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(54) Title: METHODS AND DEVICES FOR QUANTITATING BLOOD SAMPLES

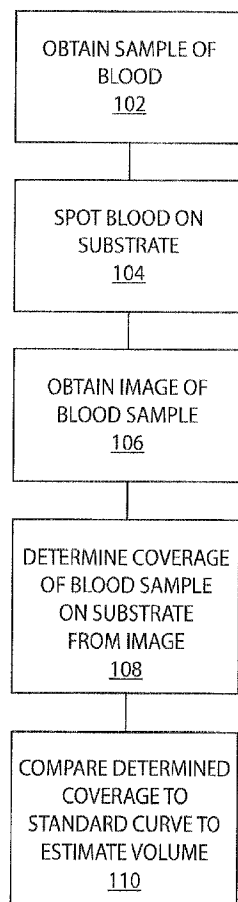


Fig. 2

(57) Abstract: Methods and devices for analysing blood sample volumes are provided. In particular, the disclosure provides a method for estimating the volume of a blood sample on a substrate including the steps of acquiring an image of the blood sample. A coverage or area of the blood sample may be obtained from the image and compared to a standard curve to obtain a volume estimate of the blood sample. The disclosure also proves a device for scanning one or more blood samples on a substrate. The device includes three layers that may be assembled to hold multiple samples between the layers. The device also includes labels and may be disassembled for decontamination and reloading of samples.



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1 **METHODS AND DEVICES FOR QUANTITATING BLOOD SAMPLES**

2 The present disclosure relates generally to methods and devices for blood
3 sampling, and more particularly to methods and devices for estimating blood sample
4 volumes. More specifically, the disclosure relates to a method for estimating the
5 volume of blood samples collected on a filter or other substrate. The disclosure also
6 more specifically relates to a device that may be used for holding filters and other
7 substrates for scanning images of the dried blood samples that will be used for
8 quantitating the collected blood.

9 Blood samples are routinely taken in clinics, hospitals or specialized labs by
10 trained professional for diagnostic purposes. A more cost effective and less invasive
11 alternative to traditional venipuncture method is collecting blood by finger stick on a
12 filter paper. The blood is then dried and sample known as dried blood spot (DBS) can
13 be stored or processed as required. The DBS can then be used for analysis of various
14 small molecules, metabolites, proteins etc. DBS is a powerful blood sampling
15 procedure as it allows collection of blood at any time or place. No special training is
16 required to collect the blood and the blood can be stored or shipped at ambient
17 temperature for a period of time.

18 One major drawback associated with DBS is the difficulty in quantitating the
19 analytes as the volume of blood loaded cannot be ascertained when directly loaded
20 onto the filter paper in non-lab settings. Volumetric application of blood is not
21 practical when collecting samples in the field. A number of factors influence the
22 spread of blood on a substrate like filter paper. The hematocrit of blood greatly
23 influences the spread of blood on the filter paper (higher hematocrit blood spreads
24 less compared to low hematocrit blood). Also there is differential spread of the blood
25 due to capillary effect (blood spreads more on thinner paper compared to thicker
26 paper). Finally, the chromatographic effect results in uneven distribution of blood
27 components (some blood components may move faster than others). So, one area of
28 the DBS may have different composition than another area. Therefore, the often used
29 practice of punching out specific sizes of blood spots may not be as accurate as
30 processing the whole blood sample entirely collected on the filter.

31 Different methods have been proposed to overcome the difficulty in
32 quantitating the analytes from DBS. One method proposes quantitating amount of

1 endogenous potassium levels to calculate the hematocrit of loaded blood. While this
2 method reports accurate estimation of hematocrit, it requires additional processing
3 and analysis of blood samples and filter paper. Another method uses diffuse reflection
4 to estimate the hematocrit of blood in the DBS to allow for sample volume correction.
5 While accurate, this method requires additional expensive lab equipment, sample
6 processing and specialized software to analyze the DBS.

7 Accordingly, there exists an urgent need in the relevant field for a technique
8 that provides accurate estimation of blood volume. Such techniques should be capable
9 of being performed in a cost effective manner, as the whole purpose of DBS
10 technology is to reduce the expenses associated with blood collection, storage and
11 shipment. Such techniques should also allow for estimation of the blood volume in the
12 entire spot, so that analytes can be quantitated accurately.

13 Furthermore, current devices known in the art used to scan blood samples,
14 including dried blood spots on filter paper, suffer from problems such as high risk of
15 contamination of samples, difficulty in keeping multiple samples organized, isolating
16 samples from human exposure, and limited ability to quickly, efficiently and
17 consistently process multiple samples or batches of samples. Accordingly, there also
18 exists a need in the relevant field for devices that overcome these limitations of the
19 prior art, including devices that may be used in conjunction with the novel techniques
20 disclosed herein.

21 Embodiments of the present disclosure provide methods and devices for
22 processing blood samples. In particular, the present disclosure provides novel
23 methods of estimating the volume of blood samples collected on filters and other
24 substrates in a consistent and accurate manner. This is essential for quantifying
25 analytes in blood samples collected in different settings, including non-laboratory
26 settings. The methods disclosed herein address the major technical problem of
27 quantification associated with an otherwise powerful blood sampling method that
28 allows collection, storage and transport of blood in the field in a minimally invasive
29 and cost-effective manner.

30 Accordingly, in one embodiment, the disclosure provides a method for
31 estimating the volume of blood samples comprising the following steps: obtaining a
32 sample of blood (e.g., by sticking a finger with lancet to get blood sample); spotting

1 the blood sample on a substrate; obtaining an image of the blood sample; determining
2 an approximate coverage or area of the image of the sample; and comparing the
3 determined approximate coverage or area to a standard curve to determine an
4 estimated volume of the blood sample.

5 In one embodiment determining the approximate coverage or area of the
6 image of the sample comprises calculating a coverage ratio of the blood sample on the
7 substrate, wherein calculating the coverage ratio of the blood sample on the substrate
8 preferably comprises counting pixels in the image of the blood sample.

9 In another embodiment calculating the coverage ratio of the blood sample on
10 the substrate comprises counting pixels in an image of a blank substrate, wherein
11 calculating the coverage ratio of the blood sample on the substrate preferably
12 comprises determining a ratio of the number of pixels counted in the image of the
13 blood sample to the number of pixels counted in the image of the blank substrate.

14 In one embodiment the standard curve comprises data from two or more blood
15 samples of known volumes plotted against data of approximate coverages or areas of
16 the two or more blood samples determined from images of the two or more blood
17 samples. In such embodiment the two or more blood samples of known volumes
18 preferably comprise samples with varying hematocrits.

19 In another embodiment the image of the sample of blood is obtained with a
20 scanner or a camera.

21 In yet another embodiment the substrate comprises a filter comprising paper.

22 The present disclosure also provides devices for holding blood samples
23 collected on filter or other substrates. The devices provide the benefits of ease of use,
24 simplified and more efficient decontamination, reduction of mistakes when handling
25 samples due to human error, the ability to easily log samples and keep records of
26 samples, and increased durability over previous devices. With respect specifically to
27 scanning of samples, the devices provide the advantages of the ability to fit virtually
28 any known scanner or similar imaging device, the ability to allow for uniform and
29 consistent sample spacing and scanning distance, and ease of cross-comparison
30 between different samples. Thus, the devices can provide the ability to perform more
31 efficient and accurate scanning in a reduced amount of time over previously known
32 devices.

1 Accordingly, in one embodiment, the disclosure provides a device for
2 scanning filters (dried blood samples) comprising: a first layer comprising one or
3 more transparent portions; a second layer comprising one or more holes, wherein said
4 one or more holes are formed through the second layer and are sized to each receive a
5 dried blood sample filter; and a third layer comprising one or more raised portions;
6 wherein said one or more transparent portions of the first layer overlap with the one or
7 more holes of the second layer and the one or more raised portions of the third layer
8 when the first, second and third layers are aligned and stacked on top of each other
9 with the second layer between the first and third layers.

10 In one embodiment each of the one or more raised portions of the third layer
11 fits into each of the one or more holes in the second layer. In such embodiment the
12 raised portions of the third layer preferably are sized to compress a dried blood spot
13 filter against the first layer and within a hole of the second layer when the first,
14 second and third layers are aligned and stack on top of each other with the second
15 layer between the first and third layers.

16 In one embodiment the first, second and third layers are configured to be
17 securely assembled to one another such that the second layer is positioned between
18 the first and third layers. In such embodiment the first, second, and third layers
19 preferably are secured by an attachment mechanism selected from the group
20 consisting of one or more screws, one or more bolts, one or more nails, a chemical
21 adhesive, a tape, one or more elastic bands, and combinations thereof.

22 In one embodiment at least one of the first, second or third layers comprises
23 plexiglass.

24 In one embodiment the first, second and third layers are substantially
25 rectangular in shape and substantially the same size.

26 In one embodiment the first layer is entirely transparent.

27 In one embodiment the one or more raised portions on the third layer comprise
28 acrylic discs.

29 In one embodiment the device further comprises one or more labels for
30 identifying the dried blood sample filters. In such embodiment the labels preferably
31 comprise one or more codes comprising one or more of letters, words, numbers,
32 colors, bar codes, and matrix bar codes, and/or are removable.

1 In yet another embodiment the one or more holes in the second layer are
2 uniformly sized and/or uniformly spaced apart from one another

3 The features, functions, and advantages that have been discussed can be
4 achieved independently in various embodiments of the present disclosure or may be
5 combined in yet other embodiments, further details of which can be seen with
6 reference to the following description and drawings.

7 *Other features, functions and advantages of the present disclosure will be or*
8 *become apparent to one with skill in the art upon examination of the following*
9 *drawings and detailed description. It is intended that all such additional systems,*
10 *methods, features, and advantages be included within this description, be within the*
11 *scope of the present disclosure, and be protected by the accompanying claims.*

12 Many aspects of the disclosure can be better understood with reference to the
13 following drawings. The components in the drawings are not necessarily to scale,
14 emphasis instead being placed upon clearly illustrating the principles of the present
15 disclosure. Moreover, in the drawings, like reference numerals designate
16 corresponding parts throughout the several views.

17 Fig. 1 shows blood spots of specific known volumes on filter paper.

18 Fig. 2 shows a flow diagram of an exemplary embodiment of a method for
19 estimating blood volume disclosed herein.

20 Fig. 3 shows images of blood spots obtained using a variety of backgrounds.

21 Fig. 4 shows two images of a dried blood spot with the spot pixels selected in
22 one image and the surrounding clear pixels selected in the other image.

23 Fig. 5 shows an exemplary graph of pixel coverage versus blood spot volume.

24 Fig. 6 shows exemplary data and corresponding standard curves of pixel
25 coverage versus blood volume generated for blood samples from three different
26 subjects.

27 Fig. 7 shows an exemplary bar graph of pixel coverage versus blood volume
28 generated from the combined results of blood samples from three different subjects.

29 Fig. 8 shows an exemplary standard curve of pixel coverage versus blood
30 volume generated from the combined results of blood samples from three different
31 subjects.

1 Fig. 9 shows a top view of an exemplary embodiment of a first layer of a plate
2 reader device disclosed herein.

3 Fig. 10 shows a perspective view of an exemplary embodiment of a first layer
4 of a plate reader device disclosed herein.

5 Fig. 11 shows a top view of an exemplary embodiment of a second layer of a
6 plate reader device disclosed herein.

7 Fig. 12 shows a perspective view of an exemplary embodiment of a second
8 layer of a plate reader device disclosed herein.

9 Fig. 13 shows a top view of an exemplary embodiment of a third layer of a
10 plate reader device disclosed herein.

11 Fig. 14 shows a perspective view of an exemplary embodiment of a third layer
12 of a plate reader device disclosed herein.

13 Fig. 15 shows a top view of an exemplary embodiment of labels of a plate
14 reader device disclosed herein.

15 Fig. 16 shows a top view of an exemplary embodiment of an assembled first
16 layer and second layer of a plate reader device disclosed herein.

17 Fig. 17 shows a top view of an exemplary embodiment of an assembled third
18 layer and labels of a plate reader device disclosed herein.

