Using a CCD Camera Imaging System as a Recording Device to Quantify Human DNA by Slot Blot Hybridization

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ABSTRACT

Slot blot hybridization of membrane-immobilized, single-stranded human DNA with the higher primate-specific alphoid probe D17Z1 is routinely used in forensic science to estimate the amount of DNA in biological samples. Typically, a chemiluminescent signal captured on film records the hybridization, and the quantity of the signal is related to the amount of immobilized DNA. Digital imaging using a cooled CCD camera offers an alternate non-film-based method for image acquisition with comparable sensitivity of detection, a greater dynamic range, enhanced capability of data interpretation, and often faster results than film. In addition, the data support the premise that more accurate and precise human DNA quantification should be obtained by not assuming a linear response of signal to known standards. Instead, quantity should be estimated using a second-order standard curve ($R^2 = 0.999$). Finally, a CCD camera imaging system offers versatility for image capture of different signal sources and analysis of samples on a variety of support media.

INTRODUCTION

The quantification of DNA is desirable for enhancing the quality of genetic typing results when carrying out the PCR. The determination of appropriate DNA template quantities for the PCR can reduce undue consumption of forensic biological evidence and reagents and can avoid potential preferential amplification that may occur, for example, with some large repeat VNTR loci. A simple, specific method for the quantification of human genomic DNA that is routinely used is based on a slot blot approach in which membrane-immobilized, ssDNA is hybridized with the higher primate-specific alphoid probe D17Z1 (4). The slot blot procedure can be completed within a day, enables the analysis of a large number of samples, and can detect subnanogram quantities of human DNA. To facilitate the implementation of the procedure, radioactive reagents have been eliminated. With probes linked directly to alkaline phosphatase, the annealed probe-target hybrid can be detected by the cleavage of the chemiluminescence substrate Lumiphos® Plus™ (Life Technologies, Rockville, MD, USA) (1). The result, after an initial ramp time, is a continuous light output. Thus, low-light signals can be collected by film over time (e.g., for increased sensitivity), and, if needed, multiple film exposures can be made. Furthermore, the continued output enables chemiluminescent-based assays to fit conveniently into any laboratory protocol and routine.

In practice, a forensic scientist views a film image of a slot blot that contains a series of known DNA standards [e.g., using the ACES™ 2.0+ Kit (Life Technologies), the standards range from 40 ng to 10 pg] and estimates in a semi-quantitative manner the amount or concentration of DNA in unknown samples. The analyst typically assumes that there is a linear response in the signal with the standards used. However, because film density is linear only within an exposure range of approximately two orders of magnitude, quantitative analysis may be problematic for some samples, such as samples that are highly concentrated and appear saturated (i.e., overloaded and black). When samples are overloaded, one may attempt to
extrapolate an estimate of the DNA quantity in a sample by considering the dimensions of the slot and/or band or by repeating the slot blot analysis on greater dilutions of the over-loaded samples. The former approach can result in unreliable estimates of the amount of DNA. The latter approach requires additional labor and cost to obtain a result. The process is accomplished visually without the use of a densitometer. However, a densitometric scan of a film image is obviously limited to the same dynamic range issues as film.

Currently images of chemiluminescent DNA slot blots are captured on film (1). While this is an effective recording device for forensic applications to estimate DNA quantity, the process can be time consuming and costly if multiple exposures are needed to evaluate a wide range of DNA quantity in different samples on one slot blot. Additionally, film presents a one-time recording, end capture of the entire exposure process.

If the intended amount of template DNA placed in a PCR is inappropriate, and as long as proper interpretation protocols are followed, no incorrect genotyping results will occur. However, overestimation of a low quantity of template DNA (e.g., less than 250 pg) may result in no interpretable data, either because of no signal or signal below an interpretational threshold, and an undesired consummation of evidentiary material. Under-estimation of large quantities of template DNA (e.g., greater than 5–10 ng) would result in amplification of too much PCR product, and re-analysis would be required, thus costing undue time, labor, and consumption of additional resources.

