National Forensic Science Technology Center
SCIENCE SERVING JUSTICE

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Evaluation of Three Parameters for Assessing DNA Quantitation

Project Information

Title: Evaluation of Three Parameters for Assessing DNA Quantitation

Evaluation Type: DNA Quantitation Methodology Comparison Study

Stakeholder: Forensic Science Community

Start Date: 7/1/2010 End Date: 10/31/2010

Manufacturer: Applied Biosystems

Kit Lot Numbers: 1004014, 1003013, 1004015, 1006016

Internet address: www.appliedbiosystems.com

Manufacturer: Promega Inc.

Kit Lot Numbers: 29407501, 29407503

Internet address: www.promega.com

Manufacturer: NIST

Standards received on dates: 05/2009, 08/2010

Internet address: www.nist.gov

Stakeholder Information

Contact Person: N/A

Phone Number: N/A

E-mail: N/A

Evaluation Team

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Evaluation Overview

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 pg 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.

The Forensic Services DNA Section of the National Forensic Science Technology Center (NFSTC) performed this comparison study to assess commercially available DNA quantitation standards to determine:

- If different quantitation standards from different manufacturers differ in performance
- If there is variation between lot numbers of standards from the same manufacturer
- How long the DNA standards, once made, are capable of giving consistent results

To achieve this, only one kit chemistry was used, but the standards were changed in the course of the experiment; they were evaluated by assessing the R^2 value, slope, Y-intercept and resulting quantities of a serial dilution of blood samples.

The following quantitation standards were evaluated:

- Applied Biosystems™ Quantifiler® Duo – Two different lot numbers
- Promega Plexor® HY – Two different lot numbers
- NIST SRM 2372 – Received in two different shipments

Plexor® HY chemistry was used on the first run along with the Quantifiler® Duo chemistry to determine if kit chemistries affect any of the factors being evaluated. If the kit chemistries were not a factor, then to limit variability only one quantitation kit chemistry was used (Quantifiler® Duo).

References

Plexor® HY System for the Applied Biosystems™ 7500 and 7500 FAST Real-Time PCR Systems Handbook [revised 2007 Nov]. Promega. 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.

Budowle, B.; Eisenberg, A.; van Daal, A. "Validity of Low Copy Number Typing and Applications to Forensic Science," Croatian Medical Journal, 50(3), (2009, June):207-217

Koukoulas, Irene, Ph.D.; O'Toole, Fiona E., B.Sc. (Hons); Stringer, Peta, Ph.D.; van Oorschot, Roland A. H., Ph.D. "Quantifiler® Observations of Relevance to Forensic Casework," Journal of Forensic Sciences, 53(1), (2007):135–141.

Grgicak, Catherine M., Ph.D.; Urban, Zena M., B.S.; Cotton, Robin W., Ph.D. "Investigation of Reproducibility and Error Associated with qPCR Methods using Quantifiler® Duo DNA Quantification Kit," Journal of Forensic Sciences, 55(5), (2010, Sept):1331–1339.

Instrument Setup Comments

N/A

Level of Operator Knowledge (Set per Manufacturer)

☐ Non-Scientist ☐ Technician ☒ Scientist

Procedure

1. Stain Whatman® stain cards with 20 µl of a blood sample.
2. After the stains have dried, package and freeze them until ready for use.
3. To assess the quantitation standards, extract the dried blood stains using the BioRobot® EZ1 Workstation Trace Tip Dance Protocol and dilute in the following series:
    - Neat – Blood Extract
    - 1:10 dilution
    - 1:20 dilution
    - 1:100 dilution
    - 1:1000 dilution
4. For every quantitation, use a new dried blood sample each time to limit the variability in quantitation that may occur due to freezing and thawing of DNA sample extracts.
5. Ensure each quantitation has at least one Applied Biosystems, NIST and Promega standard. Two quantitations are necessary to process all of the samples due to the size of the quantitation plate. Also ensure all quantitations are performed by one individual and done on the same 7500 Real-Time PCR System to limit pipetting and instrumental variations.