19 Fig. 18 shows a top view of an exemplary embodiment of an assembled plate
20 reader device disclosed herein.

21 In the following description, reference is made to the accompanying drawings,
22 which form a part hereof, and in which is shown, by way of illustration, various
23 embodiments of the present disclosure. It is understood that other embodiments may
24 be utilized and changes may be made without departing from the scope of the present
25 disclosure.

26 *Method for Estimating Volume of Blood Sample*

27 In a preferred embodiment, the methods provided herein may be used to
28 estimate the volume of blood in a dried blood spot on a paper filter or other substrate.
29 While not wishing to be bound by theory, it is believed that blood spread on filters
30 primarily is influenced by the capillary effect (i.e., the spread is inversely proportional
31 to the thickness of the paper), chromatographic effect (i.e., how fast or slow the blood
32 components spread through the filter) and the hematocrit (i.e., high hematocrit blood

1 spreads less and vice versa). In a preferred embodiment, the HemaForm™ filter (Spot
2 On Sciences, Inc.) was selected because its unique design allows spread of blood
3 evenly and the filter paper thickness is consistent. It is believed that this results in
4 more consistent blood sampling as a result of reduced hematocrit and
5 chromatographic effects. The results obtained were reproducible and consistent with
6 these filters; however, this technology may be applied to other types of filters too.

7 The present disclosure is based, in part, on the hypothesis that the spread of
8 the blood on the filter would be proportional to the volume of blood being spotted.
9 We can exploit this property to measure the volume of blood. First, specific
10 incremental volumes of fresh blood were spotted on different HemaForm™ filters and
11 visually analyzed after overnight drying. Fig. 1 depicts eight different HemaForm
12 filters with varying known volumes of blood spotted on the filters. Specifically, the
13 known volumes of blood spotted increase from left to right, top to bottom in the
14 images in Fig. 1. Thus, a quick visual inspection of the spotted filters reveals that the
15 spread of the blood on the filter is proportional to the volume of the blood spotted on
16 it. As this confirmed the correctness of the hypothesis, the next step was to actually
17 measure the spread of blood. Since there are no methods currently available to
18 correlate the spread to the volume of blood in a dried blood spot, it therefore became
19 necessary to develop a novel analytical tool that would enable the spread to be
20 quantitated.

21 Our method allows consistent and accurate estimation of blood volume loaded
22 onto a substrate (e.g., filter paper), which is essential for quantifying analytes in blood
23 samples collected in non-lab settings. This method addresses a major technical
24 problem of quantification associated with an otherwise powerful blood sampling
25 method that allows collection, storage and transport of blood in the field in a
26 minimally invasive and cost-effective manner.

27 Fig. 2 is a flow chart of an exemplary embodiment of the present invention,
28 wherein a method 100 is provided for estimating the volume of a blood sample. It
29 should be noted that any process descriptions or blocks in flow charts should be
30 understood as representing modules, segments, portions of code, or steps that include
31 one or more instructions for implementing specific logical functions in the process,
32 and alternate implementations are included within the scope of the present disclosure

1 in which functions may be executed out of order from that shown or discussed,
2 including substantially concurrently or in reverse order, depending on the
3 functionality involved, as would be understood by those reasonably skilled in the art
4 of the present disclosure.

5 As is shown in block 102 of Fig. 2, a sample of blood is obtained from a
6 human or animal patient by any method known in the art. This may include traditional
7 venipuncture, wherein blood is obtained directly from a patient's vein. The blood may
8 also be collected via a finger prick or via a "prick" of any other part of the patient's
9 body. Blood collected via this method is typically obtained from blood capillaries
10 near the surface of the skin by piercing the skin with a lancet or similar device. The
11 blood from the "finger prick" may be collected into a capillary tube and then
12 dispensed onto a filter or directly spotted onto the filter paper.

13 As is shown in block 104 of Fig. 2, the sample of blood obtained or a portion
14 of the sample of blood obtained is spotted or otherwise placed onto a substrate. The
15 substrate may be any material known in the art that is capable of retaining a sample of
16 blood. In a preferred embodiment, a paper filter may be used as the substrate. Paper
17 filters, including Guthrie cards, HemaForm filters and others, are well known in the
18 art for their use in dried blood spot sampling. In certain embodiments, the substrate
19 may be a composite material and/or may be coated, for example, with silica.

20 In block 106 of Fig. 2, an image is obtained of the spotted blood sample. The
21 image may be obtained with any imaging device known in the art, preferably after the
22 blood has dried completely. Thus, a camera, a scanner, or other similar imaging
23 devices may be used successfully with the disclosed methods. Selection of an
24 appropriate imaging device may include such considerations as ease of use, the need
25 for a fixed platform for acquiring multiple images, the ability to process multiple
26 filters, image quality, and the ability to control and managing various imaging settings
27 and controls.

28 In one embodiment, a camera may be used to obtain the image. Typically, the
29 camera will be mounted on a tripod or other device to hold it steady and to obtain
30 images that are consistent and reproducible. In an alternative embodiment, a scanner
31 may be used to obtain the image. For example, an HP Photosmart 1300 or other
32 similar device may be used to obtain the image. For any imaging device used, it may

1 be beneficial to adjust the resolution of the acquired image to a preferred or
2 standardized resolution. For example, a resolution of 600 dpi may be used. Resolution
3 may also be controlled and adjusted via software, as is discussed below. The image
4 obtained may be provided in a digital format such that it may be viewed on a
5 computing or other electronic device. This further allows the image to be viewed with
6 image editing or image analysis software.

7 In block 108 of Fig. 2, an approximate coverage or area of the blood sample
8 on the substrate is determined from the image of the blood sample. For purposes of
9 this disclosure, the coverage of the blood sample may be measured or estimated in
10 terms of absolute area or, alternatively, as a ratio of areas, such as percent coverage.
11 In a preferred embodiment, the determination of the coverage may be performed by
12 counting pixels in the image. This is preferably done with the use of image analysis
13 or image editing software. Numerous software programs for this purpose are known
14 in the art (e.g., GIMP2, Adobe Photoshop).

15 Prior to obtaining a pixel count, various settings of the software may need to
16 be adjusted to obtain an optimal image for counting the pixels. For example, it may be
17 beneficial to adjust the resolution of the images. Using a fixed resolution across all
18 images may help to ensure accurate results. Further optimization can be carried out by
19 choosing the right threshold for pixel selection with the GIMP2 image analysis
20 program. A lower threshold may often lead to non-specific selection, whereas a higher
21 threshold may interfere with color selection of pixels.

22 It may also be beneficial to optimize resolution in order to minimize the
23 shadow interference in the images. Shadows observed along peripheries of the
24 substrate (e.g., shadows along the “petals” of the HemaForm™ filter) may interfere
25 with pixel selection during pixel analysis and counting. Alternatively, or in addition to
26 adjusting settings in the scanning software for this purpose, a variety of backgrounds
27 may be used in the images in order to minimize these effects, as is shown in Fig. 3.

28 The software may be utilized to count only those pixels that correspond to the
29 portion of the image that actually depicts the blood spot. Thus, any pixel that covers
30 or at least partially overlaps with a blood spot may be counted. Alternative
31 methodologies for determining whether an individual pixel should be counted may be
32 used, so long as the methodology is consistent with respect to other images or data to

1 which the image may be compared. In certain embodiments, the absolute number of
2 pixels that correspond to the blood spot may be used to determine the area of the
3 blood spot. The pixels representing the blank areas of the filter (i.e., the portions of
4 the filter to which the blood has not spread) may also be counted. From this additional
5 count, a total number of pixels may be acquired for use in the area or coverage
6 calculation or, by adding to the pixels counted in the blood spot and comparing to a
7 known number of pixels corresponding to an entire blank filter, to ensure accurate and
8 precise pixel counting.

9 In alternative embodiments, a “percent coverage” area may be determined, for
10 example, by additionally counting the pixels representing a blank substrate (e.g.,
11 filter) in an image of the blank substrate. It is important that the image of the blank
12 substrate be acquired with the same settings (e.g., resolution) as the image of the
13 blood spot for accurate and meaningful comparison of the two images. To obtain a
14 percent coverage calculation, a ratio of the number of pixels counted in the blood spot
15 to the number of pixels counted in the blank filter is determined.

16 As is shown in block 110 of Fig. 2, the coverage or area of the blood spot
17 determined in block 108 may be compared to a standard curve, table, chart or other
18 data to calculate the volume of the blood spot. Such a standard curve, table, chart or
19 other data may include data on blood samples with known volumes. For example, a
20 series of blood samples of different known volumes may be spotted separately on
21 substrates. Images of each of the spotted blood samples may be obtained, along with
22 area or percent coverage calculations obtained from the images. These calculated
23 areas or percent coverages may then be plotted against or otherwise compared to the
24 known volumes to obtain a standard curve, table, chart, etc. A best-fit line may be
25 used to assist with comparing the data. For any subsequently obtained blood samples,
26 an estimation of the volume of the blood sample may be determined by comparing the
27 area of percent coverage of the blood sample to the standard curve or other data
28 generated in block 110. It may further be necessary to update the data in the standard
29 curve as necessary to ensure its accuracy. Moreover, it may be desirable that the
30 known samples used to generate the standard curve vary in other characteristics, for
31 example, hematocrit, to correct for possible effects of such characteristics on the
32 spreading area of the blood sample on the substrate.

1 Example 1: Generation of a Standard Curve

2 A standard curve may be generated by the following exemplary method. A
3 series of exact known volumes of fresh blood were spotted onto HemaForm™ filters
4 and dried overnight. For imaging, a camera was fixed onto a tripod. The camera
5 settings were adjusted for optimum resolution, and all of the images were then taken
6 keeping the settings fixed. Pixels were counted using the image analysis program
7 GIMP2. *This software is an ideal choice because it is freely available from the*
8 internet and it has proven successful in carrying out analyses (though other image
9 analysis programs like Adobe Photoshop etc. may work equally well). For reference,
10 an image of a blank HemaForm™ filter was acquired, and the total number of pixels
11 corresponding to the blank filter was determined from the image. The pixels in the
12 dried blood spot and the surrounding empty (clear) region of the filter around the
13 blood spot were also selected and counted, as is shown in Fig. 4.

14 Next, the pixel coverage was determined by dividing the pixels in the spot by
15 the total pixels in the blank filter. Fig. 5 shows the results of the pixel covered vs.
16 volume of blood spotted. The slope of a best-fit line fitted to the data points was
17 determined, as is also shown in Fig. 5. To test the accuracy of the line in Fig. 5,
18 estimations of the blood sample volumes obtained from the slope of the line were then
19 compared to actual known volumes. Our volume estimations were within 1-5 µl of the
20 actual volumes spotted, thereby demonstrating the efficacy of the method. However,
21 with additional refinements to the analysis, even greater accuracy may be achieved.

22 Example 2: Generation of a Combined Standard Curve

23 A combined standard curve may be obtained by the following exemplary
24 method. A scanner (e.g., HP Photosmart 3100) was selected based on the
25 considerations mentioned above. Image resolution was also optimized with the
26 GIMP2 image analysis program to improve the quality of pixel analysis and counting.

27 Bloods from three different individuals varying in age, gender and hematocrit
28 was analyzed to account for the differences in the spread of blood. Fresh blood from
29 the three subjects was spotted on filters at known different volumes. Images of these
30 spotted blood samples were acquired and used to calculate the pixels covered. From
31 this data, three separate standard curves were generated, as is shown in Figs. 6 and 7.
32 A combined standard curve, shown in Fig. 8, was also generated from which the slope

1 was determined. The combined curve was used to determine the volumes of unknown
2 samples.

3 Prediction accuracy when using the methods disclosed herein is quite high,
4 and estimated volumes may generally fall within 2-3 μ l or less of the actual volumes.
5 Hematocrit was found to have an effect on the obtained measurements. Thus, for very
6 low and very high hematocrit levels, the calculated volume may generally fall within
7 5% the actual volume spotted. Blood lower in hematocrit may tend to spread further
8 because there is a greater amount of plasma in a given volume. Conversely, a higher
9 hematocrit blood may typically spread less due to a lower amount of plasma in a
10 given sample size. Thus, measurements obtained using the disclosed methods may
11 directly correlate with the volume of plasma in the blood. Further, metabolite levels
12 measured from whole blood provides a more accurate measure of disease state or
13 progression and also allow for a more consistent comparison with plasma.

