Development of the Next Generation of Forensic DNA Standard Reference Material: SRM 2372a

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Potomac Regional Symposium
April 27, 2018

Disclaimer

I will mention commercial platforms, but am in no way attempting to endorse any specific product.

**NIST Disclaimer**: 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 it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.
Development of NIST SRM 2372a

• Review of SRM 2372a and why it benefits forensic laboratories

• Examination of the next generation of certification measurements
  • From UV absorbance to Digital PCR

• Overview of the development process of SRM 2372a

What is SRM 2372 Human DNA Quantitation Standard?

SRM 2372 was originally released in 2007

Component A: Single-source male
Component B: Multi-source female
Component C: Multi-source male/female mixture

Certified for spectroscopic traceability in units of decadic attenuance, $D_{10}$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A (ng/µL)</th>
<th>B (ng/µL)</th>
<th>C (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012 DNA Mass Concentration</td>
<td>57</td>
<td>61</td>
<td>59</td>
</tr>
</tbody>
</table>

Sold out: May 2017
Forensic Need for SRM 2372a

Is the manufacturer assigned concentration accurate?

Standard DNA within Quant Kits

- Examined 8 different lots of standard DNA within one commercial quantitation kit
  - 8 individual lots
  - Never opened/used
  - 1:5, 1:10, and 1:20 dilutions were made
    - To allow for the samples to fall within the standard curve
- SRM 2372 component A used to generate standard curve
- All commercial quantitation kit dilutions were run in triplicate and per manufacturer’s recommendations
8 Commercial DNA Standards

DNA Standard derived from a human cell line found in commercial qPCR kits
Nominal value assigned from manufacturer: 200 ng/µL

Inconsistency in manufacturer assigned concentration to commercial DNA standards

qPCR vs. Digital PCR

- Quantification of the same 8 DNA standards
- No calibration curve – absolute quantification

- Alternate target from hTERT
  - HBB1 – housekeep gene on chromosome 11

<table>
<thead>
<tr>
<th>Assay Target</th>
<th>Chromosome, Band Accession #</th>
<th>Primers and Probe^c</th>
<th>Amplicon Length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBB1 Gene HBB</td>
<td>Chr 11, p15.5 NC_000011.10</td>
<td>F gcggtagggttgaagttcac</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R ggtcaatagtagacgcgtactc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P ggcaggttccagaagagcaangga</td>
<td></td>
</tr>
</tbody>
</table>
What is Digital PCR?
Partitioning of samples into individual chambers or droplets

No need for a standard curve

Positives
(1 copy of the target)

Negatives
(0 copies of the target)

dPCR is counting accessible amplifiable targets

hTERT (qPCR) vs. HBB1 (ddPCR)

Difference between examining the hTERT target and an alternate target (HBB1) within the genome.
Example of DNA Standard Bias

- Use of cell lines for production of commercial DNA standards—deviation from wild type DNA due to characteristics of cell lines
- Example: Raji cell line used for a commercial DNA standard
  - More copies in ~85% of all tested immortalized cell lines

Practical Effect

Goal of 1:1 mixture

Assay #1: Quantifier Trio with kit standard

Assay #2: PowerQuant with kit standard

ddPCR #3: Combination of 3 assays

2.5x difference
What can laboratories do to ensure more accurate quantitation results?

Human DNA Quantitation Standard

Commercial DNA Standard

qPCR

Assigned Value: 136 ng/µL

PROPER USE OF SRM 2372A

STANDARD CURVE

DILUTION OF “UNKNOWN”

Calculate and assign the NEW concentration to the commercial DNA Standard

This is not necessarily what is written on the tube by the manufacturer.
## Assigning a value to your material