6. Evaluate the quantitations based on the Y-intercept, R^2 value and slope. Also compare the samples based on their quantitation values. Evaluate the quantitation values, R^2 values, Y-intercepts and slopes over time using the same quantitation standards. Assess the standards based on the time that the manufacturer recommends their use after making the dilution series and up to 30 days after being prepared.
7. Once results are obtained, assess them to determine if there is a correlation between the quantitation value and one of the factors being evaluated.
8. Amplify at least one set of samples with the Applied Biosystems Identifiler® Kit targeting 1 ng/µl and 200 pg/µl based on the quantitation values from each kit and run on the Applied Biosystems 3130*xl* Genetic Analyzer using the following parameters: 3 kv, 10-second injections, 8.7 µl formamide, 0.3 µl GS 500, 1 µl sample.
9. Analyze the data with GeneMapper® *ID* v3.2 software to show the downstream results of the variability in quantitation standards.

Standards, Controls and Samples Interrogated During Evaluation

Blood Standard Male Donor

Whatman® Stain Cards

NIST SRM 2372

QIAGEN

- EZ1® DNA Investigator Kit
- BioRobot® EZ1 Workstation

Promega

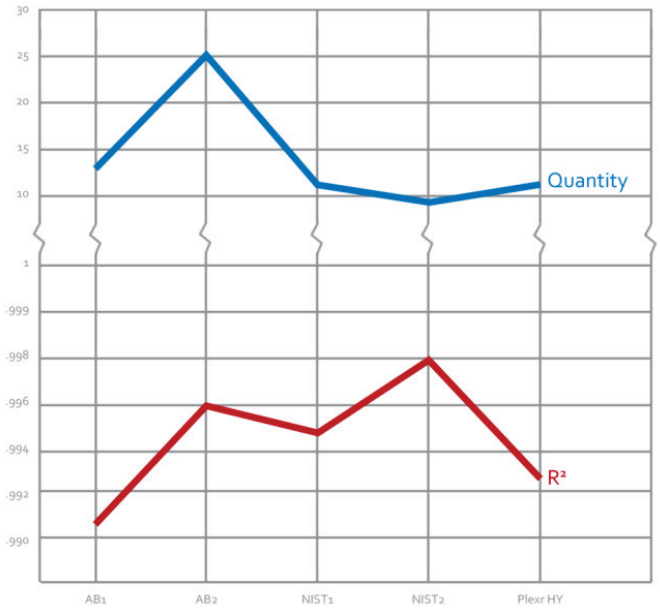
- Plexor® HY

Applied Biosystems

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

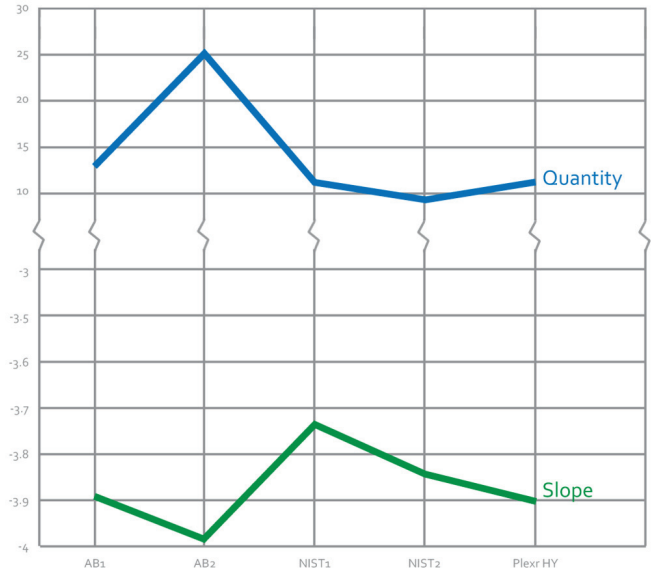
Findings

How Quantity Is Affected by R^2



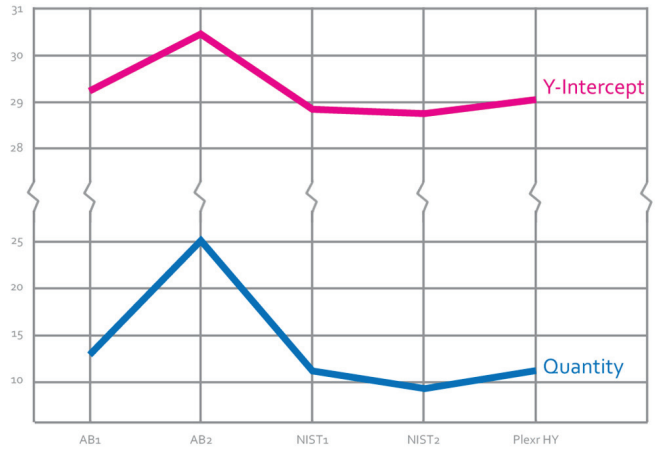
As shown in the graph above, fluctuations in the R^2 value do not correspond to changes in quantity of DNA.

