public Health Service in the United States of America. It is specified in the proposed change for asbestos in the 1968 threshold limit values of the American Conference of Governmental Industrial Hygienists.

Uniform procedures must be used for carrying out these counts if reproducible results are to be achieved. Detailed equipment and procedures used by the U. S. Public Health Service are described here.

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INTRODUCTION

For several years, a set method for counting and sizing airborne particulate matter, which has been deposited upon membrane filters, has been used. The filters used in this technique are 37 mm diameter cellulose ester membrane filters manufactured by Millipore Corporation, (Cat. No. AAWP03700). The slide mounting technique refers only to these filters, and will not work for any others.

The filters may be purchased premounted in convenient field monitor cases, and are ready for sampling. The filters are nearly 100% efficient for any asbestos dust, even for fibers with diameters much smaller than the 0.8 micron (μ) pore size. Sample preparation for electron and optical examination are both relatively simple. Slide mounts of the filters are semi-permanent.

The following procedures and recommendations will produce accurate results, if each step is performed exactly as indicated.

EQUIPMENT LIST

1. Table
2. Adjustable chair
3. Phase contrast microscope
4. Ribbon filament illuminator or (built-in illuminator)
5. Eye piece reticle (Porton type)
6. Lens tissue
7. Small camels hair brush
8. Slides
9. Cover slips
10. Spatula
11. Tweezers
12. Scalpel
13. Wheaton Balsam bottle

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LABORATORY SET-UP

It is important to provide a suitable counting area, for the room in which the counting is to be done has a great bearing upon the microscopist. It should be out of the way of normal traffic, and be kept as free from dust and smoke as possible. The tables on which the microscopes are placed should be free from vibrations and shocks as possible. An adjustable chair will do much to add to the comfort of the counter.

PRELIMINARY MICROSCOPE SET UP

Before any dust sizing or counting can be done, proper equipment must be at hand. This section describes the required equipment.

The illuminator should be small and reasonably compact. Ribbon filament illuminators are generally much easier to use. The illuminator must incorporate a condensing lens, so that the enlarged image of the lamp filament may be focused in the plane of the substage condenser diaphragm. The illuminator must also have an iris diaphragm located as near as possible to the condensing lens. The iris serves as a field diaphragm and is focused in the plane of the specimen.

The microscope used must have a substage condenser fitted with an iris diaphragm. This iris serves as an aperture diaphragm. The microscope should have a calibrated reticle inside the non-adjustable eyepiece. The reticle should be small enough that all the counting field is in focus (if the flat field objective and eyepieces are used, essentially the entire field will be in focus). The microscope must be equipped with phase contrast accessories. Using phase contrast, even objects with almost the same refractive index as the mounting media can be seen. Light waves which travel through such a specimen are retarded by a fraction of a wave-length to produce a change in phase. The phase contrast optical system reveals these phase changes as light or dark contrast against the background.

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This procedure was written by a user of Bausch and Lomb phase-equipped microscopes, so that microscope set-up procedures correspond with these. However, any good microscope with phase contrast accessories will be satisfactory.

For continual routine examination of filters, the binocular type microcope is much more comfortable. The use of a first surface mirror is recommended, but not necessary. By utilizing a second surface mirror, multiple images of the illuminator field iris are formed which will detract from the crispness of the final image.

As mentioned previously, the microscope must contain a reticle. Any reticle, such as the Porton or Patterson Globe and Circle, which projects a constant counting area and has provision for sizing, will work. The Porton reticle which we use outlines a rectangle that fits easily within the periphery of focus. The rectangle is divided into two squares. The left square is divided into six rectangles which constitutes the counting area. Above and below the large rectangle are a series of circles in which every other circle doubles in diameter. Hence the third is twice the diameter of the first; the fourth is twice the size of the second, etc. The right half of the rectangle contains a scale for extending the size above the number nine circle. The formula $D=L\ 2^N$ describes the circles sizes. The diameter D is found from L which is the unit of length, and N which represents the number of the circle. L is determined by calibration with a stage micrometer. The entire length of the rectangle is 200 L units. By measuring the length and dividing by 200, L is obtained and the circle sizes may be calculated. Whenever a microcope is disassembled or cleaned, the reticle calibration should be checked. Another fact to take into consideration is that changing the interpupillary distance of the binocular will change the calibration, so the

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microscopist may want to check the calibration at both extremes of adjustment. The counting area on the reticle should be kept as clean as possible, as dirt on the reticle is in focus and may be counted.