14 Validation of the accuracy of the standard curve may be accomplished by
15 spotting known volumes on the filter. Blinded analyses are typically performed in
16 these cases, such that the volumes are not known to the person carrying out the
17 volume analysis. Table-1 shows a sample of some volumes that were analyzed by
18 blinded analysis. As can be seen from Table 1, all estimated volumes fall within 0.2 –
19 3.5 μ l of the actual known volumes.

Subject	Calculated volume using image analysis(μ l)	Actual volume loaded on filter(μ l)
1	8.64	9.00
2	11.3	10.00
3	19.26	20.00
4	18.89	20.00
5	36.5	33.00
6	43.06	40.00
7	43.1	40.00
8	48.46	50.00
9	49.80	50.00
10	52.11	50.00

Table-1

1 Example 3: Method for Estimation of Blood Volume

2 In a preferred embodiment, estimation of blood volume may be performed as
3 follows. This technique is based on calculating the pixels in the DBS image. The
4 volume of blood in the spot is measured by comparing its pixels-against-pixel count
5 to a standard curve of DBS of known volumes. The step by step procedure is
6 described below:

7 Step 1: Pixel calculation

8 (a) Scanning DBS

9 To count the pixels in a DBS, the first step is to scan the DBS. DBS samples
10 are scanned along with a blank filter (on which blood has not been spotted) on the
11 scanner. The scanning resolution is set to a fixed setting –this can be any setting but
12 once chosen, should be kept same for all analyses. For example, a resolution of 600
13 DPI may be used.

14 (b) Image analysis using GIMP 2 image analysis software

15 This software is available for free download from the internet. The scanned
16 DBS is cropped using the selection tool and the pixels are separately counted for the
17 exact blood spot area and the blank area. Pixels are also counted for a blank filter
18 likewise.

19 (c) Actual pixel coverage

20 Percent pixels in the spot are calculated by determining the ratio of pixels in
21 the spot to the overall pixels in the blank filter.

22 Step 2: Preparation of the Standard Curve

23 Collect blood from different individuals (ideally with a varying hematocrit) in
24 heparin/EDTA tubes. The freshly collected blood is then spotted in exact known
25 volumes ranging from 5 to 60 μ l individually (in triplicate samples) to prepare DBS
26 of known volumes. These are left to dry overnight followed by scanning as described
27 in (1) above. The pixel coverage is calculated and the standard curve is created for the
28 pixel coverage against the volume. The slope is determined for the standard curve.

29 Step 3: Calculation of Blood Volume in the DBS

30 Pixel coverage is calculated for a DBS of unknown volume as described in
31 step (1) above and the volume is calculated on the basis of the slope determined as
32 discussed in step (2).

Plate Reader Device

The devices disclosed herein may be used for holding filters on which blood samples have been collected (e.g., DBS). In a preferred embodiment, this disclosure provides a device for scanning or imaging multiple blood samples quickly and without cross-contamination and with minimal scanning-related shadow artifacts. The device also provides the benefits of keeping samples organized, holding them securely in place for scanning, and isolating the sample from human exposure as much as possible. Additional advantages of the device include that it may fit virtually any scanner, is easy to use, and is durable. Because it may keep sample space and scanning distances uniform, it will allow for cross-comparison of samples scanned from one batch to the next. This may greatly reduce time spent trying to get samples scanned exactly the same way or having to adjust algorithms to calculate volumes and other characteristics of blood samples. The device is made of separate components that may be assembled for scanning and disassembled for decontamination and sample preparation. The device may also assist in logging and identification of samples as it can keep a record of which samples have been scanned via an incorporated label template.

18 In another embodiment, the present disclosure provides a device for scanning
19 dried blood spots on a filter that may be utilized with all methods disclosed herein. In
20 a preferred embodiment, the device enables scanning of multiple dried blood spots
21 simultaneously. As is depicted in Figs. 9-15, the device may include up to four parts
22 that may be assembled or otherwise positioned together.

Figs. 9-10 depict an exemplary embodiment of a first layer 10 of the device. The first layer 10 typically will comprise a flat or substantially flat piece of material. The first layer 10 may include a first surface 14 and a second surface 16 on opposing sides of the layer. Preferably, the first layer 10 will be of uniform thickness. In a preferred embodiment, the first layer may comprise a sheet of plexiglass. The first layer 10 may include one or more transparent portions 12. The one or more transparent portions 12 may be of the same or varying sizes, shapes, diameters, etc. The spacing of the transparent portions 12 may be uniform in one or more dimensions of the first layer 10. Alternatively, the transparent portions 12 may be randomly or irregularly spaced. In certain embodiments, the first layer may be entirely transparent.

1 A second layer 20 of the device is shown in Figs. 11-12. The second layer will
2 typically comprise a flat or substantially flat piece of material. The second layer 20
3 may include a first surface 24 and a second surface 26 on opposing sides of the
4 second layer. Preferably, the second layer 20 will be of substantially uniform
5 thickness. In a preferred embodiment, the second layer 20 may comprise a sheet of
6 plexiglass. One or more holes 22 are formed in second layer 20. The holes may
7 extend entirely through second layer 20 (i.e., extend from a first surface 24 to a
8 second surface 26), or alternatively may only extend partially through second layer
9 20. The one or more holes 22 may be of the same or varying sizes, shapes, diameters,
10 etc. The spacing of the holes 22 may be uniform in one or more dimensions of the
11 second layer 20. Alternatively, the holes 22 may be randomly or irregularly spaced.

12 In a preferred embodiment, the holes 22 should be sufficiently sized such that
13 each hole may receive a dried blood sample, as is shown in Fig. 16. Typically, such a
14 blood sample will be contained on a paper filter 28 or other similar substrate. Thus,
15 each of the one or more holes 22 should be large enough that a filter 28 or similar
16 substrate containing a blood sample can lay flat within the hole. In a preferred
17 embodiment, the dimensions of the hole will closely match or be slightly larger than
18 the dimensions of the filter or substrate such that any movement or shifting of the
19 filter or substrate within the hole will be limited or eliminated.

20 Figs. 13-14 show a third layer 30 of the device. The third layer 30 will
21 typically comprise a flat or substantially flat piece of material. The third layer 30 may
22 include a first surface 34 and a second surface 36 on opposing sides of the third layer.
23 Preferably, the third layer 30 will be of substantially uniform thickness. In a preferred
24 embodiment, the third layer 30 may comprise a sheet of plexiglass. This layer
25 includes one or more raised portions 32 extending upward from a first surface 34 of
26 the third layer. The raised portions 32 may be of any size, shape or color. They may
27 be formed integrally from the third layer 30 or may alternatively be separate
28 components that are attached to the third layer 30 via any adhesive mechanism known
29 in the art. The raised portions 32 may comprise any suitable material, including
30 acrylic, plastics, metals, etc. In a preferred embodiment, the raised portions 32 may
31 comprise acrylic discs. In a preferred embodiment, the raised portions 32 may be

1 sized such that each raised portion fits into a hole 22 in the second layer 20 when the
2 second and third layers are aligned and stacked on top of each other.

3 Fig. 15 shows one or more labels 40 that may be used with the device. The one
4 or more labels may each be separate, or, alternatively may be included on a template
5 42 of multiple labels. In either case the labels should be removable from the device.
6 Thus, the one or more labels, or a template containing the labels, may be configured
7 *to lie over or rest upon one or more layers of the device. Alternatively, the one or*
8 *more labels, or a template containing the labels, may include an adhesive that permits*
9 *both secure attachment of the labels to one or more layers of the device, as well as*
10 *removal of the labels from the device. The labels and/or template containing the labels*
11 *may comprise any suitable material, including paper, transfer plastic, cardboard,*
12 *plexiglass, etc. The labels may comprise a code 44 for identifying a specimen (e.g.,*
13 *blood sample, filter, substrate, etc.) held within the device. One or more types of*
14 *codes 44 may be used, including letters, words, numbers, colors, bar codes, matrix bar*
15 *codes, or any combination thereof.*

16 In a preferred embodiment, each of the first, second and third layers will
17 comprise a substantially similar size and shape. Preferably, the layers will be sized
18 such that they may be used in conjunction with a standard scanner or other imaging
19 device. Thus, in certain embodiments, the layers may be substantially the same length
20 and width as a piece of typing paper. Such a size permits the device to sit on the bed
21 of a scanner such that any specimens within the device may be scanned. The first,
22 second and third layers may comprise the same or different materials. Preferably, the
23 layers will comprise plexiglass or other plastics.

24 The layers, as well as the labels, are configured to be assembled together to
25 form the device 1, as is shown in Figs. 16-18. The layers, when assembled, may
26 simply be aligned and stacked such that the length and width of each layer aligns or
27 substantially aligns with the lengths and widths of the other layers. Alternatively, the
28 layers may be securely assembled by an attachment mechanism, including screws,
29 bolts, nails, chemical adhesives, tape, elastic bands, or combinations thereof. In one
30 embodiment, the raised portions 32 on the third layer 30 fit tightly into the holes 22 in
31 the second layer such that the layers are held snug. The assembly may also be
32 securely held on the edges with tape.

1 When the device is assembled and all parts are aligned, the various
2 components of each layer should also align with one another. That is, the transparent
3 portions 12 of the first layer 10 should align with the holes 22 of the second layer 20,
4 the raised portions 32 of the third layer 30, and the labels 40. The layers will typically
5 be stacked with the second layer between the first and third layers. Thus, each of the
6 one or more raised portions 32 of the third layer 30 will fit into a hole 22 of the
7 second layer, while *each one or more transparent portions 12 of the first layer will*
8 cover the holes 22 on the opposite surface of the hole from the surface where the
9 raised portions are inserted. Further, when the device is assembled and a filter or other
10 substrate is also inserted in a hole (as is shown in Fig. 16), the raised portions will
11 hold the filter within the hole and compress it against a transparent portion of the first
12 layer (as is shown in Fig. 18). Thus, the raised portions 32 preferably will extend the
13 full depth of the hole or nearly the full depth of the hole. Further, the insertion of the
14 raised portions in the holes also provides a secure assembly of the device by limiting
15 relative movement of the second and third layers.

16 When assembled, the labels 40, or a template 42 containing the labels, will
17 typically be attached to or otherwise lie adjacent to the third layer, as is shown in Fig.
18 18. However, the labels or template may also be attached, adhered, or otherwise lie
19 adjacent to the first or second layers also.

20 Example 4: Exemplary Characteristics of Device Components

21 In an exemplary embodiment, the components of the device may have the
22 following characteristics. The first layer comprises an 8x11" piece of plain plexiglass
23 (Fig. 9). The second layer comprises an 8x11" piece of plain plexiglass with 3/4" holes
24 drilled into it (Fig. 11). The third layer comprises an 8x11" piece of plain plexiglass
25 with 3/4" colored acrylic discs attached to it that correspond to the 3/4" holes drill in the
26 second layer (Fig. 13). The labels are included on a removable template (comprising
27 paper and/or transfer plastic) for labeling the samples (Fig. 15). The labels comprise a
28 number system.

29 Example 5: Assembly of the Device

30 In an exemplary embodiment, the components of the device are assembled as
31 follows. The first and second layers are attached together in such a way that the
32 transparent portions of the first layer correspond with the holes in the second layer

1 (Fig. 16). A blank piece of filter paper is placed a hole of the second layer and the
2 remaining holes are filled with filters containing dried blood spots. The third layer and
3 label template are assembled (Fig. 17) and placed on top of the first and second
4 layers, such that each label corresponds to the appropriate filter or blood sample.
5 Also, the raised portions (acrylic discs) of the third layer compress the filters down
6 within each hole so than an accurate scan can be made of 100% of the surface area of
7 each *specimen*. The whole assembled device (Fig. 18) is then placed in the scanner
8 and scanned.

