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# DNA Quantification by Gel Densitometry with Norgen DNA Ladders

## Introduction

Analytical gel densitometry has become a mainstream technique for quantifying DNA. Unlike other methods such as spectrophotometry and the colorimetric diphenylamine assay, gel analysis provides both qualitative and quantitative assessments of a DNA preparation. A gel picture, for example, provides a wealth of information such as quality of the DNA, and, to a certain extent, the contamination by RNA and proteins. The gel can then be digitized and the image used for quantification. With the availability of hardware for gel imaging and software for data acquisition, as well as standard DNA markers specifically designed for quantification, gel densitometry has become a method of choice by many investigators.

Highly accurate quantification can be achieved with gel densitometry, yet there are important issues that must be considered to obtain consistent and reliable results. This document serves as a guideline for achieving the desired results with gel densitometry in general, and using Norgen's quantitative DNA molecular weight markers in particular. Some key issues that are often ignored in routine analyses are explored. Finally, certain common lab practices that are not compatible with the technique are cited and explained as to why they should be avoided.

## Ethidium bromide staining and gel densitometry

Pre-staining of agarose gels refers to adding the stain (ethidium bromide) to the molten agarose and casting it to form the gel. During electrophoresis, the stain intercalates with the DNA and they migrate as complexes. If pre-stained agarose gels are used, it is advised that the electrophoresis buffer should also contain the stain in the same concentration as in the gel. If the stain is present in the gel but not in the buffer, the gel will result with uneven staining because the free ethidium bromide migrates towards the top of the gel leaving the bottom part with no stain. Therefore, the background noise caused by the ethidium bromide on the gel becomes non-uniform. The uneven staining will produce incorrect results in densitometry.

Post-staining of gels is the preferred method for purposes of quantifying by densitometry. After electrophoresis, the gel is soaked in the running buffer containing ethidium bromide at a concentration of 0.5 µg per mL for a prescribed time of 30 to 60 minutes depending on the thickness of the gel. Post-staining is preferred over pre-staining because uniform

staining is easily achieved. In addition, DNA with intercalated ethidium bromide has altered electrophoretic mobility.

Gels should be stained so that the DNA bands bind the stain to saturation levels while nonspecific staining of the gel itself is kept at a minimum. Since the amount of bound ethidium bromide is more or less proportional to the amount and length of the DNA, then it is important that each band is sufficiently saturated with the stain. Under-staining can greatly affect the densitometry of small DNA bands below 500 bp. One easily detects under-staining in gels because the raw pixel count, for example, of a 200bp band would be identical to one that is 300bp in size.

Over-staining of gels is mainly caused by high concentrations of ethidium bromide. It dramatically reduces the signal-to-background ratio since the gel itself will nonspecifically bind the stain. Faint bands are specially affected by over-staining as they tend to blend with the background. To avoid over-staining, use ethidium bromide concentrations below 0.5 µg per mL. De-staining gels that are over-stained is not recommended for densitometry purposes.

**Agarose concentration and gel thickness**

Gel concentration and gel thickness affect the rate of ethidium bromide staining since both retard the rate of diffusion of the stain. In general, however, staining with 0.5 µg ethidium bromide per mL of buffer for a period of 40 minutes is sufficient for gels made with up to 2.5% agarose and a thickness of up to 70 mm.

**Digital Imaging**

Pixel saturation is the single most important factor that affects the results of gel densitometry. For this reason, most software bundled with gel documentation systems contain features to prevent this source of error. Pixel saturation is caused by overexposure during capture of the digital image. Camera settings for aperture and time of exposure can be adjusted to compensate for pixel saturation.

It should be noted that the intensity of a stained band on a gel diminishes with time due to damaging effects of UV illumination. For this reason, two digital images of a gel taken just 15 minutes apart under continuous UV exposure may not be identical It is suggested digital imaging should be done immediately on a freshly stained gel.

**Gel Linearity and Saturation**

**A. Gel calibration**

Gel calibration is an important initial step to undertake to provide the highest precision in the quantification process. Gel calibration simply means determining the linear capacity of the user's gel system (for a specified agarose gel concentration, buffer, gel dimension, gel thickness, ethidium bromide staining) with respect to the amount of a specific DNA ladder. Working in the linear range of the gel simplifies all subsequent calculations.