How Quantity Is Affected by Slope



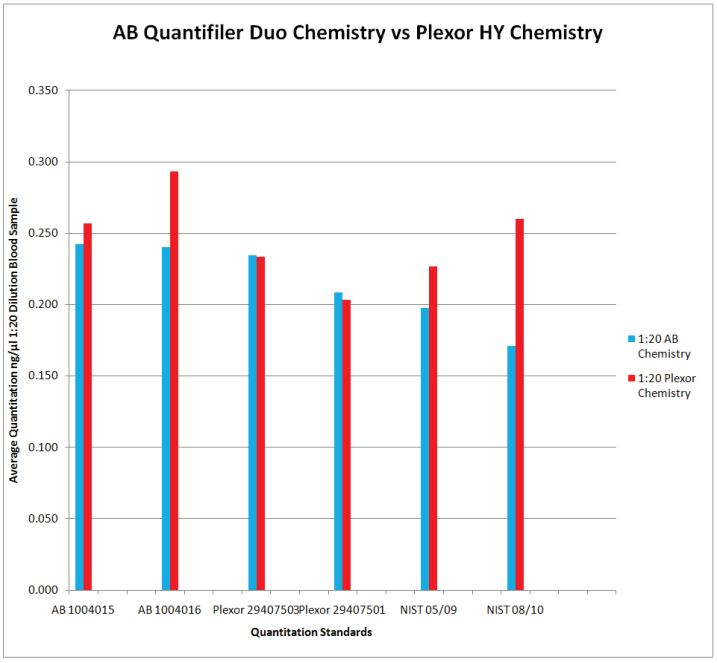
Changes in the value of the slope of the curve and changes in quantity do not show any correlation to each other, as shown in the graph above.

How Quantity Is Affected by Y-Intercept



As shown in the graph above, 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.

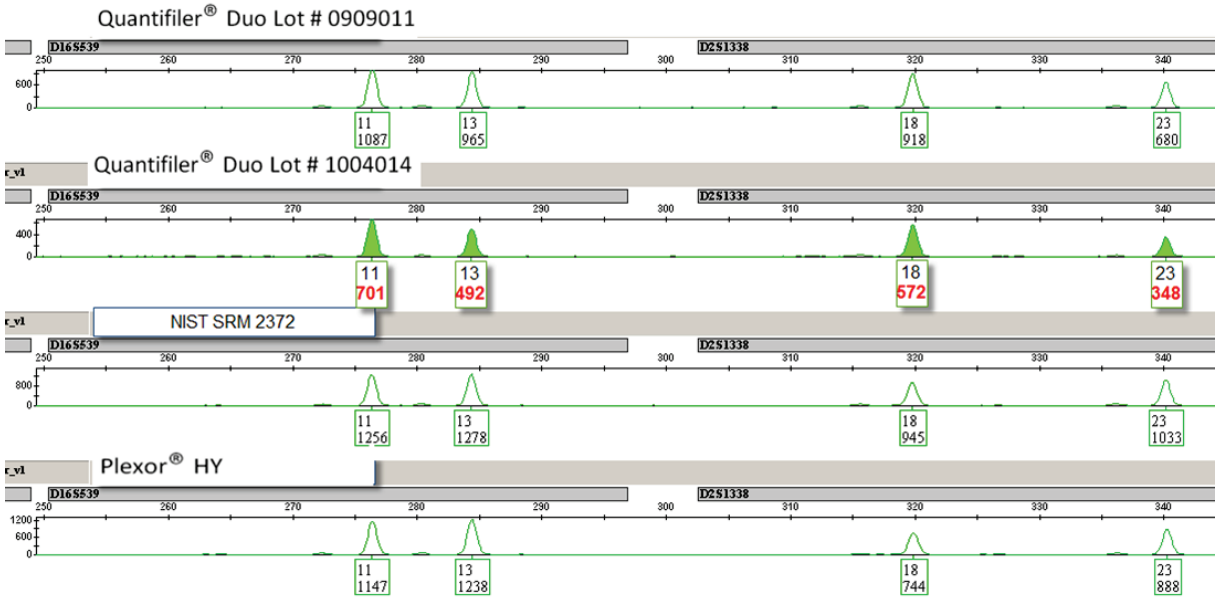
AB Quantifiler® Duo Chemistry vs. Plexor® HY Chemistry



The standards were run using a different chemistry to determine if the Applied Biosystems chemistry affects the performance of the quantitation standards. The results are shown in the graph above.