9 It should be emphasized that the above-described embodiments of the present
10 disclosure, particularly, any “preferred” embodiments, are merely possible examples
11 of implementations, merely set forth for a clear understanding of the principles of the
12 disclosure. Many variations and modifications may be made to the above-described
13 embodiment(s) of the disclosure without departing substantially from the spirit and
14 principles of the disclosure. All such modifications and variations are intended to be
15 included herein within the scope of this disclosure and the present disclosure and
16 protected by the following claims.

17

CLAIMS

2 What is claimed is:

1. A method for estimating the volume of a blood sample comprising the following steps:

5 obtaining a sample of blood;

6 spotting the sample of blood on a substrate;

7 obtaining an image of the sample of blood;

8 determining an approximate coverage or area of the image of the sample; and

9 comparing the determined approximate coverage or area to a standard curve to

10 determine an estimated volume of the sample of blood.

2. The method of claim 1, wherein determining the approximate coverage or area of the image of the sample comprises calculating a coverage ratio of the blood sample on the substrate, wherein calculating the coverage ratio of the blood sample on the substrate preferably comprises counting pixels in the image of the blood sample.

3. The method of claim 1 or claim 2, wherein calculating the coverage ratio of the blood sample on the substrate comprises counting pixels in an image of a blank substrate, wherein calculating the coverage ratio of the blood sample on the substrate preferably comprises determining a ratio of the number of pixels counted in the image of the blood sample to the number of pixels counted in the image of the blank substrate.

4. The method of claim 1, wherein the standard curve comprises data from two or more blood samples of known volumes plotted against data of approximate coverages or areas of the two or more blood samples determined from images of the two or more blood samples and wherein the two or more blood samples of known volumes preferably comprise samples with varying hematocrits.

26 5. The method of any one of claims 1-4, characterized by one or more of the
27 following featuers:

28 (a) wherein the image of the sample of blood is obtained with a scanner or a
29 camera; and

30 (b) wherein the substrate comprises a filter comprising paper.

31 6. A device for scanning filters for dried blood samples comprising:

32 a first layer comprising one or more transparent portions;

1 a second layer comprising one or more holes, wherein said one or more holes are
2 formed through the second layer and are sized to each receive a dried blood sample
3 filter; and

4 a third layer comprising one or more raised portions;

5 wherein said one or more transparent portions of the first layer overlap with the
6 one or more holes of the second layer and the one or more raised portions of the third
7 layer when the first, second and third layers are aligned and stacked on top of each
8 other with the second layer between the first and third layers.

9 7. The device of claim 6, wherein each of the one or more raised portions of the
10 third layer fits into each of the one or more holes in the second layer.

11 8. The device of claim 7, wherein the raised portions of the third layer are sized
12 to compress a dried blood spot filter against the first layer and within a hole of the
13 second layer when the first, second and third layers are aligned and stack on top of
14 each other with the second layer between the first and third layers.

15 9. The device of any one of claims 6-8, wherein the first, second and third layers
16 are configured to be securely assembled to one another such that the second layer is
17 positioned between the first and third layers.

18 10. The device of claim 9, wherein the first, second, and third layers are secured
19 by an attachment mechanism selected from the group consisting of one or more
20 screws, one or more bolts, one or more nails, a chemical adhesive, a tape, one or more
21 elastic bands, and combinations thereof.

22 11. The device of any one of claims 6-10, characterized by one or more of the
23 following features:

24 (a) wherein at least one of the first, second or third layers comprises
25 plexiglass;

26 (b) wherein the first, second and third layers are substantially rectangular in
27 shape and substantially the same size;

28 (c) wherein the first layer is entirely transparent; and

29 (d) wherein the one or more raised portions on the third layer comprise acrylic
30 discs.

31 12. The device of any one of claims 6-11, further comprising one or more labels
32 for identifying the dried blood sample filters.

1 13. The device of claim 12, wherein the labels comprise one or more codes
2 comprising one or more of letters, words, numbers, colors, bar codes, and matrix bar
3 codes.

4 14. The device of claim 12, wherein the one or more labels are removable.

5 15. The device of any one of claims 6-14, wherein the one or more holes in the
6 second layer are uniformly sized and/or uniformly spaced apart from one another.

7

1/15

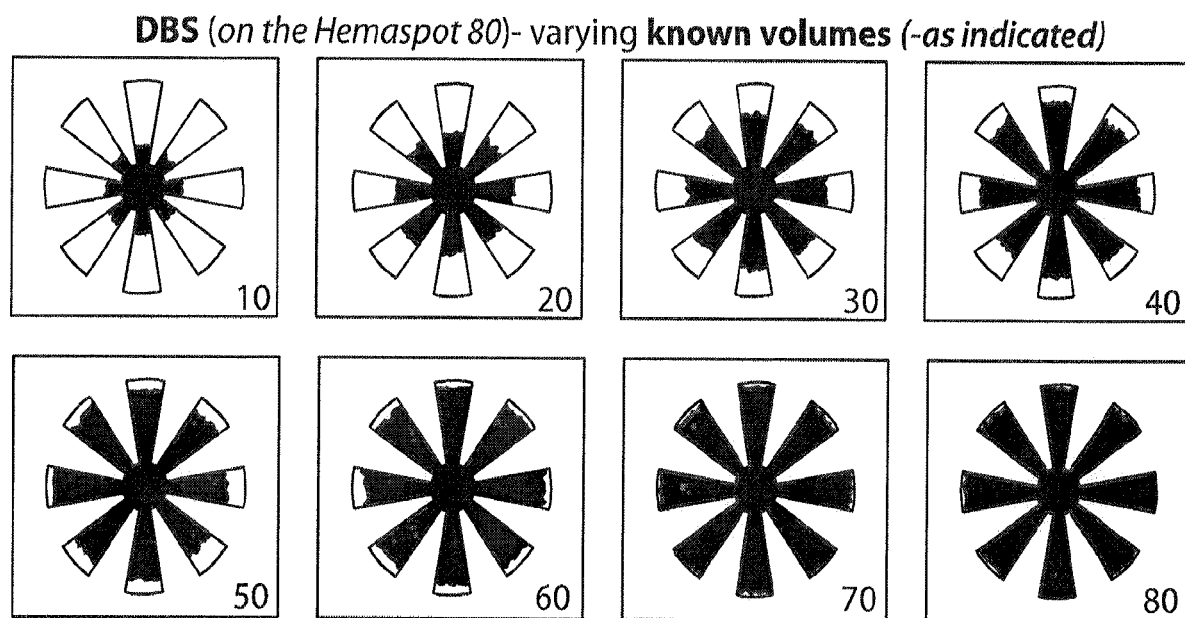


Fig. 1

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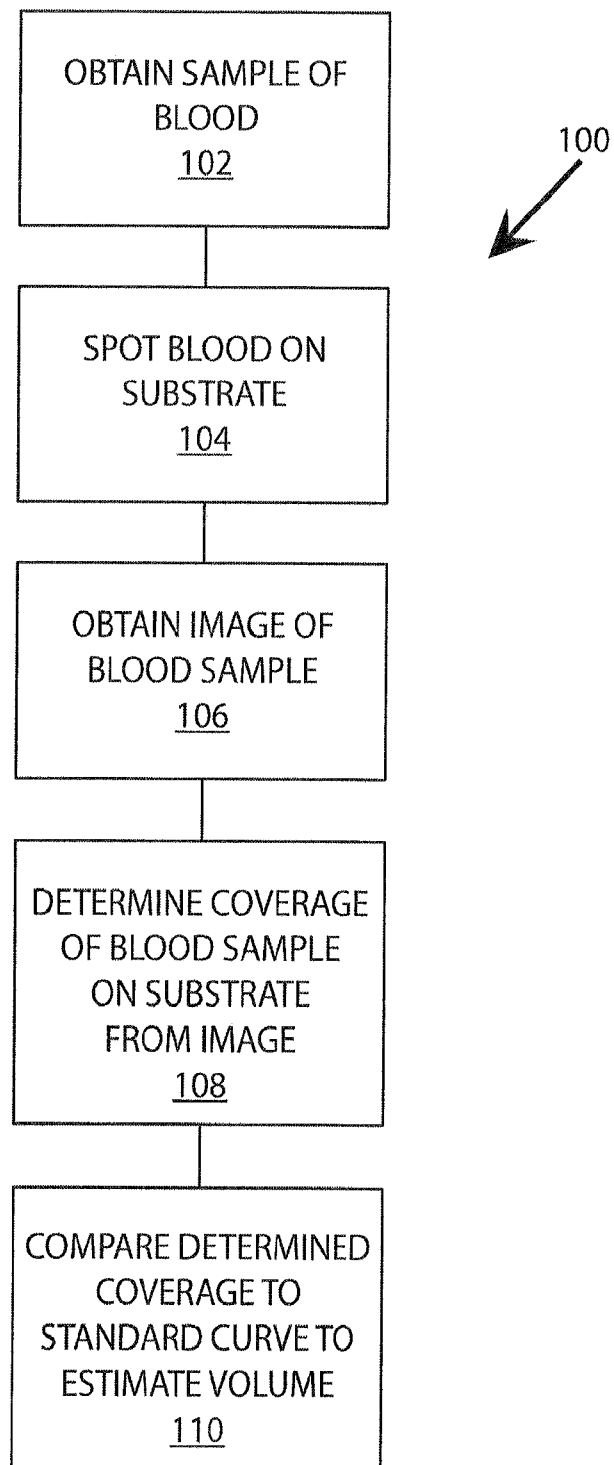


