

Introduction:

Quantitation of DNA is a quality assurance step to ensure that there is enough DNA in a forensic sample to give optimal results based on the specifications noted for each amplification kit used. The Quality Assurance Standards for Forensic DNA Testing Laboratories (issued by the FBI), states under Standard 9:

“STANDARD 9.4: *The laboratory shall quantify the amount of human DNA in forensic samples prior to nuclear DNA amplification*”.

Low copy number (LCN) samples have been defined as those containing 200 picograms of DNA or less. There are also restrictions for entering LCN analysis results into CODIS. This makes it very important that laboratories' quantitation methods are accurate and reliable to ensure that they know how much of a sample they are working with. Accurate quantitation results will also allow a laboratory to choose the best analysis method for characterization.

Testing has shown that depending on the quantitation kit used, the quantitation does not always provide accurate results. The DNA standard used in these quantitation kits can over- or under-estimate the quantity of DNA in a sample by two- to three-fold.

Apart from the reasons listed above, accurate and reliable quantitation results affect all aspects of the DNA analysis process. It has a definite impact on all laboratory-conducted DNA validations, especially when attempting to evaluate sensitivity of downstream methods and instrumentation. Accurate quantitation results will also ensure laboratories do not waste time and resources repeating processes because the results were not what were expected based on their quantitation. As laboratories obtain the actual quantitation results of the samples they are processing, they can take the necessary measures to correct any inconsistencies and optimize their methods.

Materials:

Blood Standard Male Donor

Whatman® Stain Cards

NIST SRM 2372

QIAGEN

EZ1® DNA Investigator Kit
BioRobot® EZ1 Workstation

Promega

Plexor® HY

Applied Biosystems™

TE Buffer
Quantifiler® Duo Kit
AmpF!STR® Identifiler® Kit
Running Buffer 10X
16-capillary array 36cm
POP-4™ polymer for 3130xl
Internal Lane Size Standards
Hi-Di™ Formamide
96-Well GeneAmp® PCR 9700
7500 Real-Time PCR System
3130xl Genetic Analyzer

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Methods:

Stains were made on Whatman® stain cards with 20µl of a blood sample. After the stains were dried, they were packaged and frozen until ready for use.

To assess the quantitation standards, the dried blood stains were extracted using the BioRobot® EZ1 Workstation Trace Tip Dance Protocol and diluted in the following series:

Neat – Blood Extract

1:10 dilution

1:20 dilution

1:100 dilution

1:1000 dilution

For every quantitation, a new dried blood sample was used each time to limit the variability in quantitation that may occur due to freezing and thawing of DNA sample extracts.

Each quantitation had at least one Applied Biosystems™, NIST and Promega standard. Two quantitations were necessary to process all of the samples due to the size of the quantitation plate.

All quantitations were performed by one individual and done on the same 7500 Real-Time PCR System to limit pipetting and instrumental variations.

The quantitations were evaluated based on the Y-intercept, R^2 value and slope. The samples were also compared based on their quantitation values.

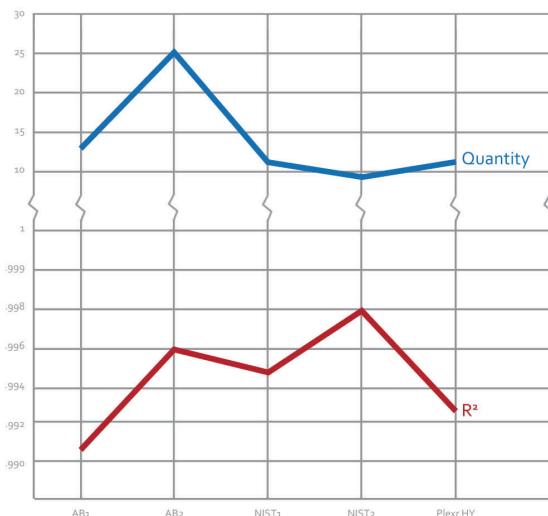
Quantitation values, R^2 values, Y-intercepts and slope were evaluated over time using the same quantitation standards. Standards were assessed based on the time that the manufacturer recommends their use after making the dilution series and to up to 30 days after being prepared. Once results were obtained, they were assessed to determine if there was a correlation between the quantitation value and one of the factors being evaluated.

At least one set of samples was amplified with the Applied Biosystems™ Identifiler® Kit targeting 1ng/µl and 200pg/µl based on the quantitation values from each kit and run on the Applied Biosystems™ 3130xI Genetic Analyzer using the following parameters:

- 3kv, 10-second injections, 8.7µl formamide, 0.3µl GS 500, 1µl sample and analyzed with GeneMapper® ID v3.2 software to show the downstream results of the variability in quantitation standards.