MICROSCOPE SET-UP AND THE PROCEDURE FOR OBTAINING KOHLER ILLUMINATION

By utilizing equipment that meets the requirements just mentioned, any counter may achieve comparable counts after some practice. This section will deal with setting the microscope up and obtaining Kohler illumination. It is extremely important that every step be followed exactly as indicated.

1. Place the microscope on a flat, level surface at a height such that the eyepieces may be observed without strain or discomfort.
2. Place a moderate dust sample upon the stage.
3. Place the illuminator directly in front of the microscope. Sight across the two and be sure they are aligned. For coil filament bulbs the illuminator iris should be ten inches from the center of the microscope mirror. For ribbon filament bulbs, the front of the filter holder should be seven inches from the center of the plane side of the mirror. Never use the curved side.
4. Remove all diffusing filters from the system and insert a neutral density filter and a clear blue filter. Blue or green colored filters may be used at the discretion of the microscopist.

NOTE: Where the microscope includes a built-in illuminator to give Kohler illumination, the above steps will not all be necessary.

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REVIEW

A. Check microscope and illuminator alignment.

B. Check the distance between the microscope and the illuminator.

C. Be sure that you are using the flat side of the mirror.

D. Make sure that there are only two filters in the system.

5. By means of the focusing knob on the side of the substage condenser assembly, raise it until the upper lens nearly touches the bottom of the slide. Remember that the free working distance of the condenser is in the order of 1.2 mm and that the slide is about 1 mm thick.

6. Turn on the illuminator. Using the tilt controls, direct the beam onto the center of the mirror. Then, by tilting the mirror direct the beam upward into the condenser.

7. Close the substage iris completely. Open the illuminator iris all the way.

8. By means of the focusing controls on the side of the illuminator, focus the image of the filament on the bottom of the substage iris. This may be done by leaning over the microscope and observing the substage iris in the microscope mirror. (On such models as the Bausch and Lomb PR-27, there is a fine focus knob at the rear of the illuminator. In that case, move the condensing lens all the way forward and trim the focus with the fine focus knob.)

9. Open the substage iris about half way.

REVIEW

A. Check the distance between the slide and condenser.

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B. Be sure that the beam is striking the center of the mirror, and that it is being deflected upward into the condenser.

C. Please repeat step 7.

D. Check step 8. If the image is not in focus now, correct it. Do not continue until the filament is correctly focused.

E. Please repeat step 9.

10. Put the 10X objective in place and focus on the sample. This is done as follows: First, looking from the side of the microscope, lower the objective until it gets very near to the slide. Then, by looking through the eyepieces, focus up. Never focus down with coarse focus when looking through eyepieces.

A word of caution: The objective lenses are very expensive, so be careful not to grind them through the slide. Always focus up. Now trim the focus with fine focus knob. Focus sharply on the sample. Secondly, close the field iris fully. If the consenser is nearly in focus, you should see a bright spot of light. By using the condenser focus knob, bring the field iris into sharp focus. Now, both the field iris and the objective are in focus on the sample. If there appear to be multiple images of the field iris, it is because of the second surface mirror. Some of the light waves are reflected from the first surface, resulting in secondary images.

12. If the image of the field iris is not centered, re-center it by tilting the mirror. If the color around the field iris is not uniform, recheck the tilting of the illuminator.

REVIEW

A. Check and be sure you are focused on the sample. 10X objectives have a relatively long focal length and it may be possible to focus on: (a) the top of the cover slip. (b) the sample. (c) the bottom of the filter. (d) the top of the slide. (e) the bottom of the slide. (f) the top of the condenser. So be sure of the focal plane.

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B. Be sure that the field iris is focused in the sample plane. Check this often when examining samples. As the sample focus changes, so does the condenser focus.

C. Recheck the centering and tilt of the illuminator.

DETAILED INSTRUCTIONS FOR BAUSCH AND LOMB BINOCULAR MICROSCOPES

1. Leave the microscope phase ring on C. Insert the telescope that is supplied with the kit into the right eyepiece tube. Focus on the dark phase ring. Open and close the substage iris to be sure that it is centered in relation to the objective phase ring. If it is not in the center, loosen the set screw on the condenser mount and properly seat the condenser. Then re-tighten the set screw. This should very seldom be necessary. Do not force anything and be careful and patient. When you are satisfied that the condenser is aligned, open it all the way.