Gel linearity for a specific marker is established by running varying amounts of the marker on a gel system of choice. After electrophoresis, the digital image of the stained gel[1] is taken and densitometric readings obtained. Figure 1 illustrates this process by running the Norgen Low Ranger DNA Ladder on a 13cm-wide by 15cm-long gel. The DNA marker was loaded with varying amounts of DNA (250, 500, 750 and 1,000 ng).

The total pixel density for each lane was determined by drawing a rectangle around the bands, as illustrated in Panel B. The rectangles are of equal sizes, including one that serves as the background drawn in a blank area of the gel.

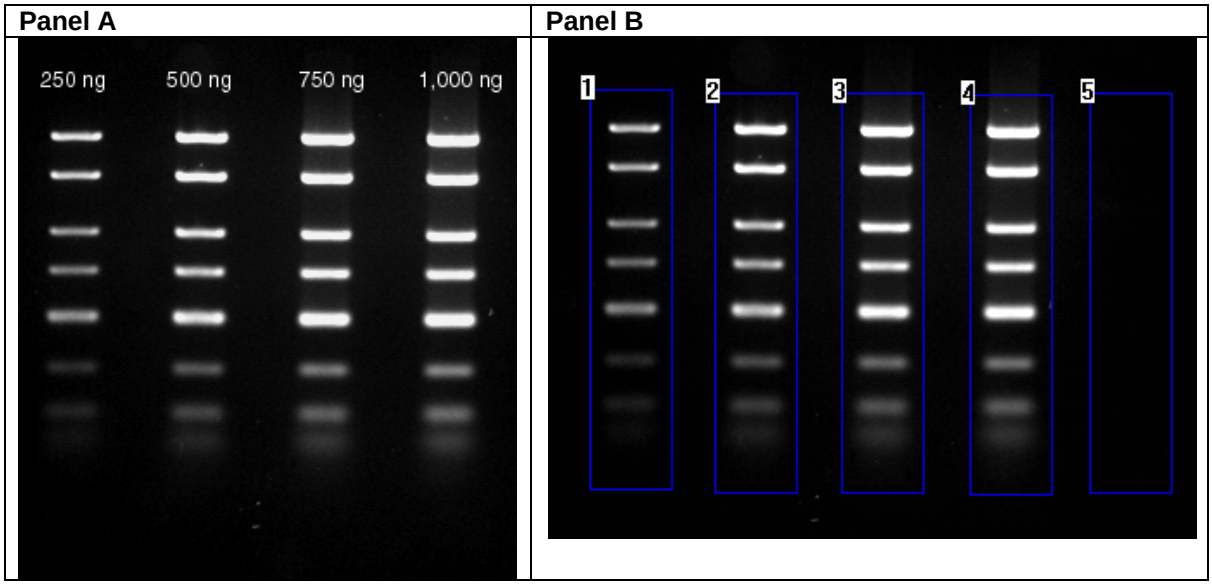


**Figure 1 .** Electrophoresis of the Norgen Low Ranger DNA Ladder at varying amounts (Panel A) and the densitometric method to quantify pixel density of each lane (Panel B).

**Table 1** shows the actual pixel densities of the defined areas for the different lanes in **Figure 1** before and after removing the pixel count for the background. The plot of the amount loaded per lane versus pixel density is shown in **Figure 2**. It is clear from the example that the amount of the marker loaded per lane shows a trend that has an initial linear portion and approaches saturation at higher amounts.

[1] It is assumed that the user is familiar with their gel-doc system for obtaining digital images of gels. Aperture and exposure times determine the final image; they should be set to avoid pixel saturations.

**Table 1 Pixel density determination**

| Amountof DNA (ng) | Pixel Density | Density minus Background |
|---|---|---|
| 250 | 111,972 | 83,979 |
| 500 | 167,958 | 139,965 |
| 750 | 223,944 | 195,951 |
| 1,000 | 242,606 | 214,613 |
| background | 27,993 | |