Fig. 2

3/15

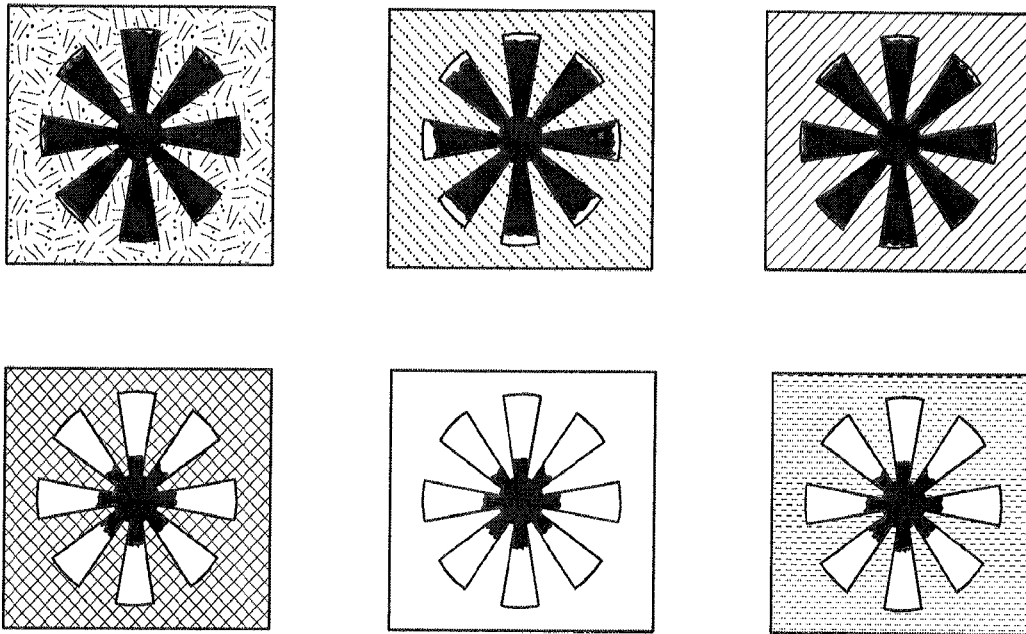


Fig. 3

4/15

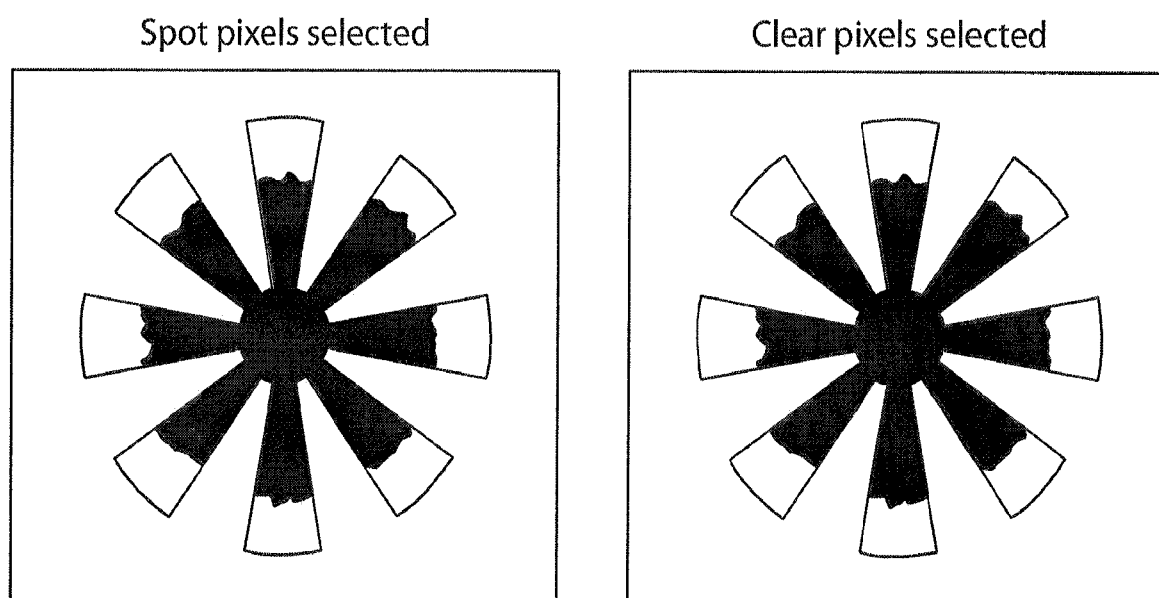


Fig. 4

5/15

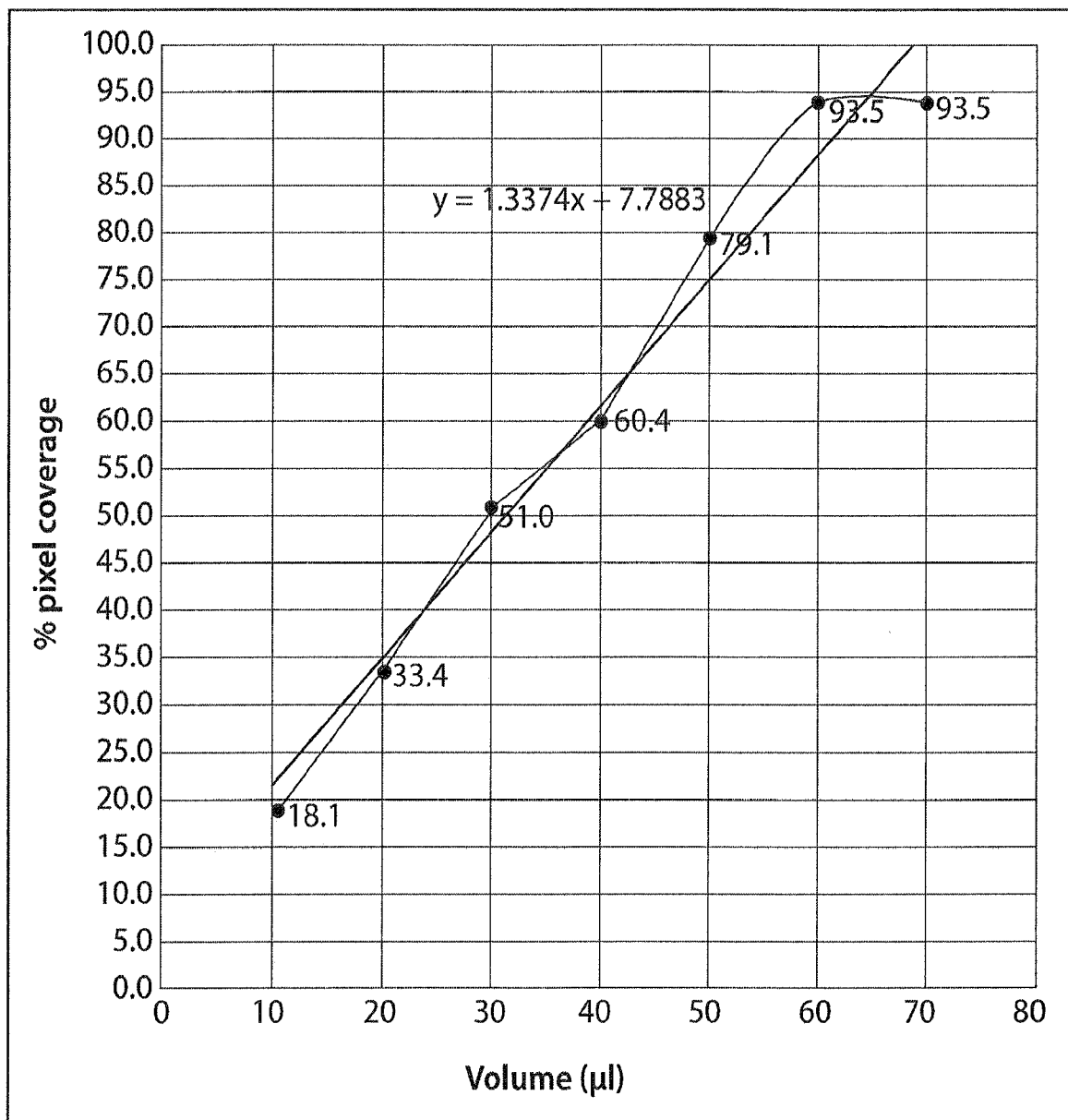


Fig. 5

6/15

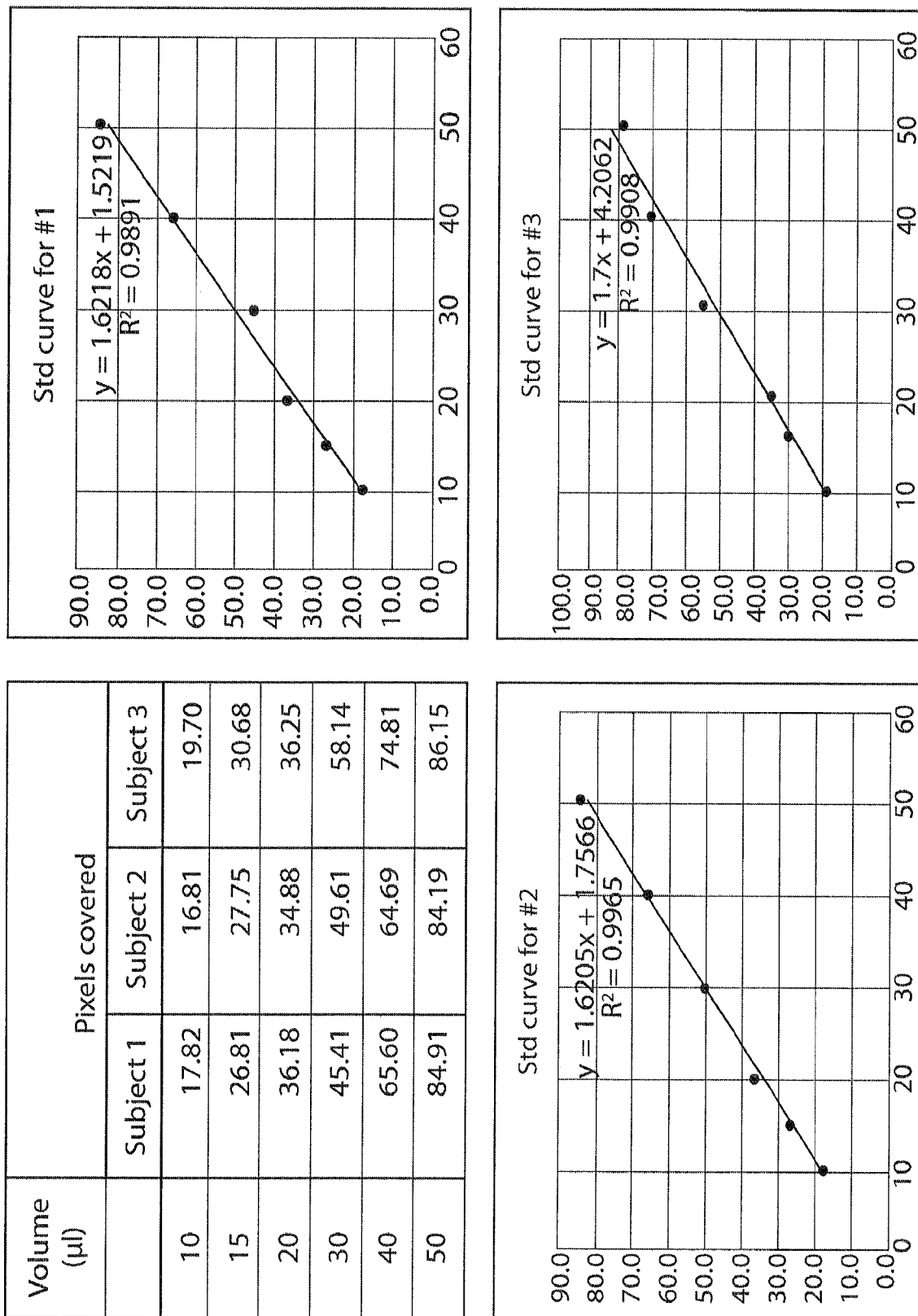


Fig. 6

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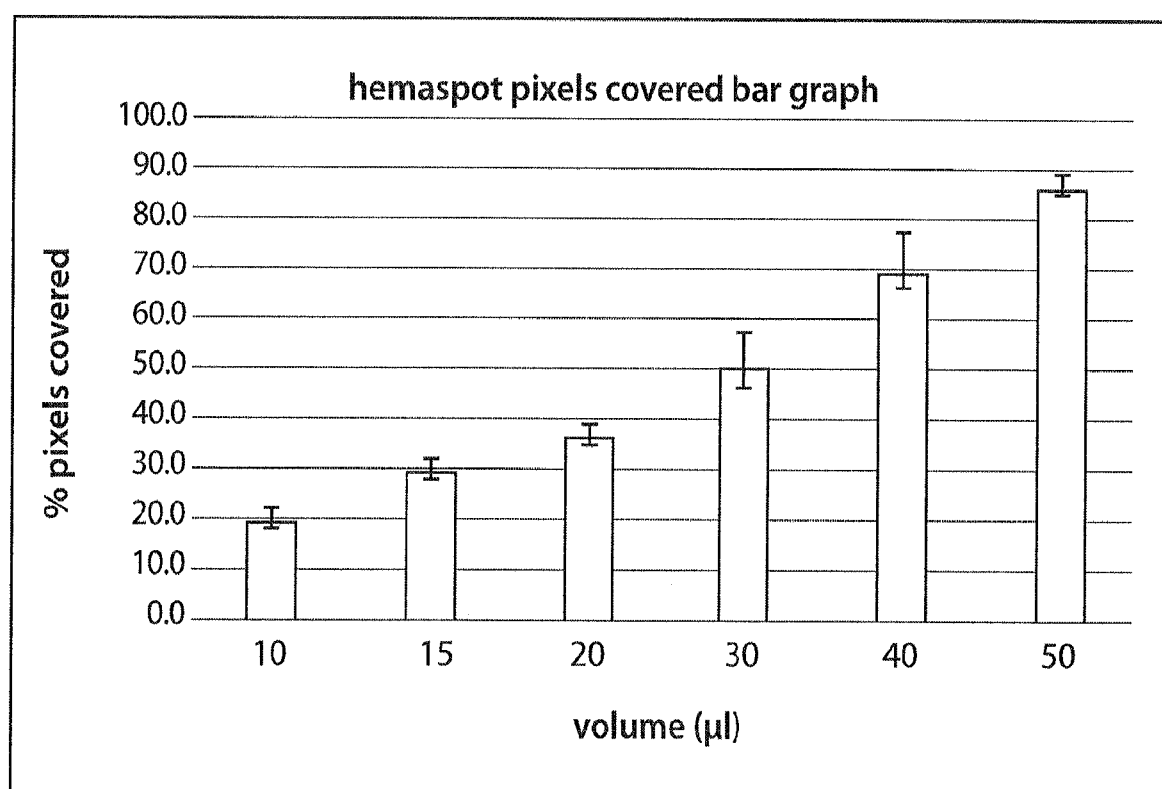