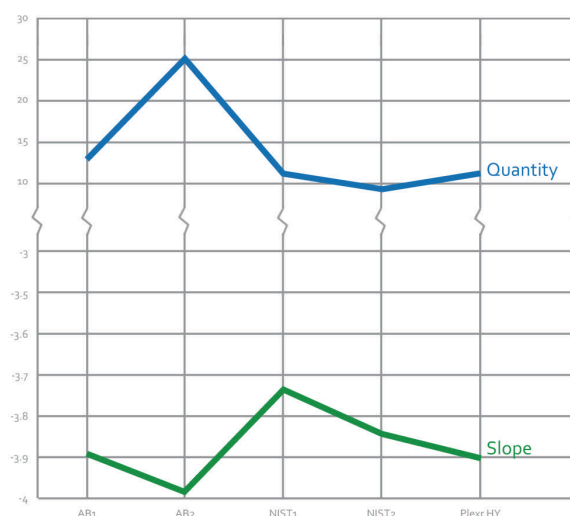
Results:

How Quantity is affected by R^2



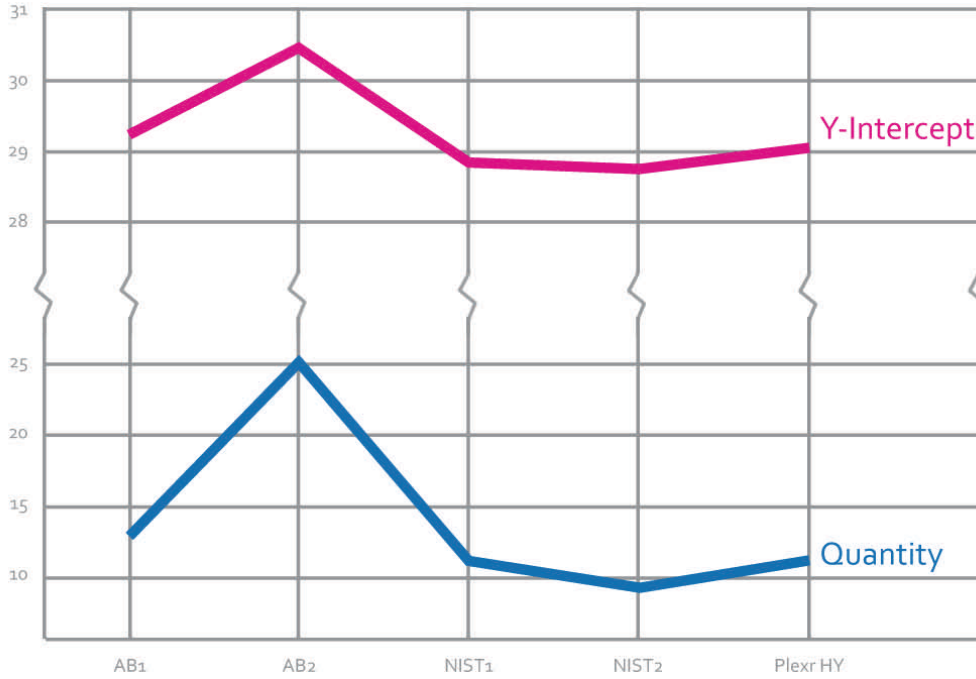
As shown in the graph above, fluctuations in the R^2 value of the slope do not correspond to changes in quantity of DNA.

How Quantity is affected by Slope



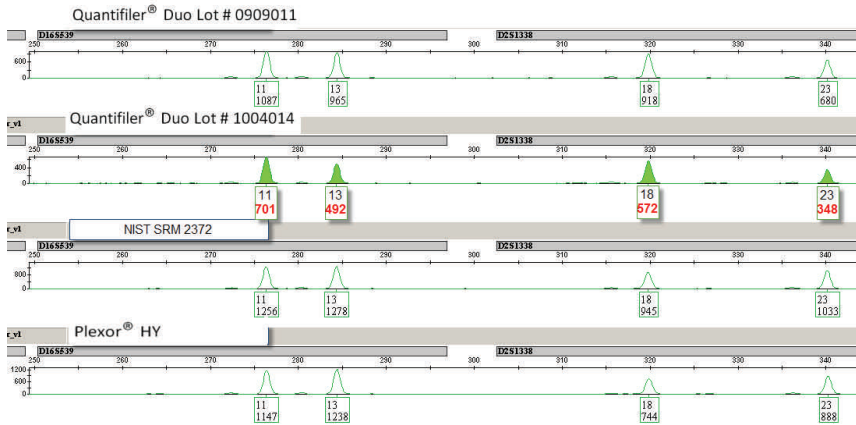
Changes in the value of the slope of the curve and changes in quantity do not show any correlation to each other.