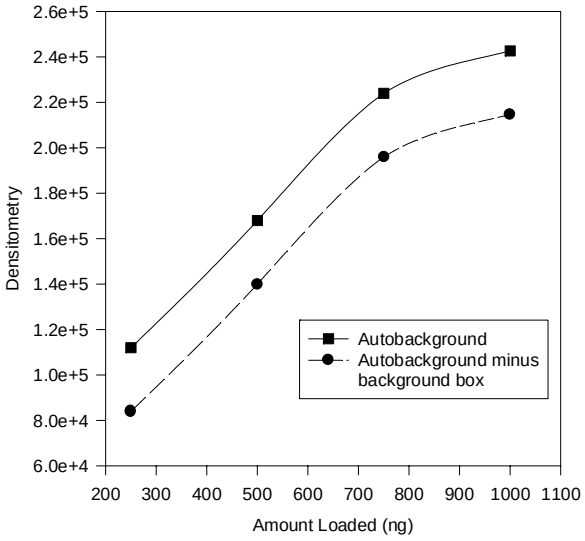


**Figure 2. Plot of total lane density versus the amount of marker.**

Another approach to establishing gel linearity is to first obtain pixel counts for individual bands of the marker, then add them together to obtain the total pixel count for all bands in the marker. The sum of the band densities count is then plotted against the amount loaded. The result of this type of analysis is shown in **Figure 3**, which is similar to that shown in **Figure 2**.

| Panel A | Panel B |
|---|---|

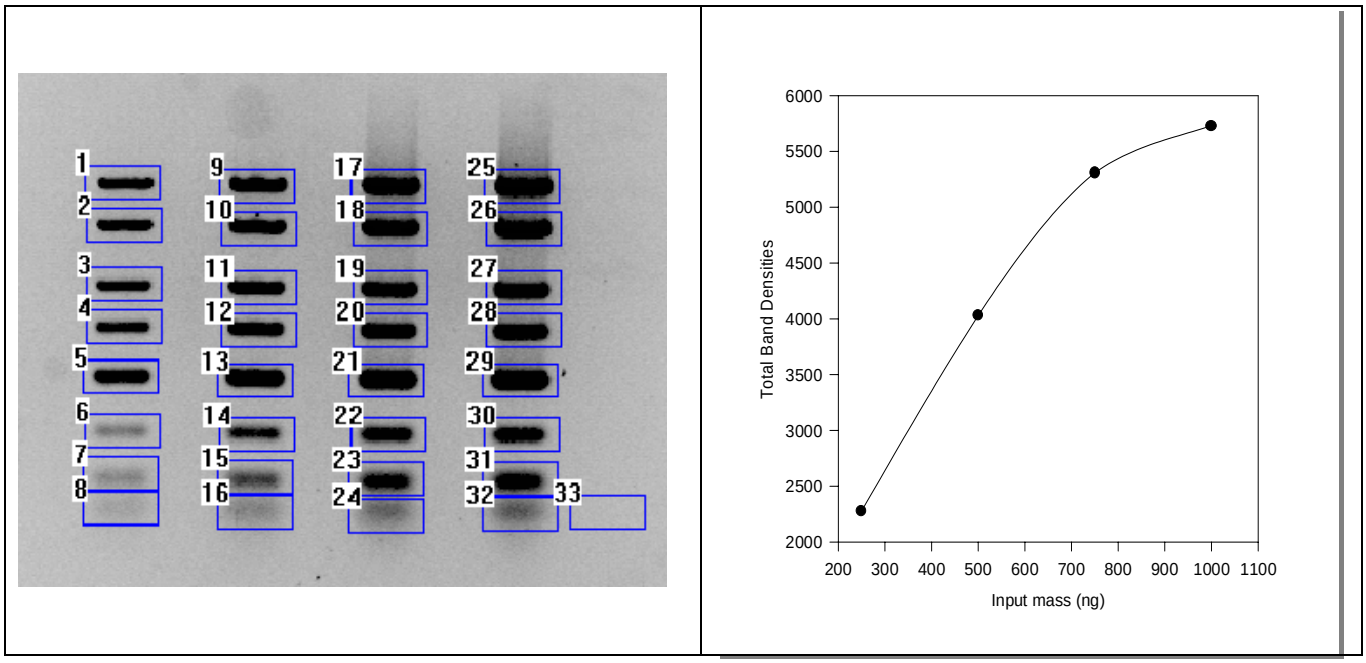


**Figure 3 Densitometry of individual bands and plotting of the sum of each marker's bands against input mass of the marker. Each band is enclosed by a rectangle whose pixel count is determined (Panel A). Input mass for the marker plotted against the sum of pixels for each band (Panel B).**

In the two methods of densitometry to test gel linearity, the input amounts of 250, 500 and 750 ng are arguably in the linear range, but 1,000 ng is not. The importance of taking into account the proper amount of the DNA marker to use for a particular gel system is most apparent when one starts assigning masses for each of the bands of the marker. Band mass assignment is a necessary step for establishing a standard curve to be used for predicting unknown DNA masses.

