# Development and Usage of a NIST Standard Reference Material for Real Time PCR Quantitation of Human DNA


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**P-303**

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NIST SRM 2372 Human DNA Quantitation Standard has been produced to support the need for a human-specific DNA quantitation standard in forensic casework and calibration of new quantitative polymerase chain reaction (qPCR) assays. The conventional DNA concentration has been assigned with one of the U.S. National Reference UV/Visible Spectrophotometers, assuming an absorbance of 1.0 at 260 nm equals 50 ng/µL of double stranded DNA. In addition, an interlaboratory study has been conducted, to verify that the SRM 2372 materials perform well in currently used DNA quantitation assays by the forensic DNA community. Each unit of SRM 2372 consists of three well-characterized DNA extracts. Component A is a single-source human male material derived from blood. Component B is a multiple-source human female material derived from blood. Component C is a multiple-source male and female material extracted from human placentas purchased from Sigma (St. Louis, MO). SRM 2372 is intended to enable the comparison of DNA concentration measurements across time and place. Manufacturers can use SRM 2372 to validate the values assigned to their own reference materials. Individual forensic laboratories can use SRM 2372 to validate DNA quantitation methods and to verify the assigned concentration of in-house or commercial DNA calibration standards.


## SRM 2372 Human DNA Quantitation Standard

Components

A: Male/single donor/RNased/NIST
B: Female/multiple donors/NIST
C: Mixture/male & female/commercial

Quantities supplied:
110 µL of Human Genomic DNA ≈ 50ng/µL

Certification

Decadic Attenuance (**Absorbance**) by a U.S. National Reference Spectrophotometer
Homogeneity by a Cary 100 Bio Spectrophotometer
**Validation of conventional [DNA] by Interlaboratory Study and NIST qPCR studies.**

## Requirements for NIST SRM 2372 Human DNA Quantitation Standard

**The material must be fit for purpose:**

- **Homogeneity** Tested Random Samples
  All tubes contain the same materials (concentration and quality)
- **Stability** Sarstedt Tubes (2.0 mL)
  DNA solutions will withstand shipping and normal storage
- **Recoverability** Interlaboratory Study & Tube Study
  What went in the tubes comes out
- **Traceability** Analysis by a Reference Spectrophotometer
  Values assigned are traceable to the designated certification method.

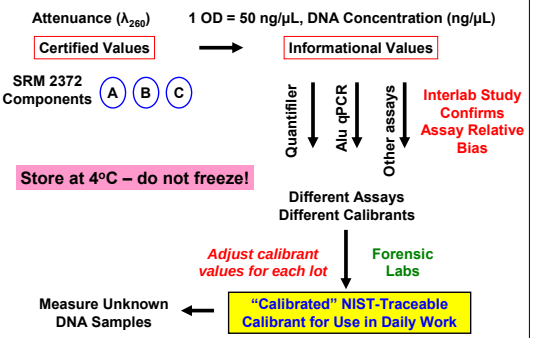
## Selection of DNA Sources

Based on previous work, more consistent DNA calibration solutions are obtained from human Buffy coats than from cell lines.

- When the Calibrant DNA is 'different' than the 'unknown', a bias between the methods is observed
- When the Calibrant DNA and 'unknown' are similar, there is less Method Bias observed
- This makes cross comparisons of Methods difficult when using different calibrants
- For practical purposes: a consistent method/calibrant may be optimized to give acceptable STR results **(within a lab)**
- However new calibrants (and new lots) should be performance checked for consistency

*Presented at 58th Annual AAFS meeting:*
*http://www.cstl.nist.gov/biotech/strbase/pub_pres/Vallone_AAFS2006_qPCR.pdf*

## Overview of SRM 2372 Values and Use




The workflow above illustrates the intended use of the SRM 2372 components. The SRM materials should be used to calibrate existing qPCR standards. It is **not** intended for everyday qPCR use or to replace existing kit standards.


These projects were and are supported by **Grant Number 1999-IJ-R-A094 and 2003-IJ-R-029**, which is an interagency agreement between NIJ and the NIST Office of Law Enforcement Standards, awarded by the National Institute of Justice, Office of Justice Programs, US Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position or policies of the US Department of Justice. Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.


## Component Preparation

- Components A and B were prepared from Buffy coats at NIST using a modified "Salt out" procedure. *Miller, S.A., Dykes, D.D., and Polesky, H.F. (1988) Nucleic Acids Res. 16 (3): 1215.*
- After the initial extraction and EtOH precipitation, the material was re-extracted to assure purity.
- Additionally Component A was treated with RNase prior to the second extraction.
- All components were solubilized from an air dried state in $TE^{-4}$ buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) that had been autoclaved.
- Volume prepared was from 210 mL to 250 mL of each component in Teflon containers.
- Materials were allowed to equilibrate several days prior to initial [DNA] determination, which was performed by scanning from $A_{220}$ to $A_{345}$ and measuring $A_{260}$.

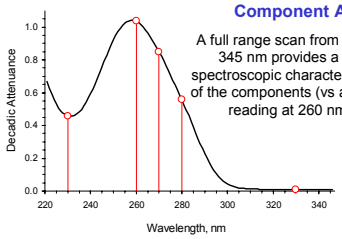
## Isolated DNA of Component B in Ethanol

DNA extracted from Buffy coats from anonymous female donors. The image is the re-extracted DNA in the final ethanol wash. The extracted DNA is floating toward the top of the 50 mL polypropylene centrifuge tube.