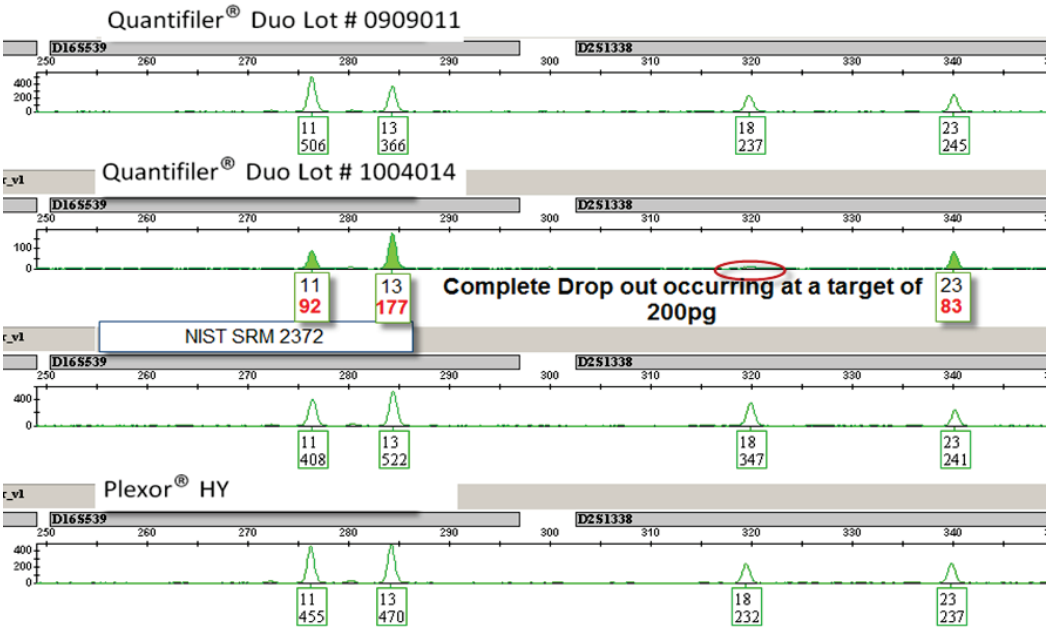
Quantities estimated using Promega's Plexor® HY standards were the most consistent with each other when different quantitation chemistries were used. The same consistency was seen when using the Quantifiler® Duo chemistry.

Target 1 ng/µl



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

Target 200 pg/µl



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

Conclusions

Of the three parameters assessed (R^2, slope 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' Quantifiler® Duo quantitation standards showed the greatest variability over time and between lot numbers.
- Promega's Plexor® HY standards over time showed the least amount of variability.
- NIST SRM 2372 standards, though accurate, if stored in a diluted state over a long period of time may lose accuracy.
- Quantities estimated using Promega's Plexor® HY standards were the most consistent with each other when different quantitation chemistries were used.

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 be based not only on the NIST SRM 2372 results but also on the RFU values that are observed when the manufacturer's suggested target amount (usually 1 ng/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 wasting time and money by needlessly making new standard curves.

Strengths

- Applied Biosystems quantitation standards gave accurate results when used within one week of preparation.
- NIST standards gave accurate results when used within one week of preparation.
- Plexor® HY standards gave accurate and consistent results within 4 weeks of preparation.

Areas for Improvement

- Applied Biosystems standards need to give more consistent results from one lot number to the next.
- NIST standards need to be more stable for a longer period of time once diluted.

Limitations

- Stability and accuracy of quantitation standards will be affected by length of time in diluted states and any changes in the instrument.

Training Requirements

- No additional training is necessary for a trained DNA analyst to use these products.

Health and Safety Issues

- Normal laboratory practices are required.

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.

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