2. Turn the phase wheel to 10. This shows that the 10X phase ring is in place. Look through the telescope again. By using the adjustment screws on the side of the condenser, move the rings until they are perfectly concentric. When changing phase rings, move them carefully, so as not to knock the condenser out of alignment.

3. Turn to the 43X objective. Carefully turn the wheel to the 43 phase ring. Align the two rings in the same manner as performed in step 2.

4. Remove the telescope and replace the eyepieces. Again, check the condenser focus. You are now ready to examine the sample at 430X, assuming that you are using 10X eyepieces.

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FILTER MOUNTING

Set-up Prior to Mounting

Have available at hand a quality scalpel and a supply of No. 10 curved blades, a pair of tweezers (fine pointed are preferred), a spatula or fire polished glass rod and a box of lens tissue. Slides (1" x 3" or 25 x 75 mm) with frosted ends are best because one may label the slide with the date, sample number, etc. It is advisable to use quality number 1-1/2 cover slips. Most dry objectives are corrected for this thickness cover glass. The mounting media should be kept in a Wheaton balsam bottle. The edge around the lid should be coated with stopcock grease to keep out the surrounding air.

The mounting solution may now be prepared. A one to one solution by volume of dimethyl phthalate and diethyl oxalate is poured into the balsam bottle. Then add 0.05 gram of filter material for each milliliter of solution. The added filters increase the viscosity of the solution. Before using the solution, all of the filter material must be dissolved and the solution must be of uniform consistency. This may require a day or so with frequent stirring. Refrigeration may add to the shelf life of the solution.

Mounting Procedure

Keep in mind that cleanliness is important. First, lay down two pieces of lens tissue; one directly in front of you and the other to the left or right for the mounting tools. Next, wipe the scalpel, tweezers and glass rod or spatula with lens tissue. Holding a slide by the frosting, wipe it clean. Lay it down with the frosted end toward you. Even pre-cleaned slides must be cleaned this way. Hold a cover slip by the edges between the index finger and the thumb, and clean it in the same manner as the slide.

Place the cover slip so that one edge rests on the unfrosted end of the slide and the other edge rests on the lens tissue. Be sure that the lower surface does not come into contact with the lens tissue

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Using the glass rod, or dropper, dispense a small drop of the mounting media onto the center of the slide. Replace the rod or dropper into the solution bottle and cover it. Using the spatula, spread the solution into a triangular shape. While the fluid is spreading, cut a wedge of corresponding size from the filter. Place it upon the mounting media, sample side up. Pick up the cover slip and place it on top of the filter. Press lightly on the cover slip to be sure the mounting media has made contact with it. Label the slide before completing any other slide. Sometimes, after an hour or so, the filter may not have cleared. Usually, this can be cured by pressing lightly on the cover slip with a pencil eraser that has been wrapped with lens tissue. If the filter has been used to filter liquids or is damp, it will require drying, for it will not clear unless completely dry.

Use only enough solution to clear the filter. If too much solution is used the sample may spread and give an erroneous count per unit area. The filter should be counted as soon as possible because the solution may eventually form crystals that look like fibers and may be mistaken for part of the sample.

After a little practice and experience, several samples may be mounted at the same time with excellent results.

Remember to keep the area in which mounting is done as clean as possible to prevent contamination of the filter.

MICROSCOPE PRECAUTIONS AND MAINTENANCE

Quality lens tissue is a must. The microscopist should use it for any kind of lens cleaning. It should be as lint free as possible. It may be wrapped around the blunt end of a small camels hair brush and used to clean the eyepieces. Never wipe the inside of the microscope body. If it is necessary to remove dirt from the inside of the tubes, blow it out with

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Using the glass rod, or dropper, dispense a small drop of the mounting media onto the center of the slide. Replace the rod or dropper into the solution bottle and cove. it. Using the spatula, spread the solution into a triangular shape. While the fluid is spreading, cut a wedge of corresponding size from the filter. Place it upon the mounting media, sample side up. Pick up the cover slip and place it on top of the filter. Press lightly on the cover slip to be sure the mounting media has made contact with it. Label the slide before completing any other slide. Sometimes, after an hour or so, the filter may not have cleared. Usually, this can be cured by pressing lightly on the cover slip with a pencil eraser that has been wrapped with lens tissue. If the filter has been used to filter liquids or is damp, it will require drying, for it will not clear unless completely dry.