Fig. 7

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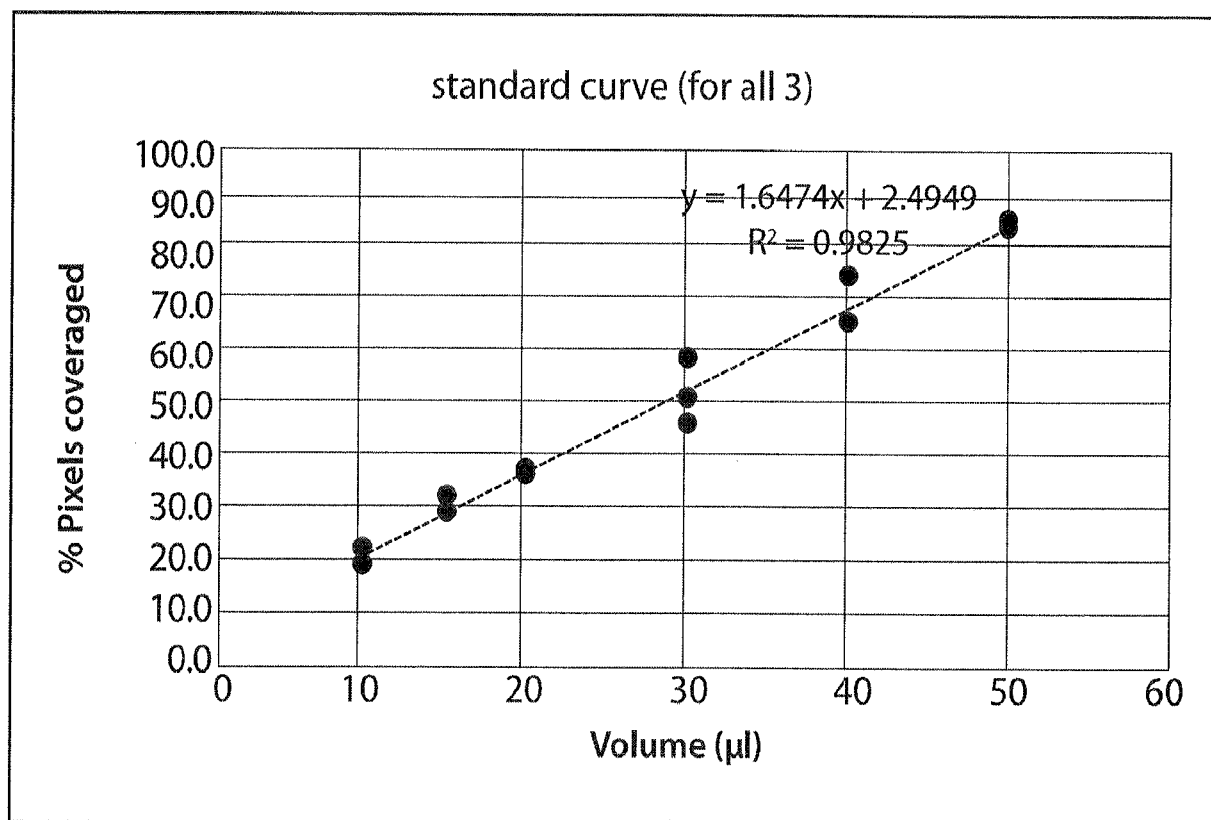