Digital imaging offers an alternate non-film-based method for image acquisition with enhanced capability of data interpretation and often with faster results than film. Cooled CCD cameras offer comparable sensitivity to film (3), particularly under the conditions described in our study. They offer a wider dynamic range and, over long-term use, can be less costly and time consuming than film records and a film developer. This is true even when the price of the camera is considered. The dynamic range can be up to five orders of magnitude (i.e., 16 bits or 65 536 gray scales). The acquired image can be analyzed visually at different gray scales, which simulate exposures of lesser time durations. (Note that adjusting the gray scale only changes the display; the quantitation results remain the same regardless of gray level adjustment). Data can be transmitted electronically without the limitations of film capture, stored with a minimum cost and computer disk space, and easily imported into documents or printed. Lastly, with the accompanying integration software, a more objective evaluation of DNA quantitation can be obtained than by eyeing film or by using film densitometry, which requires an additional step of digitization followed by correction for nonlinear response. Thus, a cooled CCD camera-based imaging system may be better suited than film for image capture and analysis of slot blots for human DNA quantitation.

This study evaluates the use of the CCDBIO™ 16HC Imaging System (MiraiBio, Alameda, CA, USA) as a replacement for film detection of chemiluminescent signals generated on human DNA quantitation slot blots. The data demonstrate that a 16-bit CCD camera system offers several advantages over film for estimating the quantity of human DNA.

MATERIALS AND METHODS

We used purified human DNA of known quantity provided in ACES 2.0® Human DNA Quantitation System (Life Technologies). Slot blot hybridization was performed as previously described (1). After an initial ramp period (typically 3 h) to obtain a continuous glow at maximum signal, images were captured on X-ray film (BioMax Light-2; Eastman Kodak, Rochester, NY, USA). A CCDBIO 16HC Imaging System (MiraiBio) was used to capture chemiluminescent signals on the same blots, according to the manufacturer’s recommendations. For more information regarding the camera system, visit www.miraibio.com. Quantitation analysis of the CCD-captured images was performed using the image acquisition software GeneSnap™ and the data analysis software SlotQuant™ (both from MiraiBio).

Gray Level Adjustment—Optimizing Gray Level Settings

Proper setting of the gray level adjustment parameters enhances the signal and suppresses noise, resulting in clean, readable images. The improper setting of these parameters can result in either saturated or faint images. The gray level adjustment parameters modify the signal intensity of each dot, or pixel, in the scanned image. A pixel is the smallest unit of an acquired image. Each CCDBIO 16HC image file contains 16 bits per pixel, or 65 536 gray scale levels. Most computer monitors are 8-bit and can only display 256 shades of gray. The SlotQuant software translates the image for the 8-bit display. Each of the 8-bit divisions in the translated image is composed of 256 additional gray scale divisions that are present in the 16-bit digital file. These 256 values are averaged to give a single 8-bit gradation value. The gray level adjustment tool enables the best display of the image. It allows for the optimization of the presented image by assigning these 256 shades of gray only to the region containing the sample signal.

Cutoff Thresholds

SlotQuant assigns a background cutoff threshold and a high signal cutoff threshold for each image. Cutoff thresholds are applied to the range of 65 536 shades of gray generated during a scan. Background, or noise, lies at the low end of this range; signal lies near the high end of this range. Signals below the background cutoff threshold appear white in the image; signals above the high signal cutoff threshold appear black. The range between the background and the high signal cutoff thresholds signals is divided into the 8-bit 256 gradations. To obtain greater clarity of an entire image or certain selected bands within it, the range of gray levels in each image can be modified. In the gray level adjustment window, cutoff threshold values can be adjusted. Alternatively, reference background and high signal areas on the image can be designated. Using these reference areas, SlotQuant automatically adjusts all gray levels on the image. By adjusting the cutoff thresholds, the range between the highest and the low est acceptable gray shades is adjusted. When the high signal cutoff is too low, dark band images may be blurry with indistinct edges. When the background cutoff is too high, faint images are lost. Because signal intensities can vary greatly from one scan to the next, it is difficult to assign default values for high signal and background cutoff thresholds. Images that contain only faint bands against a clean background can be enhanced with high signal and background cutoff thresholds set to relatively high values. Images that contain heavily
stained regions may suffer from a substantial loss of content, even with low threshold values.