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an aspirator bulb. Whenever you are using immersion oil, be sure that you wipe all surfaces that were in contact with the oil.

Generally, solvents should not be used when cleaning lenses. Alcohol, like most solvents will dissolve the lens mounting cement, which may ruin the lens system. Xylene should be used sparingly to remove immersion oil.

Use a camels hair brush to clean first-surface mirrors and non-critical lens surfaces. Never wipe first-surface mirrors with lens tissue.

Avoid touching a lens surface with fingers as the fingerprint has a tendency to etch into the lens surface.

COUNTING PROCEDURE

Place the sample on the stage and focus on it. Since the sample is retained in the top ten to fifteen microns of the filter, be sure that you are focused in the proper plane. The reticle in the left eyepiece is used to define the counting area. The reticle should be calibrated prior to being used. The total fiber count (a fiber is anything three times as long as it is wide) should be at least 100 fibers, or twenty fields, whichever is less. It is advisable to make as large a count as practical to get an accurate average. If a fiber crosses the limits of the counting field, only those crossing either or both of two adjacent sides are counted. For example, a fiber crossing the top or right, or possibly both sides would be counted. The sides chosen are at the discretion of the counter, but any counter must always use the same sides. The fibers counted are estimated as to length, using the circles at the top of the reticle. Our practice has been to record all fibers seen, all fibers longer than five microns and all fibers longer than 10 microns. However, for comparison with the ACGYH TLV (1968), only the longer than 5μ fibers need be counted.

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FIELD MONITOR DISCUSSION

Membrane filters may be purchased pre-loaded in plastic holders (Aerosol Analysis Monitors). The Monitors consist of three sections. The filter is held between the middle and bottom sections. Between the membrane filter and the bottom section lies a support pad. The calibrated pump, designed to draw a known and constant supply of air, is always connected to the bottom of the field monitor case. When many loaded cases are taken into one particular area for sampling, a blank or control filter (meaning a loaded field monitor case) should be taken out to the area, brought back unused and counted. This will determine what degree of general contamination is present on the filters. We have found that banding the field monitor cases with cellulose bands greatly reduces the general contamination of the filters. The bands may be purchased from Walter H. Jelly and Company, Inc. They are white, opaque, size 41 x 25 and come packed in solution S-132. Upon removal from the solution, the bands are slipped around the middle and bottom joints, or around all joints, depending upon whether the sample is going to be drawn through the top section plug hole, or the entire top section removed for "open" sampling. The latter procedure is recommended for asbestos dust sampling since it results in a more even sample distribution over the entire exposed filter surface.

Convenient personal sampling pumps, such as those distributed by Mine Safety Applicances Co., Willson Products Division or Union Industrial are excellent for short term, low rate (around 2 lpm) sampling. A sampling pump is connected to a 3 foot length of non-collapsable, one quarter inch, rubber tubing with a male Leur slip adapter (stock number LH/L, available from Becton, Dickinson & Co.) inserted into the other end. This enables one to connect the hose directly to the case by simply inserting the adapter into the plug hole at the bottom of the case.

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Field monitor cases may easily be re-used. The plugs and all sections are separated and placed in warm detergent water. The parts should be scrubbed thoroughly and rinsed in tap water. However, if the filters are to receive any chemical analysis, the cases should also be rinsed in distilled water. The cases should be allowed to dry in a clean area, (a clean room is preferable), to reduce contamination. The clean area is equally important when loading the filters into the cases. The filter pad (the thicker, fibrous disc) is placed in the bottom section. The filter is placed directly on the pad. The middle and top sections are added, the case is pressed tightly together, and the top plug is inserted. Replacement of the bottom plug is not necessary. A cellulose band is then placed around the case and after the band dries, the unit is ready for sampling. Sample code numbers, etc. may be written on the band or upon the filter case, first making sure the markings can be removed when and if it is necessary.

Additional information concerning filters and field monitors may be obtained from Millipore Corporation.

CALCULATION OF CONCENTRATION

When a sample is taken, pertinent information about the sample should be recorded at the sampling site. First, the sample number should be written on the field monitor case and the sample ticket. Next, enter a description of his operation in the appropriate space. If the sample is a general air sample and does not pertain to any particular employee, write "general air sample" in the employee's name space. Information about the area may be written in the "description of operation" space. If you have several pumps which are identical in appearance, but have different flow rates, it would be a good idea to assign them instrument numbers. This enables you to keep accurate records of maintenance and calibration. When instrument

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numbers are kept it is not necessary to enter the flow rate at the sampling site. It may be entered later from the record cards. Otherwise, enter the flow rate at the sampling site. When a number of filters are taken into a general area a control (or blank) is taken along. This should be assigned a number and entered in the blank number space. Do not assign blank filters duplicate numbers.