Fig. 8

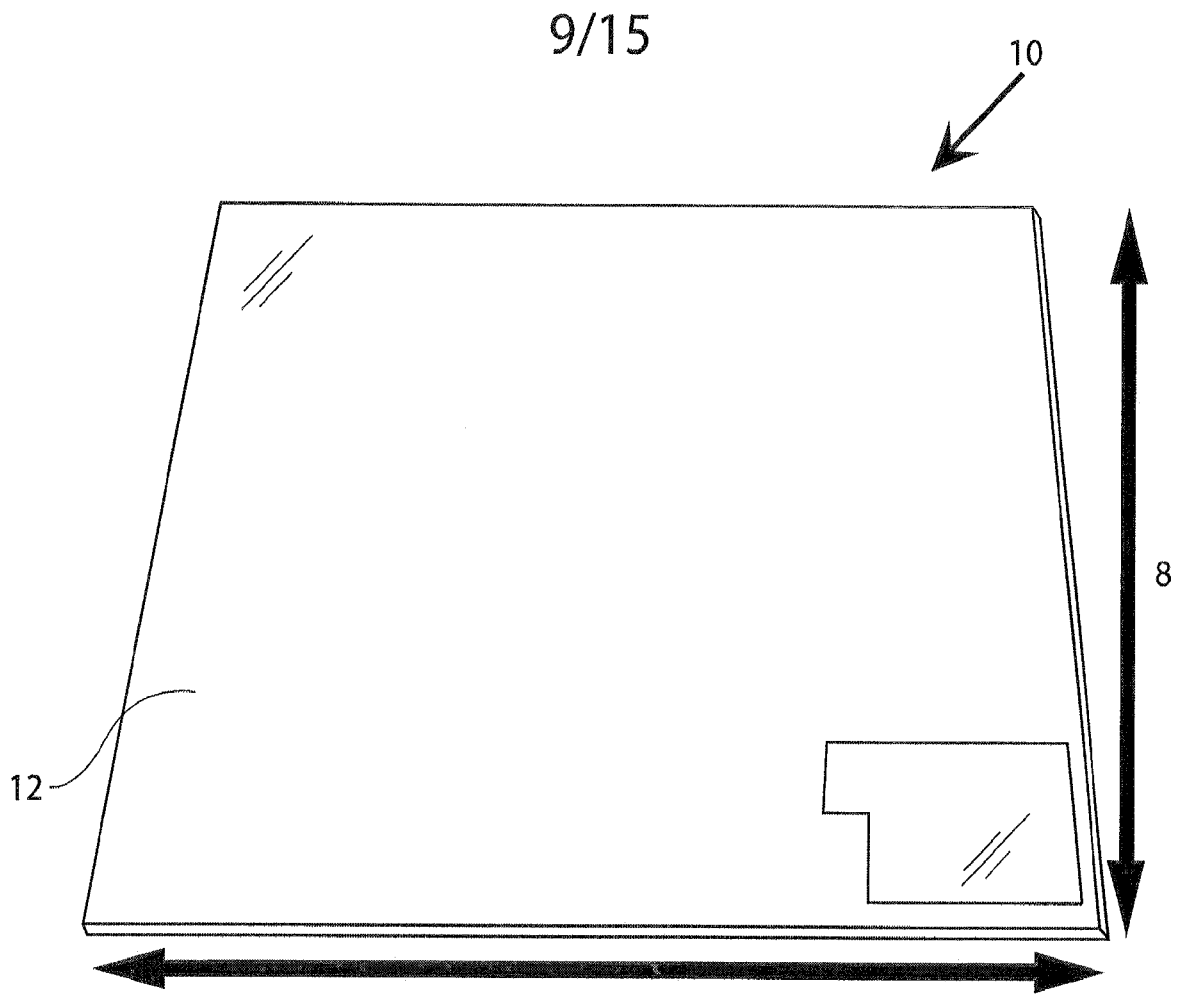


Fig. 9

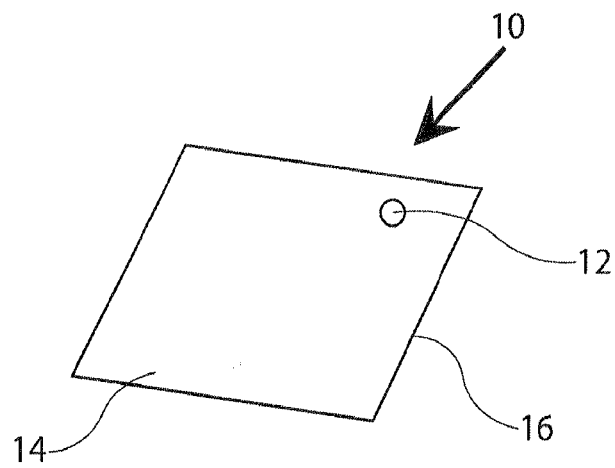


Fig. 10

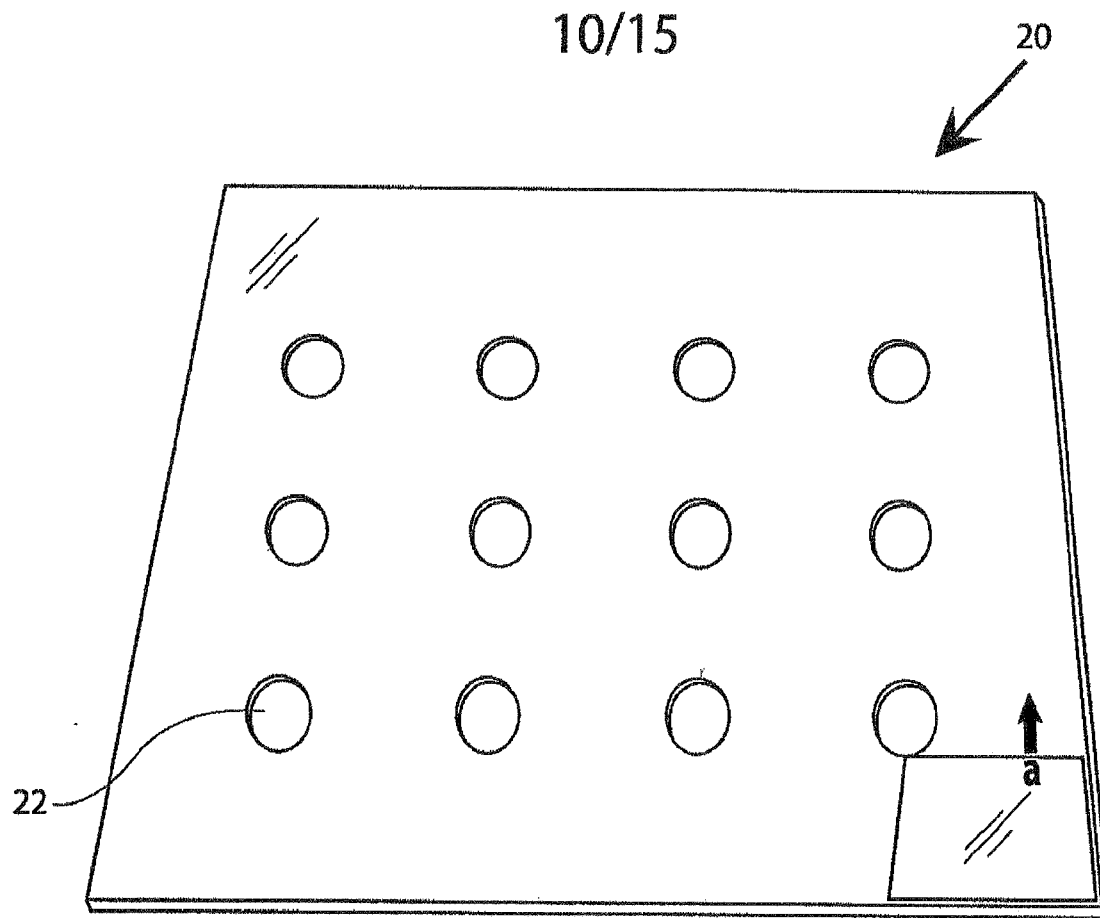


Fig. 11

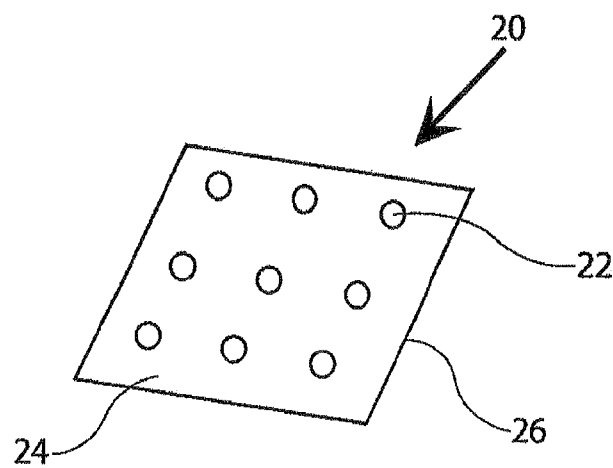


Fig. 12

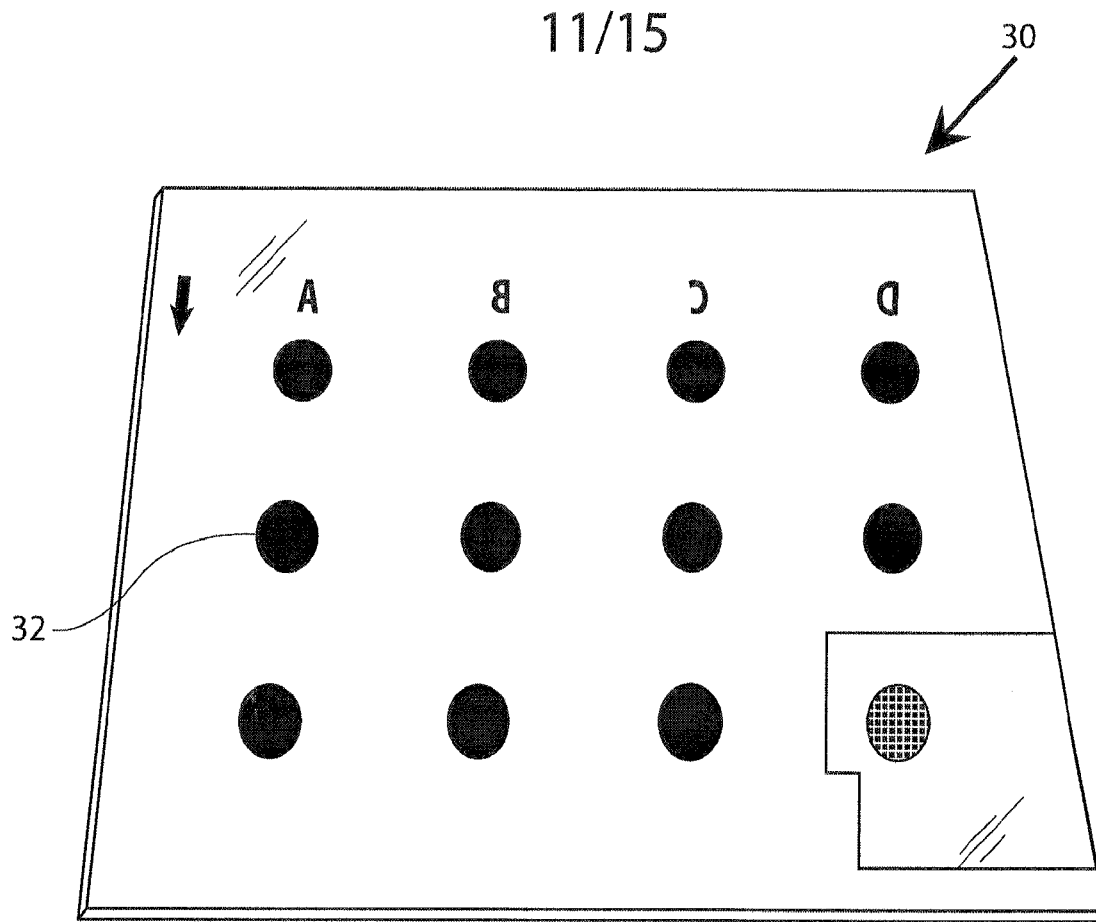


Fig. 13

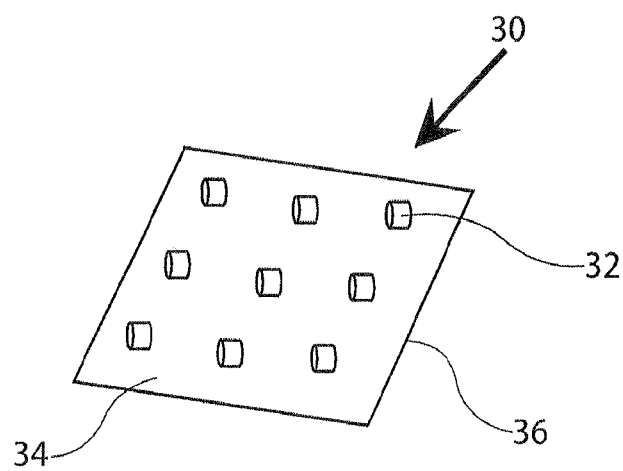


Fig. 14

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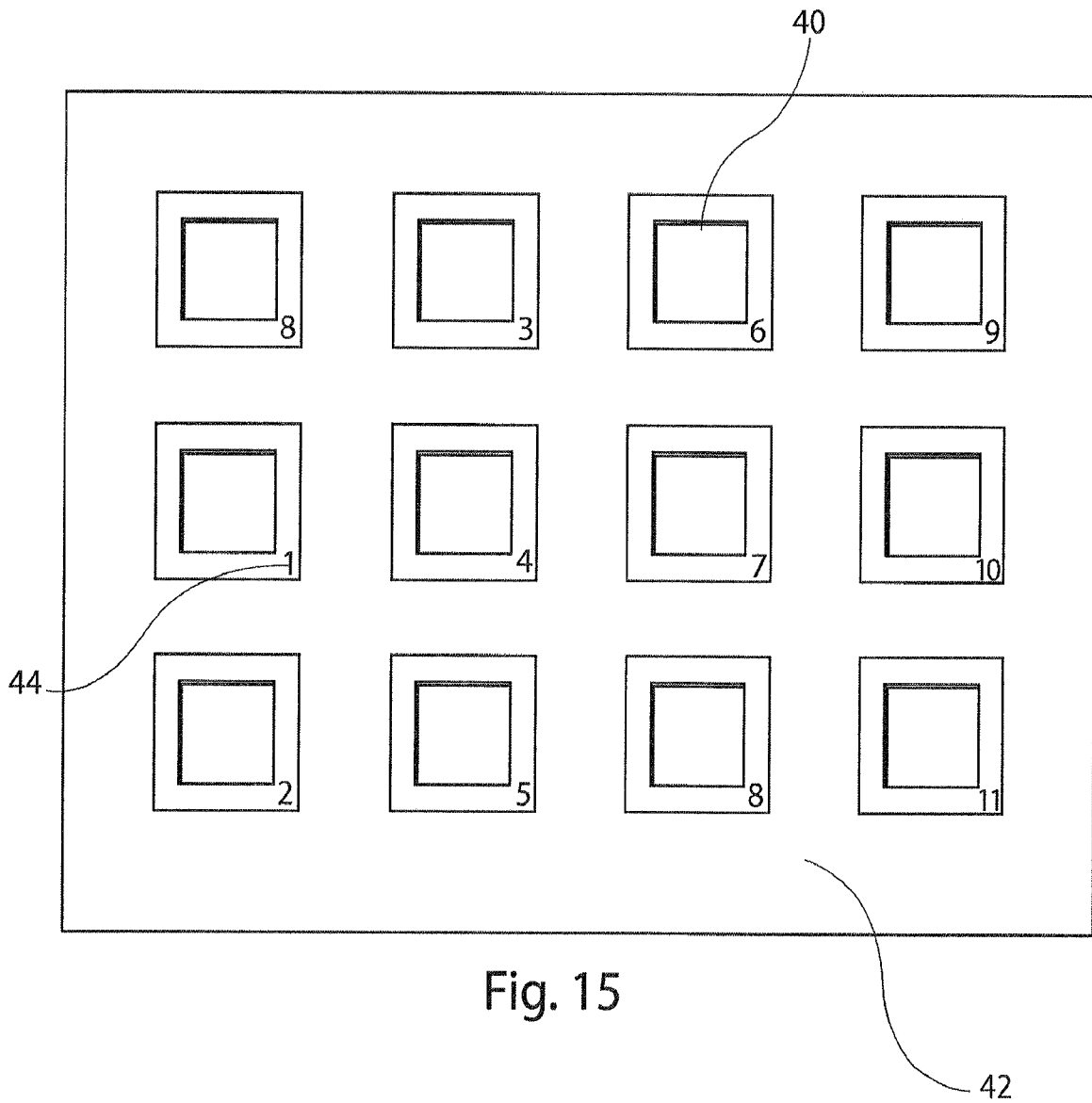