RESULTS AND DISCUSSION

Results were comparable across the membranes, showing reproducibility of results for sensitivity of detection and dynamic range within each recording system. Although the naked eye method is generally effective (and can also be performed on images recorded with the CCD camera), the process is improved with digital data. Generally, the sensitivity of detection is related to the duration of exposure, unless the light output is too low to record an image on film. Masucci et al. (3) reported that the sensitivity of detection between a CCD camera system and film is equivalent within the linear response region of each. Our study using the CCDBIO 16HC camera imaging system demonstrated similar findings. Figure 1 displays the signal response from a chemiluminescent slot blot after a 10-min exposure using the CCDBIO 16HC imaging system. As little as 100–200 pg human DNA could be detected visually, although 40 pg were detected on the computer. As capture time increases to 30 min, the maximum time routinely used in our study, the sensitivity of detection improves to 10–40 pg. Shorter exposures of only 3 min enable detection of the 200–400 pg human DNA. These detection results are similar to the sensitivity of film, even though film exposure times vary and the sensitivity of the film depends on many factors, for example, the emulsion and silver grain size. Typically, a 10-min exposure on film detects a 100-pg standard (data not shown). In many forensic applications, a 3-min image captured with the CCDBIO 16HC should be sufficient. Figure 2 shows a densitometric scan of the human DNA quantity standards of a film-captured image (that is equivalent to an image captured by a 30-min exposure in the CCDBIO 16HC). As expected, given the limited dynamic range of film, saturation of signal occurs at the 1-ng sample. Thus, extrapolation of the quantity of DNA is difficult for samples containing at least 1 ng.

Software analysis enables more effective evaluation of the data. This includes localized background correction, an automated process that identifies the DNA chemiluminescent bands, a grid for all standards and unknown samples, and then, based on the standards, a standard curve to quantify the unknown samples. The automated data analysis and integration for all slots and bands on a membrane occur in a matter of minutes. A plot of DNA quantities versus signal output from a blot containing known standards is displayed in Figure 3. A similar plot with film-recorded results was not carried out for comparison purposes because of the more limited dynamic range of film. The data support the premise that more accurate and precise DNA quantitation can be obtained by not...
assuming linearity and instead estimating quantitation using a second-order standard curve (R² = 0.999). These results are similar to those observed by Masucci et al. (3), who analyzed dilutions of horseradish peroxidase-labeled antibody with luminol as the substrate. The assumption that there is a linear response of signal to quantity, combined with the subjectivity of visual estimates, may explain, in part, the variation in estimating DNA quantity by slot blot analysis among forensic laboratories (2).

An even greater value of the use of a 16-bit CCD camera is a wider dynamic range (at least four orders of magnitude), which enables the processing of a wide range of signal intensities. Figure 4A shows a 30-min exposure of a slot blot membrane. The 20-pg standard is visible, and, although not visible on the image, the 10-pg standard was detected by the camera. However, it is evident, as it is with film, that the 1-ng and greater standards are saturated. With film-based detection, a new film would have to be placed in contact with the membrane for a shorter exposure time to obtain an interpretable result. However, by adjusting the gray scale, a 30-min exposure can be presented to simulate an equivalent from 8- to 10- or 2- to 3-min CCD camera capture time (or any time frame less than 30 min can be considered) (Figure 4, B and C). These three different gray scale images are similar to three different film exposures. Thus, with the use of a CCD camera system, only one exposure is needed to capture an image, better estimation of DNA quantity can be made for quite disparate samples with one exposure, better extrapolation is achieved to estimate DNA quantities typically considered overloaded with film, and the cost of film is eliminated.

Kline et al. (2) reported that there is variation in the ability to accurately determine the quantity of DNA results among forensic laboratories. Factors that contribute to the variation among laboratories include but are not limited to (i) variation in film recording, (ii) subjective estimation of the amount of DNA present, both for chemiluminescence, film, or colorimetric assays, (iii) pipetting errors, (iv) DNA binding efficiency within and between membranes, (v) hybridization efficiency, and (vi) the assumption of a linear response from the chemiluminescent signals and range of DNA quantities. While it is difficult to control these parameters so that there is no variation in performance, the recording device should not contribute substantially to such a variation. To assess the impact of the CCD camera imaging system on the variation of quantity estimates or the degree of reproducibility, the same