When the sampling pump is turned on, enter the time in the "time on" space. While the sample is being taken make notes of controls, i.e., exhaust hoods, fans, window ventilation, etc. These notes should be entered in the "description of controls." The sampler should then sign his name or initials at the bottom of the sheet.

After sufficient time has passed, the sampling instrument should be turned off and the time recorded in the "time off" space. The difference between "time on" and "time off" should be expressed in minutes and should be entered in the "total time" space. The "total air volume" may be found by multiplying the flow rate (expressed in liters per minute) by the total time and recorded in the appropriate space.

After the sample has been taken, brought back into the lab and mounted, the counting procedure is next.

The microscope must be properly set up. Place the slide to be counted on the mechanical stage. Focus on the sample a short distance away from the edge. Never count close to the edges of the filter because the filter will spread a little and lower the count. Now, enter the date, sample number, blank number and field area on the count sheet. The counter should initial the form in the "initial" space. The sized fibers are entered from right to left across the form, first entering the $>10\mu$ fibers, then the $>5\mu$ fibers and finally, all fibers seen, called the total. After 100 fibers or 20 fields are counted and recorded, all of the vertical columns are added up and the totals are entered under their corresponding columns. Divide the

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totals at the bottom by the number of fields counted to determine the average number of fibers per size range per field. By using information on these two sheets the concentration in fibers per cubic milliliter may be calculated and entered in the "conc." space on the count sheet.

$$\text{Concentration} = \frac{(\text{Av. Fiber Cnt.})(\text{filter area})}{(\text{field area})\ (\text{Sample volume})\ (1000)}$$

Av. Fiber Count:	Average fiber count per size range
Filter area:	855 mm^2 for 37 mm dia. membrane filters
Counting area:	Reticle calibrated in mm^2
Sample volume:	Total time x flow rate, express in liters
1000:	Converts liters to milliliters

If blanks are used, subtract the average blank count of the same size range from the average fiber count.

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ADDRESSES

Millipore Field Monitor Cases (complete)
Cat. No. MAWP 037AO
Millipore filters (W. Pads)
Cat. No. AAWPO3700
Millipore Filter Corporation
Bedford, Massachusetts 07130

Portor Reticle
Cat. No. 30084
Patterson Globe & Circle Reticle
Cat. N. 30083
Edmund Scientific Co.
701 Edscorp Building
Barrington, New Jersey 08007

Wheaton Balsam Bottle
Cat. No. 2244
Arthur H. Thomas Co.
P. O. Box 779
Philadelphia, Pennsylvania 19105

Adapters, hose end for 1/4" tubing to male leur slip
Cat. No. LH/L
Becton, Dickinson and Company
Rutherford, New Jersey