## US National Spectrophotometer HAS II

This 2nd generation High Accuracy Spectrophotometer is one of two National Level Reference Instruments at NIST

## Spectrophotometric Determination




**UV/Visible spectrum of Component A**

A full range scan from 220 to 345 nm provides a full spectroscopic characterization of the components (vs a single reading at 260 nm)

The **primary** wavelength readings were performed at **260 and 280 nm.**

The measurement at 260 nm allows for an estimation of the conventional DNA concentration.
An optical density (OD) reading equal to 1 can be converted to:

**OD = 1** **≈ 50 µg/mL dsDNA**
≈ 40 µg/mL ssDNA
≈ 33 µg/mL short DNA oligomers

The **ratio of 260 / 280** provides an estimate of contaminating protein. This ratio should be in the acceptable range of **≈ 1.8 to 2.0**.

Monitoring **additional** wavelengths can be used to further gauge purity.

Significant absorbance at **230 nm** can be an indication of phenolate ion, thiocyanates, and other organic absorbing compounds. Water saturated with phenols absorbs at **270 nm. A ratio of 260 / 270** ~ 1.2 indicates a preparation free of phenol. *Stulnig and Amberger 1994 BioTechniques;16:403-404* Significant absorbance at **330 nm** is typically caused by light scattering, indicating the presence of particulate matter.

## HAS II Certified Values of Decadic Attenuance for SRM 2372

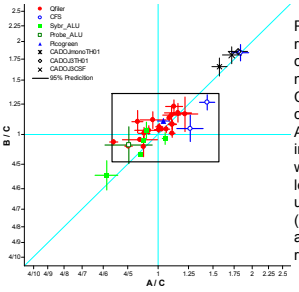
| Component | 260 nm | error at 260nm | Nominal [DNA], ng/µL |
|---|---|---|---|
| **A** | 1.049 | ± 0.025 | 52.5 |
| **B** | 1.073 | ± 0.030 | 53.6 |
| **C** | 1.086 | ± 0.028 | 54.3 |

5 mL were required to fill 2 cuvettes per component, each run in duplicate (4 replicate measurements).
The nominal DNA concentration was estimated *Using 1 OD = 50 ng/µL double stranded DNA.* ***We do not know the uncertainty in this conversion.***

## Interlaboratory Study

32 laboratories participated

Net result of the study: the SRM materials are appropriate for use with different qPCR methods

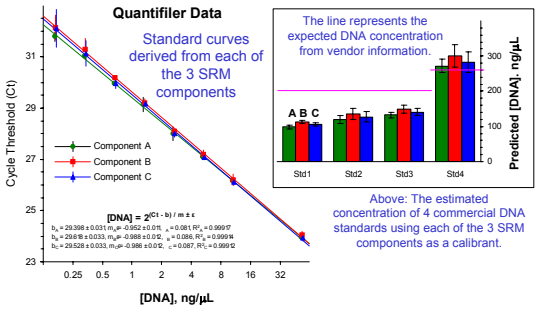



Participants were asked to run their qPCR method of choice using the SRM materials. SRM component C was used to determine the concentration of components A and B. Samples were run in duplicate on a plate. Labs were also asked to run at least 2 plate experiments under different conditions (new dilution series, different analyst, instrument, reagents, day of analysis)

*While the Interlaboratory data for Component B looks good, it failed homogeneity testing resulting in all 1700 vials being* ***disposed*** *of and a* ***new lot*** *of Component B was produced.*

## Value Assignment

- As a practical example we calibrated **4 different commercial DNA standards**
- Standards were serially diluted 10, 50, 100 and 200 fold
- All samples were analyzed in duplicate (Quantifiler qPCR assay)
- Standard curves derived from **all 3 SRM components** were used to determine the concentrations of the commercial standards




Above: The estimated concentration of 4 commercial DNA standards using each of the 3 SRM components as a calibrant.

**The table below is a summary of the results using Component A as the calibrant. Results obtained using components B and C were similar. Deviations from the stated DNA concentration were as high as almost 50%**

| Standard | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|
| Dilution | [DNA] | SD | [DNA] | SD | [DNA] | SD | [DNA] | SD |
| 10x | 105 | 3.2 | 122 | 1 | 126 | 5.8 | 256 | 10.1 |
| 50x | 105 | 3.3 | 122 | 7.3 | 145 | 0.8 | 272 | 7.8 |
| 100x | 99 | 6.2 | 113 | 11.6 | 138 | 0.5 | 270 | 10.5 |
| 200x | 100 | 1.7 | 137 | 18.5 | 137 | 3.9 | 311 | 3.7 |
| Average | 102 | | 123 | | 136 | | 277 | |
| Stated | 200 | | 200 | | 200 | | 260 | |
| Deviation | -49% | | -38% | | -32% | | 6% | |

DNA concentrations listed above are in units of ng/µL

**Conclusions:**
**SRM 2372 will be made available in the fall of 2007. Manufacturers can use SRM 2372 to validate the values assigned to their own reference materials. Individual forensic laboratories can use SRM 2372 to validate DNA quantitation methods and to verify the assigned concentration of in-house or commercial DNA calibration standards. The materials are NOT intended for everyday usage or to replace commercial DNA standards.**

*Poster available for download from STRBase http://www.cstl.nist.gov/biotech/strbase/pub_pres/ValloneISFG2007poster.pdf*