In the above example, band masses have been assigned by proportional distribution of the total input mass to each of the bands according to their pixel densities. For example, if a particular lane was loaded with 500 ng of the marker, then a band in that lane whose pixel density represents 10% of the sum of all the bands will be assigned 50 ng. This is done for all bands for the different lanes noting that each lane has a different but known amount of the marker. For added accuracy, the pixel density of each band has been corrected for the background. Thus a data set can be generated so that each band will have an "apparent" mass and pixel density.

If one plots the apparent mass against its density for all the bands of a single lane, then a perfect linear relationship is obtained. Two lanes with amounts in the linear range of the gel are expected to yield two coincident lines. Any divergence, therefore, will be indicative of nonlinearity.

**Figure 4** shows the regression analysis of the data points for the bands that were quantified by densitometry in the manner illustrated in **Figure 3.** The regression line for data points from lanes loaded with 250, 500 and 750 ng, respectively, revealed a high degree of correlation ($r^2$ = 0.9660). These amounts of the DNA marker are, therefore, within the linear range. The data points from the lane loaded with 1,000 ng of DNA marker reveal a regression line that is distinct from the other lanes. As stated previously, the lane loaded with 1,000 ng of marker falls outside the linear range.

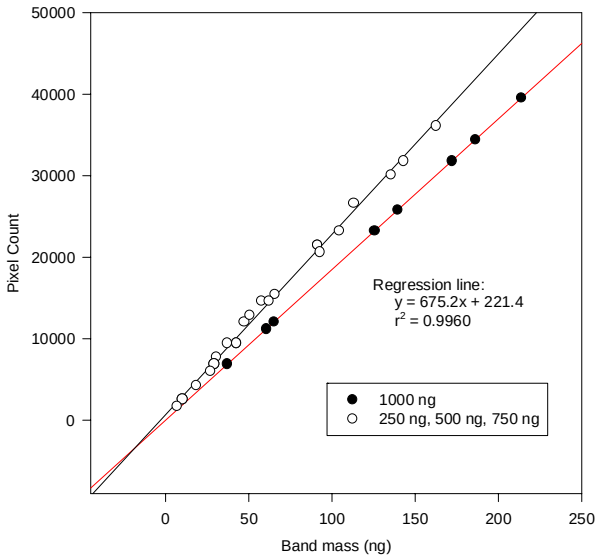


**Figure 4. Band mass assignment and gel linearity.** Data pairs from lanes loaded with 250, 500 and 750 ng, respectively, are indicted by hollow circles, while those from the 1,000 ng loading are represented with solid circles. The indicated regression function and its $r^2$-value are calculated from the hollow circle data pairs.

It is apparent that band mass assignment is readily affected when the amount of the marker loaded is outside the linear range of the gel. This could result in an inappropriate standard curve for use in predicting unknown DNA masses.

## B. Generating Molecular Mass Standard Curves

For predicting the mass of DNA bands on gels, standard curves (regression curves) that relate pixel counts to DNA mass must be generated. There are two ways to do this; each has its own advantages and either one can be used.

### *B1. Standard curves based on assigned band masses*

Standard curves based on the assigned or "apparent" band masses of the markers are generated from regression lines for the bands' assigned masses and their corresponding pixel densities. **Table 2** shows the pixel densities of the bands comprising the Norgen

Low Range DNA Ladder loaded at 250, 500, 750 and 1,000 ng. Regression analysis between the assigned mass and pixel densities for the bands in the lane loaded with 500 ng shows a high degree of correlation ($r^2$ = 0.9797, **Figure 6**). The *estimated regression function* can then serve to predict the masses of other DNA bands whose pixel densities were determined from the same digitized image. Statistical methods are available to estimate errors of prediction, or express the predicted values in terms of confidence intervals.