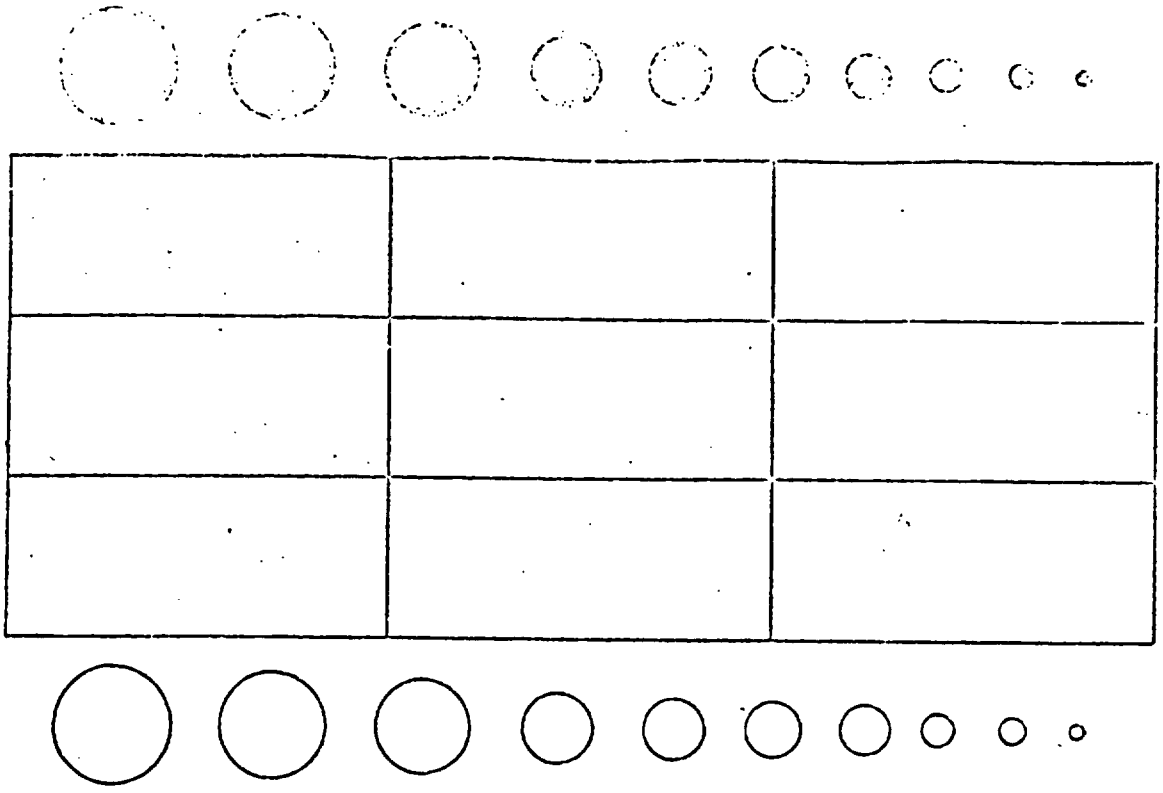
White, opaque, cellulose bands, 41 x 25
No. 28
Walter H. Jelly & Co., Inc.
2822 Birch Street
Franklin Park, Illinois 60131

Personal Sampler Mk. II
Willson Products Division
Reading, Pennsylvania

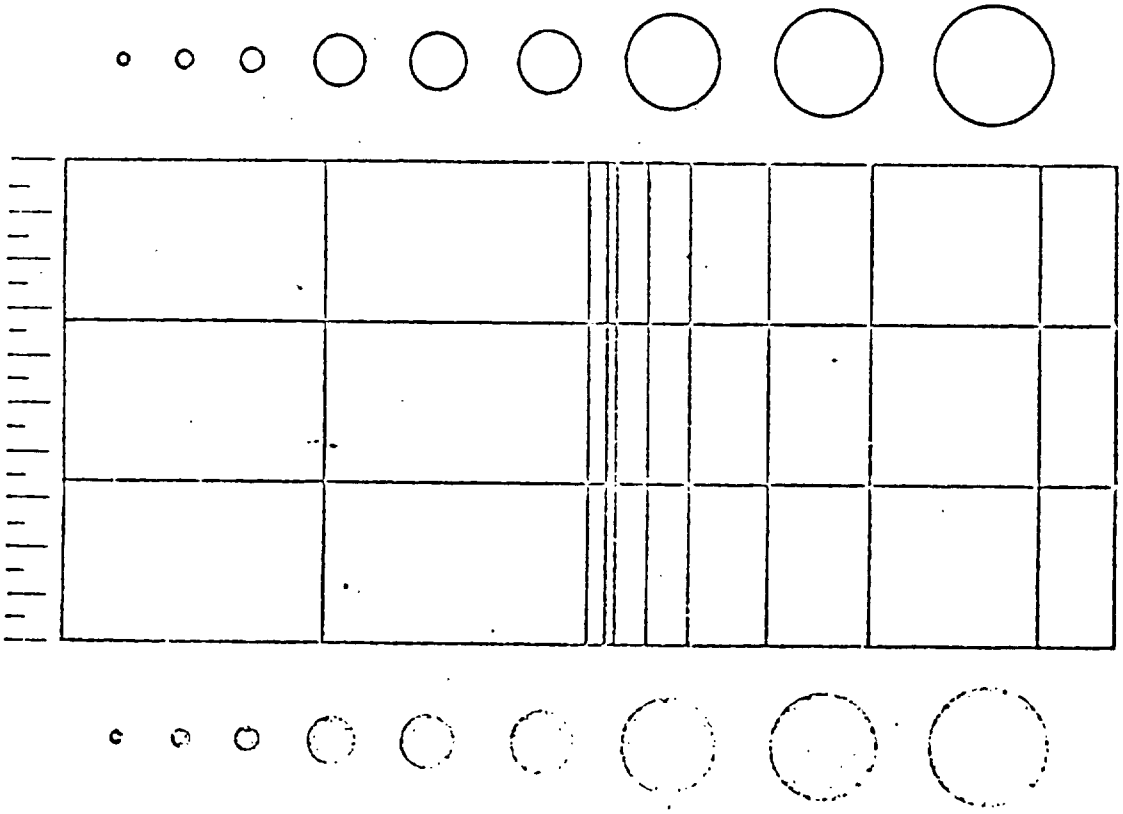
Monitaire Sampler
Mine Safety Appliances Co.
Pittsburgh, Pennsylvania