How Quantity is affected by Y-Intercept



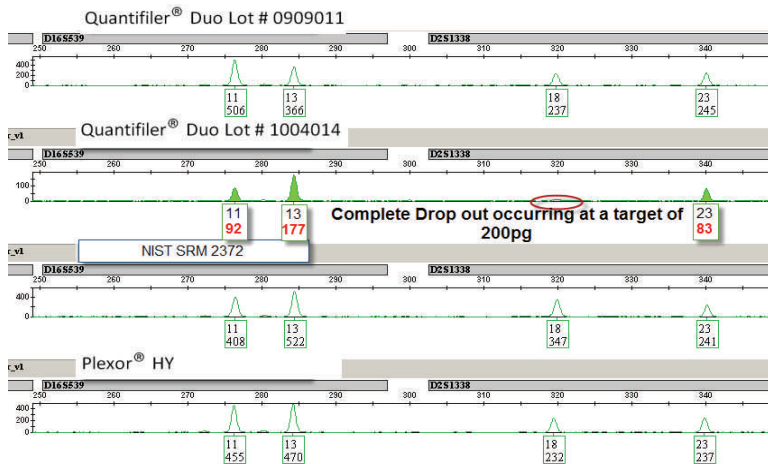
Fluctuations in the value of the Y-intercept correlate exactly with changes in the quantity of the samples. This proves that the Y-intercept directly affects the calculated value for the Quantity of DNA.

Target 1ng/μl



In the electropherograms to the left, the quantity of DNA targeted was 1ng/μl based on the quantitation values from the respective kits listed. From the peak heights, the estimation of 1ng from Quantifiler® Duo Lot #1004014 was not as accurate as the other standards.

Target 200pg/μl



A quantity of **200pg/μl** should yield a full profile with no evidence of stochastic effects. In the electropherograms to the left, when a sample that was estimated by Quantifiler® Duo Lot #1004014 to have a quantity of 200pg, the resulting profile showed the effects of low level amplification Peak Height Imbalance and Drop Out.

Conclusions:

Of the three parameters assessed (slope, R^2 and Y-intercept) the accuracy of quantitation most strongly correlates to the Y-intercept. The Y-intercept is the best indicator of the accuracy of the quantitation result.

- An increase of 1 point of the CT value on the Y-intercept, 28 to 29 for example, correlates to a two-fold increase in the estimated quantitation value.
- In general, Applied Biosystems™ quantitation standards showed the greatest variability over time and between lot numbers.
- Promega's Plexor® HY standards showed the least amount of variability over time.
- NIST SRM 2372 standards, though accurate, may lose accuracy if stored in a diluted state over a long period of time.
- Quantities estimated using Plexor's standards were the most consistent with each other when different quantitation chemistries were used.

Discussion:

Laboratories can employ several methods to ensure the accuracy of their quantitation results.

Suggested Solutions:

1. Using the NIST SRM 2372 standard to determine the accuracy of the quantitation results. The NIST SRM 2372 standard should be diluted so it falls within the dynamic range of the slope. These dilutions should be made periodically to ensure that the diluted NIST samples are accurate.
2. Based on validation studies, an acceptable range for the Y-intercept should be determined. A correction factor can then be applied to curves whose Y-intercept falls outside of this range. This validation should be repeated if any changes are made to the instrument during preventative maintenance or if any changes to the optics or chemistry are made.
3. Validation studies should not only be based on the NIST SRM 2372 results but should also be based on the RFU values that are observed when the manufacturer's suggested target amount (usually 1ng/μl) of DNA is amplified. Laboratories should also set an acceptable RFU range when this amount of DNA is amplified.
4. Laboratories should validate the length of time a diluted standard maintains its accuracy to ensure that over time the accuracy is not drifting. This will also help to ensure that the laboratory is not needlessly wasting time and money by consistently making new standard curves.

Benefit to the Forensic Community:

Even though qPCR is considered an estimate, the accuracy of the results are very important. LCN analysis is being defined by the quantity of DNA, and there are restrictions regarding the upload of LCN DNA profiles into CODIS. Laboratories should take steps to ensure the quantitation results are accurate; if they do not, they may inadvertently perform LCN analysis, since the true quantity of DNA is not known. By ensuring the accuracy of the quantitation results, laboratories can minimize variability that is often seen downstream during the characterization step. This will increase efficiency and manage costs by reducing the time required for unnecessary concentrating or diluting of extracts and, in some cases, of re-extracting and repeating the entire analysis process.

References:

Plexor® HY System for the Applied Biosystems™ 7500 and 7500 FAST Real-Time PCR Systems Handbook. [Revised 2007 Nov] Available from <http://www.promega.com/tbs/tm293/tm293.html>

Quantifiler® Duo DNA Quantification Kit User's Manual [2008] Applied Biosystems™. Available from http://www3.appliedbiosystems.com/cms/groups/applied_markets_support/documents/generaldocuments/cms_049050.pdf

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