**Table 2. Densitometry of individual bands using Method 1**

| | | | Pixel Density | | | |
|---|---|---|---|---|---|---|
| **Band No.** | **Size (bp)** | **Mass (ng)** | **250 ng** | **500 ng** | **750 ng** | **1,000 ng** |
| 1 | 2,000 | **105** | 6912 | 13824 | 19008 | 19584 |
| 2 | 1,500 | **87** | 6336 | 12096 | 16128 | 17280 |
| 3 | 1,000 | **68** | 4608 | 8640 | 12096 | 13248 |
| 4 | 750 | **59** | 4032 | 7488 | 10944 | 11520 |
| 5 | 500 | **94** | 6336 | 10944 | 14976 | 15552 |
| 6 | 300 | **27** | 1728 | 3456 | 5184 | 5760 |
| 7 | 150 | **34** | 1728 | 4032 | 5184 | 5760 |
| 8 | 50 | **25** | 576 | 2304 | 3456 | 3456 |
| | | **$r^2$** | **0.9771** | **0.9797** | **0.9776** | **0.9685** |

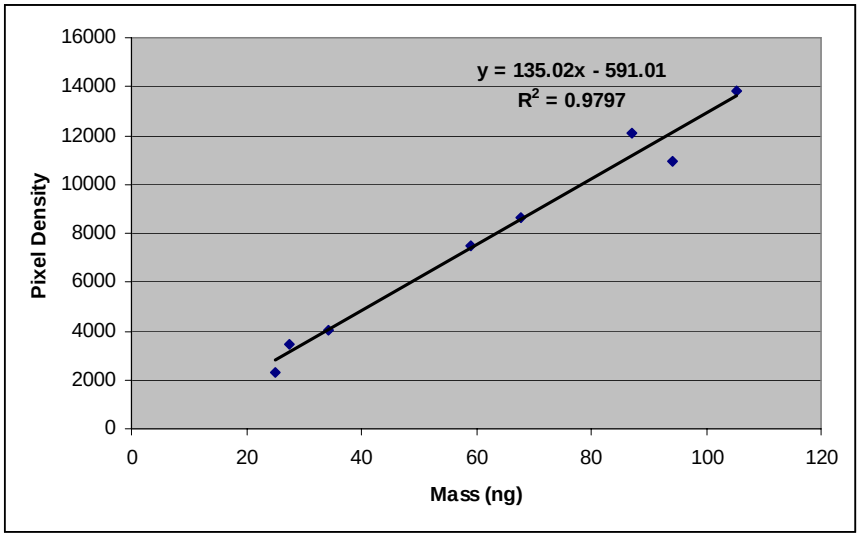


**Figure 5. Linear regression of band mass versus pixel density. Data set used is from lane loaded with 500 ng of DNA marker. Densitometry is by Method 1.**

## *B2. Standard curves based on proportional distribution of total marker mass to individual marker bands*

The second method for generating standard curves for estimating unknown masses depends mainly on the amount of the marker that is loaded on a lane. The amount of DNA marker per volume is specified as 500 ng per 10 μL. After electrophoresis, densitometry is used to obtain pixel densities of each band.

**Table 3** shows the densitometry of a lane containing 500 ng of the test marker. All bands of the marker have been accounted for and their pixel densities are noted. The sum of all pixel densities is then calculated. The proportional mass assigned to each band is simply the ratio of a particular band's pixel density to the total densities multiplied by the amount of the marker. For band 1, the pixel density was determined to be 13, 824 units, which corresponds to 22% of the total density. Since it is known that 500 ng of marker was loaded, it can be determined that this band contains 110 ng of DNA. Shown on **Table 3** are the proportional masses of the marker's bands.