<table>
<thead>
<tr>
<th>Diluted sample</th>
<th>Serial Dilution</th>
<th>qPCR Result ng/µL</th>
<th>Std dev ng/µL</th>
<th>Dilution Factor</th>
<th>[DNA] ng/µL</th>
<th>Std dev ng/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unk 1</td>
<td>1→10</td>
<td>12.6</td>
<td>0.58</td>
<td>x10</td>
<td>126</td>
<td>5.8</td>
</tr>
<tr>
<td>Unk 2</td>
<td>1→5</td>
<td>2.9</td>
<td>0.02</td>
<td>x50</td>
<td>145</td>
<td>0.8</td>
</tr>
<tr>
<td>Unk 3</td>
<td>1→2</td>
<td>1.4</td>
<td>0.01</td>
<td>x100</td>
<td>138</td>
<td>0.5</td>
</tr>
<tr>
<td>Unk 4</td>
<td>1→2</td>
<td>0.7</td>
<td>0.02</td>
<td>x200</td>
<td>137</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Newly assigned value to your daily use calibrant is the mean of the [DNA] column = 136 ng/µL

When using this calibrant in the future, the starting concentration will be 136 ng/µL

## Concentrations of commercial DNA standards needs to be performed with each lot of material

## Conclusions

- Multiple sources of bias exist in qPCR, some of which cannot be remediated
  - Bias from commercial DNA standards can be remediated with calibration to SRM 2372a
  - Additionally, SRM 2372a may aid in identifying forms of bias within qPCR technologies during internal validation within laboratories

- SRM 2372a should be used to make an outside material NIST Traceable for everyday use within a laboratory to limit the bias between commercial DNA standards

- It is important to keep in mind that using DNA quantitation as a gate keeper is impacted by new qPCR targets and STR kit PCR buffer formulations
  - Insensitive qPCR assays or inaccurate DNA standards may not accurately reflect the ability of new, more sensitive STR kits to obtain results
SRM 2372a: Human DNA Quantitation Standard

To be used as a qPCR calibrant OR to assign a value to a ‘pot’ of DNA – in house or commercial

<table>
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<tr>
<th>Component</th>
<th>Copy Number (per µL)</th>
<th>DNA (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (red cap)</td>
<td>15.1 ± 1.5</td>
<td>49.8 ± 5.0</td>
</tr>
<tr>
<td>B (white cap)</td>
<td>17.5 ± 1.8</td>
<td>57.8 ± 5.8</td>
</tr>
<tr>
<td>C (blue cap)</td>
<td>14.5 ± 1.5</td>
<td>47.5 ± 4.8</td>
</tr>
</tbody>
</table>

Table 1. Certified Values of Number and Mass Concentration for SRM 2372a

The copy number values are metrologically traceable to the natural units count 1 and ratio 1 and International System of Units (SI) derived units of volume. The DNA mass concentration values are metrologically traceable to the natural units count and ratio 1 and SI derived units of mass and volume.

SRM 2372a became available for purchase March 26, 2018
What has changed with SRM 2372a?

UV Absorbance   Digital PCR (dPCR)

How did we get here?

Steps to Develop SRM 2372a

Development and validation of digital PCR assays and platforms → File paperwork with Human Subjects (MTA, SRI, Determination) → With approval, buffy coat samples are ordered and extracted manually

Preliminary screening of bulk material, UV measurements, dilution to target concentration → Bottling and labeling → Homogeneity Testing, Certification, and Stability Studies performed

Report of Analysis, NIST Special Publication, Certificate of Analysis complete and approved
Why use dPCR for certification?

- No need for an external calibrant
- Multiple dPCR assays can be used for characterization
  - Establish reasonable estimates of uncertainty
- More accurate form of concentration measurement for end user

dPCR platforms at NIST

Droplet Digital - ddPCR

Chamber Digital - cdPCR

BIO-RAD QX200

Fluidigm BioMark
“Absolute” Quantitation at NIST

NIST has developed and optimized >10 dPCR assays for absolute quantitation.