Unico Telematic Model C-110
Unico Environmental Industries, Inc.
Fall River, Massachusetts

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PATTERSON GLOBE AND CIRCLE



PORTON RETICLE

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SAMPLE _____ DATE _____

INST. _____ FLOW RATE _____ BLANK _____

TIME ON _____ TIME OFF _____

TOTAL TIME _____

TOTAL AIR VOLUME _____

EMPLOYEES NAME _____

JOB TITLE _____

DESCRIPTION OF OPERATION

DESCRIPTION OF CONTROLS

_____

INITIALS _____

SAMPLE TICKET

DATE _____ SAMPLE _____

FIELD AREA _____ BLANK _____

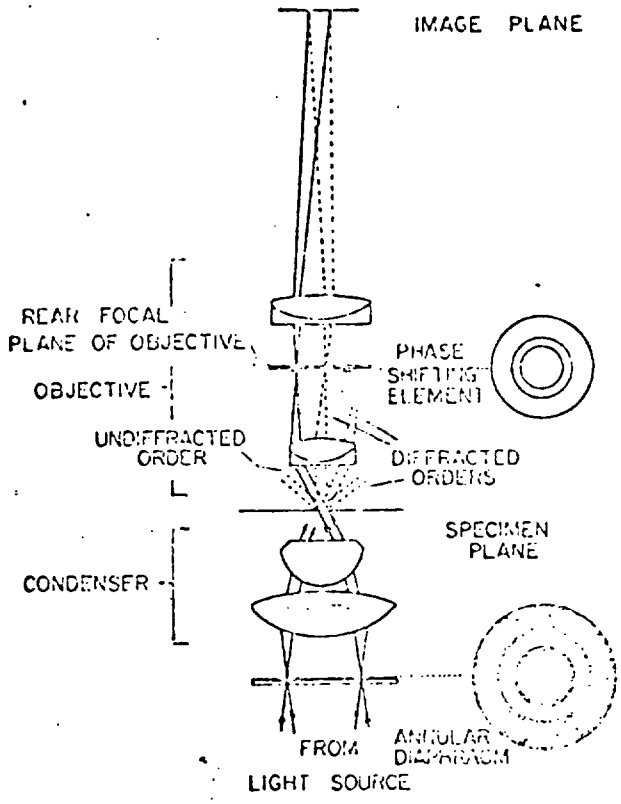
INITIALS _____ CONCENTRATION _____

	TOTAL	FIBERS >5 μ	>10 μ
TOT.			
AV.			

COUNT SHEET

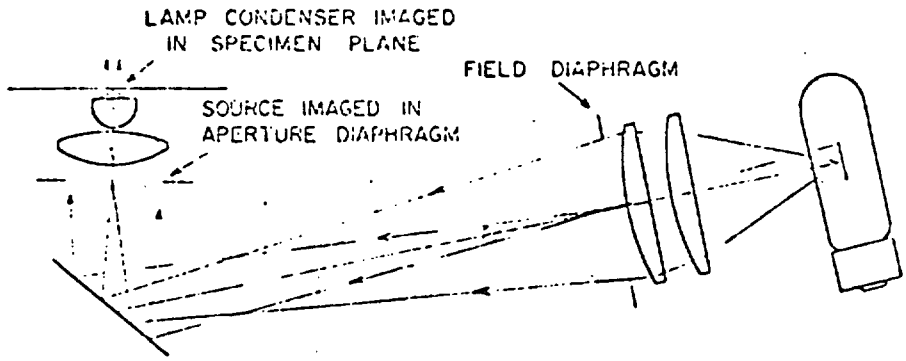
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PHASE CONTRAST MICROSCOPE

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KOEHLER ILLUMINATION

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