**Table 3. Band Mass Assignment by Proportional Distribution**

| Band No. | Size (bp) | Pixel Density | % of Total Pixel Density | Proportional mass (ng) | Mass (ng) | Mass Diff. | % Diff. |
|---|---|---|---|---|---|---|---|
| 1 | 2,000 | 13,824 | 22% | 110 | **105** | 4.7 | 4% |
| 2 | 1,500 | 12,096 | 19% | 96 | **87** | 9.2 | 11% |
| 3 | 1,000 | 8,640 | 14% | 69 | **68** | 1.1 | 2% |
| 4 | 750 | 7,488 | 12% | 60 | **59** | 0.6 | 1% |
| 5 | 500 | 10,944 | 17% | 87 | **94** | 6.9 | 7% |
| 6 | 300 | 3,456 | 6% | 28 | **27** | 0.2 | 1% |
| 7 | 150 | 4,032 | 6% | 32 | **34** | 2.2 | 6% |
| 8 | 50 | 2,304 | 4% | 18 | **25** | 6.7 | 27% |
| | | 62,784 | 100% | 500 | | Mean=3.9 | Mean=7% |

Plotting the pixel densities against proportional masses gives the required linear function to use for subsequent calculations of unknown band masses. For the above example, the constant of proportionality (or slope) for pixel density to mass is 125.57. For example, if an unknown band has a pixel density of 10,000 units, then its mass is 10,000/125.57, or about 80 ng.

Note:
The proportional mass obtained from densitometry in the above example (**Table 3**) is identical to the method used to assign band masses that appear on the marker's labels. Since gel densitometry is subject to a wide range of variability, the differences between these masses should be regarded as experimental, and therefore can be treated statistically. **Table 3** shows the deviations from the indicated band masses and their equivalent percent differences. The absolute difference between two masses ranges from 0.6 to 9.2 ng, with a mean of 3.9. If expressed as percentages, deviations range from 1% to 27%, with a mean of 7%. Naturally, a small absolute mass difference will have a high percentage equivalent if the mass of the band under consideration is small.

To better see deviations between the two sets of numbers, one may apply Pearson correlation analysis. A pair-wise plot of the two sets is shown in **Figure 6**. The high correlation coefficient indicates: 1) high accuracy in establishing the band masses as indicated on the marker label, and 2) appropriate conditions were used in gel preparation, staining and densitometry for quantitative analysis.

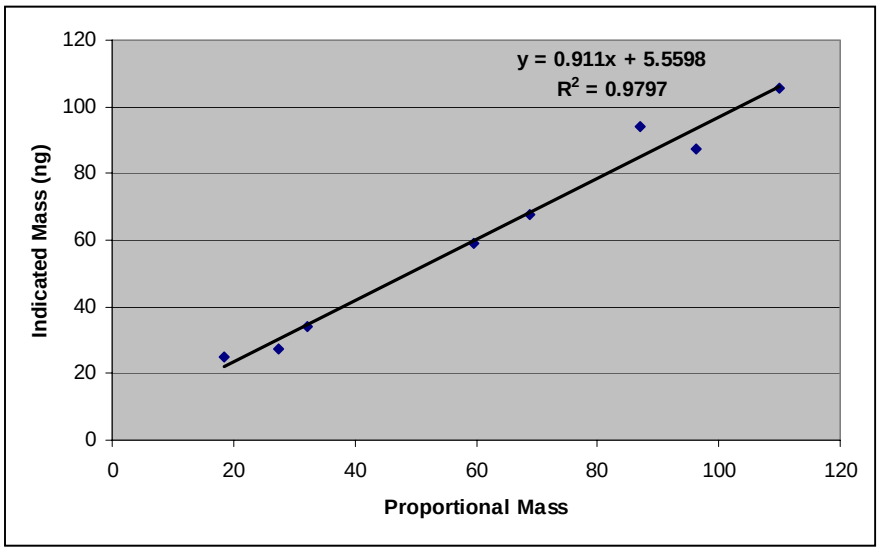


**Figure 6. Regression analysis of proportional mass determination versus the indicated band masses.**

The main advantages of using the manufacturer's indicated band masses in establishing a standard curve (Method B1) is that a) the quantification results can be expressed statistically; b) a partial set of bands comprising the DNA marker can be used in establishing the standard curve and from it determine unknown bands by interpolation; and c) non-linearity of the gel system used can be judged from a poor fit of the data points to a linear relationship.

Method B2, which is assigning the molecular mass by proportionality, is fast and is also quite accurate for mass determinations. However, there are no built-in features to the method that allows checking for gel linearity since the generated standard curves are all derived from pixel density counts and not independently derived. It is important, therefore, to fully calibrate a gel system for use with a specific marker. Also, one has to be able to account for all the marker's bands so that the total mass input can be proportionately assigned. A missing band can render subsequent mass predictions inaccurate.