Figure 4. Time course of slot blot images captured with the CCDBio 16HC camera system. (A) A slot blot image captured in a 30-min exposure. The first two columns of samples are the range of standards typically placed on the membrane (labeled from 40 to 0.01 ng). The last four columns are the same samples as the standards ranging from 0.1 to 20 ng. The arrow indicates the 20-pg sample, which is the lowest level visible to the eye on this blot at this exposure. Although invisible to the eye, the 10-pg sample was detected with the CCD camera. (B) The same image as in Figure 4A, but the gray scale has been adjusted to simulate an 8–10-min exposure with the CCD camera. The arrow indicates the 200-pg sample, and the lowest level visible to the eye is the 100-pg sample on this blot at this exposure. Although invisible to the eye, the 40-pg sample was detected with the CCD camera. (C) The same image as in Figure 4A and B, but the gray scale has been adjusted to simulate a 3–5-min exposure with the CCD camera. The arrow indicates the 200-pg sample, which is the lowest level visible to the eye on this blot at this exposure. Although not visible by eye, the 100-pg sample was detected with the CCD camera.

Figure 5. Two-dimensional protein gel of an E. coli cell lysate (50 μg total protein) stained with SYPRO Ruby and image-captured with only a 1.6-s exposure with the CCD camera. The image on the left is the fully captured image. The image on the right is the same image with the gray scale adjusted. The gel was kindly provided by Nancy Kendrick of Kendrick Laboratories, Madison, WI, USA (www.kendricklabs.com).
blot was imaged five times in one day (so that chemiluminescent signal decay would not be a significant factor), each time with a 30-min exposure. Each sample on the blot was prepared individually so that no two samples were from the same dilution. Thus, the results enable an assessment of both sample manipulation and image capture within one blot. The amount of variation caused by the CCD camera can be evaluated for eight different preparations of four quantities that were imaged five times. The average estimates for the eight preparations for 0.10 ng ranged from 0.08 to 0.12 ng (0.02 SD); for 1.0 ng ranged from 0.92 to 1.03 ng (0.05 SD); for 4 ng ranged from 3.54 to 4.60 ng (0.35 SD); and for 20 ng ranged from 15.51 to 27.2 ng (4.00 SD). However, the SD within a sample imaged five times was no larger than 0.02, 0.02, 0.08, and 0.31, respectively. Thus, the variation caused by repeat analyses of the same sample with the camera is small.

In a second experiment, eight preparations of the same quantity of DNA ranging from 0.10 to 10 ng were placed on two blots. The amount of variation observed between blots was comparable to the variation observed within one blot; the range and SD were similar. One might predict that the data should vary more between blots than within a blot. The similarity of results is most likely due to the methods of preparing the samples. Each sample in each slot was individually diluted from a stock, and it may be that the pipetting error, typically encountered in routine laboratory manipulations, may be the main contributing factor to the variation in DNA quantitation by slot blot analysis (2). Regardless, the data demonstrate that the CCDBIO 16HC camera imaging system yields reproducible results and does not contribute substantially to the variation in quantitation.

In conclusion, a CCD camera-based imaging system is an attractive alternative for image capture compared to film. The features include comparable sensitivity of detection, wider dynamic range, less subjective interpretation of DNA quantities, automated quantitation analysis, low-cost storage and processing of data, and a reduction in the cost of analysis, particularly for high-volume laboratories. Additionally, artifacts peculiar to film, such as those generated from static electricity, are eliminated (data not shown).

In addition to chemiluminescence, the camera can detect fluorescent and colorimetric signals. Thus, proteins and nucleic acids (labeled with other dyes) can be imaged; signals on agarose, polyacrylamide, and membrane supports in microarray and macroarray formats can be analyzed. As an example, Figure 5 shows two representations, taken with 1.6-s exposure, of a two-dimensional protein gel of an E. coli cell lysate (50 µg total protein) that was stained with SYPRO® Ruby (Molecular Probes, Eugene, OR, USA). The sensitivity of detection was greater than silver staining. With the increased dynamic range, spots that represent low levels of protein that are typically masked by overloaded spots can now be analyzed. Thus, an ancillary benefit of a CCD camera imaging system is its versatility for the image capture of several different signal sources, for the analysis of samples on a variety of support media, and for the recording of results for a number of different applications.

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REFERENCES


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