Fig. 15

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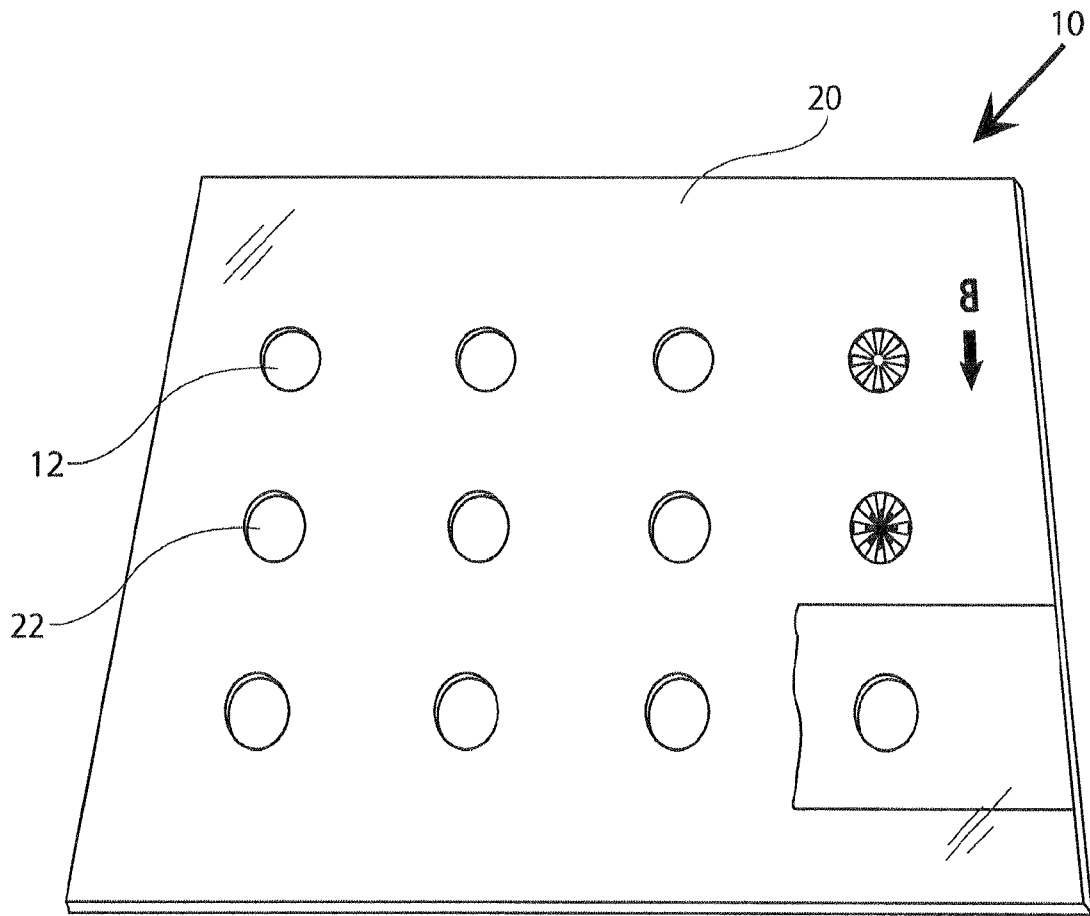
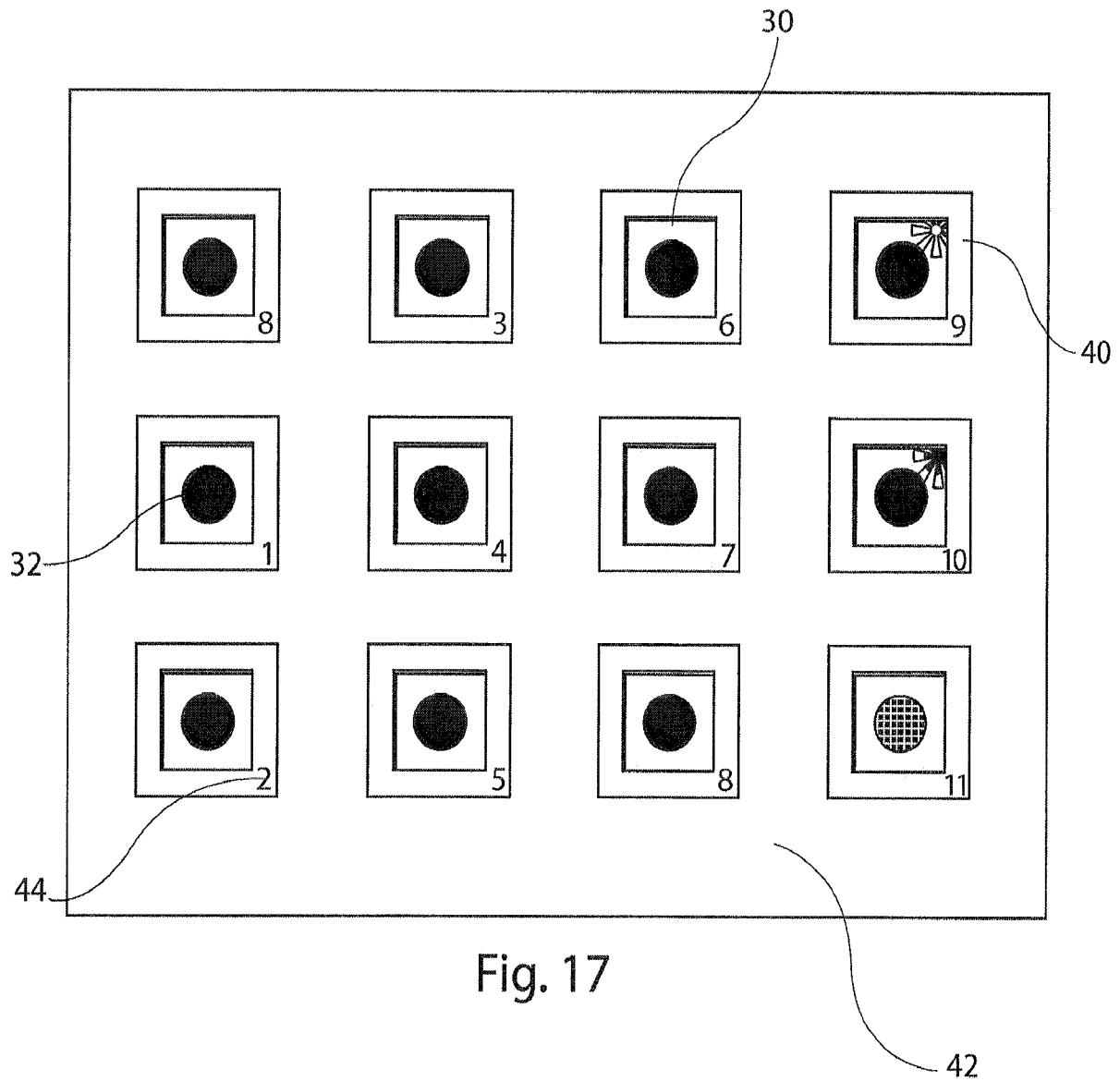


Fig. 16

14/15



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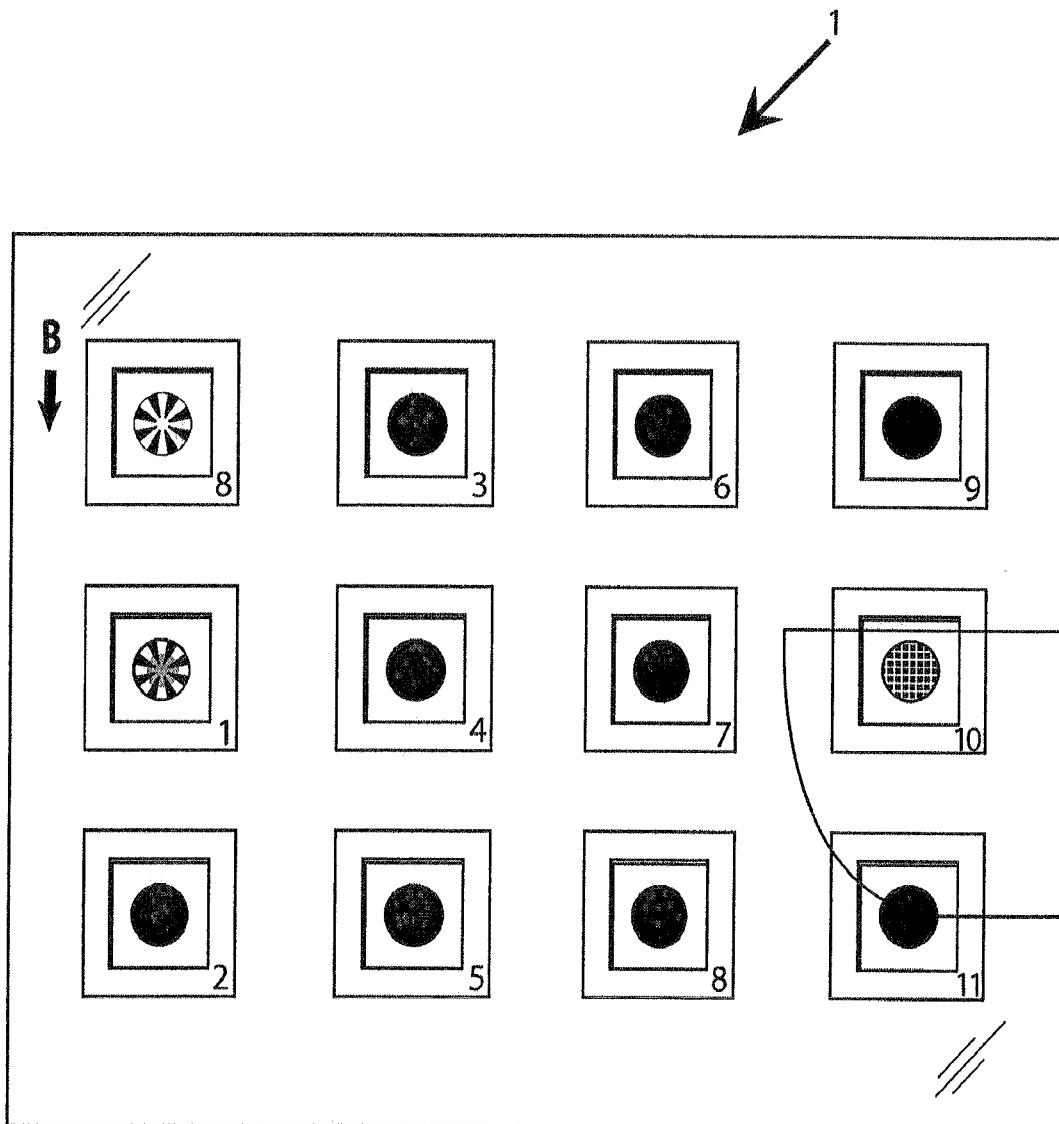


Fig. 18

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/30226

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5 and 11-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-4, directed to a method for estimating the volume of a blood sample.

Group II: Claims 6-10, directed to a device for scanning filters for dried blood samples.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

****please see the continuation at the end of this form****

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/30226

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 31/00 (2016.01)

CPC - G01N2496/05; G06K9/00; G06T7/602; A61B5/14535; B32B7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): G01N 31/00 (2016.01)

CPC: G01N2496/05; G06K9/00; G06T7/602; A61B5/14535; B32B7/00; G01N21/00; G01N33/525; G01N33/96; B01L3/50; H04N5/23222

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC: 36/16; 600/322; 436/11; 435/288.7; 356/433; 382/134; 356/128; 348/135; 356/440; 436/165; 382/128; 494/10; 356/246; 436/173; 435/287.1; 435/283.1; 435/4; 428/98; 264/299; 264/1.1; 422/400

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Pat Base (AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO), Google Patent, Google Scholar; Search terms: scanning image filter sample substrate blood dried transparent hole layer align stacked raised estimate volume known compare count pixel hematocrits varying grooves ridges paper dried powder solid transmissive pore porous voids

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 2013/0011042 A1 (SATISH et al.) 10 January 2013 (10.01.2013) para [0019]-[0020], [0024], [0026], [0033], [0034]; abstract; claims 1 and 10	1-2 ----- 3-4
Y	US 2011/0115905 A1 (BEUMER et al.) 19 May 2011 (19.05.2011) para [0024]	3
Y	US 6,006,119 A (SOLLER et al.) 21 December 1999 (21.12.1999) col 7, ln 1-4	4
A	US 2008/0102535 A1 (CHACE et al.) 1 May 2008 (01.05.2008) para [0013]-[0014]; abstract	6-10
A	US 6,255,061 B1 (MORI et al.) 3 July 2001 (03.07.2001) col 4, ln 8-16; figure 3; abstract	6-10
A	US 2004/0028875 A1 (VAN RIJN et al.) 12 February 2004 (12.02.2004) para [0200]; figure 24A	6-10
A	US 2013/0301901 A1 (SATISH et al.) 14 November 2013 (14.11.2013); the entire document	1-4
A	WO 2013/016038 A1 (CONSTITUTION MEDICAL, INC.) 31 January 2013 (31.01.2013); the entire document	1-4
A	US 2012/0309636 A1 (GIBBONS et al.) 6 December 2012 (06.12.2012); the entire document	1-4



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 September 2016 (26.09.2016)

Date of mailing of the international search report

07 OCT 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/30226

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/0151637 A1 (DAVIN) 5 August 2004 (05.08.2004); the entire document	6-10
A.	US 2007/0227967 A1 (SAKAINO et al.) 4 October 2007 (04.10.2007); the entire document	6-10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/30226

****continuation of Box III (Lack of Unity)******Special Technical Feature:**

Group I requires method for estimating the volume of a blood sample comprising the following steps: obtaining a sample of blood; spotting the sample of blood on a substrate; obtaining an image of the sample of blood; determining an approximate coverage or area of the image of the sample; and comparing the determined approximate coverage or area to a standard curve to determine an estimated volume of the sample of blood, not required by Group II.

Group II requires a device for scanning filters for dried blood samples comprising: a first layer comprising one or more transparent portions; a second layer comprising one or more holes, wherein said one or more holes are formed through the second layer and are sized to each receive a dried blood sample filter; and a third layer comprising one or more raised portions; wherein said one or more transparent portions of the first layer overlap with the one or more holes of the second layer and the one or more raised portions of the third layer when the first, second and third layers are aligned and stacked on top of each other with the second layer between the first and third layers, not required by groups I.

Common technical features:

Groups I-II share the technical feature of a blood sample. However, these shared technical features do not represent a contribution over prior art, because the shared technical feature is being anticipated by US 2008/0102535 A1 to Chace et al. (hereinafter "Chace"). Chace teaches a providing a test sample that was obtained by treating a dried blood sample (abstract; claim 1).

As the shared technical features were known in the art at the time of the invention, they cannot be considered common technical features that would otherwise unify the groups. Therefore, Groups I-II lack unity under PCT Rule 13.

Note: claims 5 and 11-15 are determined unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

摘要

本發明提供了用於分析血液樣品體積的方法和裝置。具體而言，本發明提供一種用於估算基底上的血液樣品體積的方法，包括獲取血液樣品圖像的步驟。可以從圖像中獲得血液樣品的覆蓋範圍或面積，並將其與標準曲線比較以獲得血液樣品體積的估算值。本發明還提供用於掃描基底上一個或多個血液樣品的裝置。該裝置包括可以組裝以在層之間保持多個樣品的三個層。該設備還包括標籤，並且可進行拆卸以進行樣品的淨化和重新裝載。