Optimized Assays for SRM 2372a

10 assays across 8 different chromosomes

All assays are single copy, and Human, or Primate specific.
Importance Assay Design

- Single copy target assays only (for Abs quant)
  - NCBI BLAST search to assess genomic targets
- Not expecting all assays designed to give the same target number (genome accessibility)
Converting copies per nanoliter to nanograms nuclear DNA per microliter

This allows for SRM 2372a to be certified for ng/µL

\[
\text{nDNA}_{\text{ng/µL}} = \left( \frac{C_{\text{copies}}}{\text{µL mixture}} \right) \left( \frac{µL}{\text{droplet}} \right) \left( \frac{mL}{\text{sample}} \right) \left( \frac{V}{µL} \right) \left( \frac{r_{\text{target}}}{\text{mol base pairs}} \right) \left( \frac{6.022 \times 10^{23} \text{ base pairs}}{\text{mol}} \right) \left( \frac{10^{3} \text{ ng}}{10^{9} \text{ g}} \right)
\] (1)

where \( r \) is the number of assay targets per human haploid genome equivalent (HHGE), \( n \) is the number of nucleotide base pairs (bp) per double-stranded HHGE, and \( m \) is the average molar mass of a bp in the DNA polymer.

For independent multiplicative factors such as these, the combined relative uncertainty of their product can be estimated from the square root of the sum of squares of the individual relative uncertainties [4, Section 5.1.6]:

\[
\frac{u(\text{nDNA})}{\text{nDNA}} = \sqrt{\left( \frac{u(C)}{C} \right)^2 + \left( \frac{u(V)}{V} \right)^2 + \left( \frac{u(m)}{m} \right)^2 + \left( \frac{u(n)}{n} \right)^2 + \left( \frac{u(mHGE)}{mHGE} \right)^2}
\] (2)

Duewer DL, Kline MC, Romsos EL, Toman B. Anal Bioanal Chem. 2018 May;410(12):2879-2887

Converting copies per nanoliter to nanograms nuclear DNA per microliter

This allows for SRM 2372a to be certified for ng/µL

Copies per nanoliter

\[
\text{[DNA]}_{\text{ng/µL}} = \text{[DNA]}_{\text{HHGE}} \times \frac{3.301 \text{ ng}}{\text{HHGE}}
\]

Table 1. Certified Values of Number and Mass Concentration for SRM 2372a(a)

The copy number values are metrologically traceable to the natural units count 1 and ratio 1 and International System of Units (SI) derived units of volume. The DNA mass concentration values are metrologically traceable to the natural units count and ratio 1 and SI derived units of mass and volume.

<table>
<thead>
<tr>
<th>Component</th>
<th>Copy Number(a) (per nL)</th>
<th>DNA(a) (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (red cap)</td>
<td>15.1 ± 1.5</td>
<td>49.8 ± 5.0</td>
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Duewer DL, Kline MC, Romsos EL, Toman B. Anal Bioanal Chem. 2018 May;410(12):2879-2887
Mitochondrial DNA Quantification

- Challenging to create a commutable standard
  - Degradation of plasmids
  - Contamination
  - Inefficient amplification
  - Cell line vs. genomic DNA

<table>
<thead>
<tr>
<th>Population (US)</th>
<th>n</th>
<th>Mean (mtDNA/gDNA)</th>
<th>SD (mtDNA/gDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>27</td>
<td>115</td>
<td>22</td>
</tr>
<tr>
<td>Hispanic</td>
<td>30</td>
<td>106</td>
<td>22</td>
</tr>
<tr>
<td>African American</td>
<td>26</td>
<td>130</td>
<td>22</td>
</tr>
<tr>
<td>Cell Lines</td>
<td>30</td>
<td>457</td>
<td>176</td>
</tr>
</tbody>
</table>

Mitochondrial DNA Quantification

Mitochondrial to genomic DNA ratio information included in SRM 2372a

Optimized 3 qPCR assays into digital PCR assays

<table>
<thead>
<tr>
<th>Component</th>
<th>mtDNA/gDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (red cap)</td>
<td>174 ± 4</td>
</tr>
<tr>
<td>B (white cap)</td>
<td>206 ± 5</td>
</tr>
<tr>
<td>C (blue cap)</td>
<td>279 ± 7</td>
</tr>
</tbody>
</table>

SRM 2372a provides the ratio of mtDNA to gDNA
Steps to Develop SRM 2372a

Development and validation of digital PCR assays and platforms → File paperwork with Human Subjects (MTA, SRI, Determination) → With approval, buffy coat samples are ordered and extracted manually

Preliminary screening of bulk material, UV measurements, dilution to target concentration → Bottling and labeling → Homogeneity Testing, Certification, and Stability Studies performed

Report of Analysis, NIST Special Publication, Certificate of Analysis complete and approved

Before Beginning SRM 2372a

There was Human Subject Protections paperwork

Excluded Human Data/Specimens Form

Complete this form when your research study fits into one of the categories below and this is the primary use of the specimens and/or data. The form should be routed through your OU for approval and submitted to the HSPO for acknowledgement and tracking before beginning the work on this study.

NREL-16-0003-EXCL

And approval

This HSP has received your proposed project using only excluded specimens and/or data that meet the criteria for exclusion: subject research as defined in Department of Commerce regulations; 31 CFR 47, also known as the Common Rule (45 CFR 46, Subpart B) for the protection of human subjects; as indicated in your documentation. More specific information can be found in (insert individual). The additional information on the form has been supplied by the investigator, and the form has been completed by the investigator. The investigator is responsible for determining the appropriate level of information to be included in the form. The investigator is also responsible for obtaining any necessary approvals for the research, as indicated in your documentation.
Acquiring Materials and Extraction

Buffy coat samples were purchased from a blood bank.

DNA was extracted using a manual method for high quality and high yield.

The beginning stages

DNA was allowed to solubilize and reconstitute in Teflon pots in TE-4.

Preliminary screening and UV measurements
The beginning stages cont.

- UV measurements were made on the bulk pots of the four DNAs
  - DNA were diluted to ~50 ng/µL

- Component C mixture was gravimetrically prepared as a 1→3 mixture (Male:Female)
  - Preliminary testing was repeated

- Once material is proven to behave as expected with ddPCR the **bottling process begins**
Bottling and Labeling Process

Labelers check the caps, add a SRM label, and verify volume of product.

Completed units for testing
Homogeneity, stability and certification

2100 units of each component produced
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SRM 2372a became available for purchase March 26, 2018

Homogeneity and Stability

• Homogeneity: 22 vials in duplicate with 2 assays

• Stability:
  • 3 temperatures (4 °C, 22 °C, 37 °C)
  • Two vials in duplicate with 2 assays
Certification Measurements

• Once materials were determined to be homogeneous and stable certification measurements were made.

10 genomic DNA assays and 3 mitochondrial DNA assays
6 independent measurements

Value Assignment

• Data from the homogeneity, stability, and certification measurements were compiled for value assignment.
  • Uncertainty assigned to all measurements

Table 25: Recommended Values and 95% Uncertainties for Certification

<table>
<thead>
<tr>
<th>Component</th>
<th>Units</th>
<th>Value</th>
<th>Un(Value)%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Copies per nanoliter</td>
<td>15.1</td>
<td>1.5</td>
</tr>
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</tr>
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<td>5.0</td>
</tr>
<tr>
<td>B</td>
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<td>C</td>
<td>ng/μL</td>
<td>45.9</td>
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</tr>
<tr>
<td>A</td>
<td>mtDNA/nDNA</td>
<td>174</td>
<td>4</td>
</tr>
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<td>B</td>
<td>mtDNA/nDNA</td>
<td>206</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>mtDNA/nDNA</td>
<td>279</td>
<td>7</td>
</tr>
</tbody>
</table>
Paperwork Stage

Internal Report of Analysis

NIST Special Publication 260-189

This details the production, evaluation, and certification measurements for SRM 2372a

SRM 2372a became available for purchase March 26, 2018
SRM 2372a provides the ratio of mtDNA to gDNA

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SRM 2372a provides the ratio of mtDNA to gDNA

**Digital PCR at NIST**

- Digital PCR has become our ‘go to’ method for the quantification of nucleic acid-based materials
- Replacing UV spectroscopy (indirect method)
- The typical downstream application of our reference materials is PCR or sequencing-based
  - We care about intact (and accessible) genomic targets
- SRM 2372a provides a certified value for DNA concentration in ng/µL
  - mtDNA/nDNA ratio now provided
Acknowledgments

NIST Team for This Work

Margaret Kline  Dave Duewer  Blaza Toman  Pete Vallone

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301-975-5107

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