Application of a DNA Quantitation Standard for Human Identity Testing

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Outline

- What is SRM 2372?
- Why is it used?
- How should it be used within a laboratory?

Steps in Forensic DNA Analysis

Steps Involved

- Collection
- **Extraction**
- **Quantitation**
- **Multiplex PCR**
- **STR Typing**
- **Interpretation of Results**

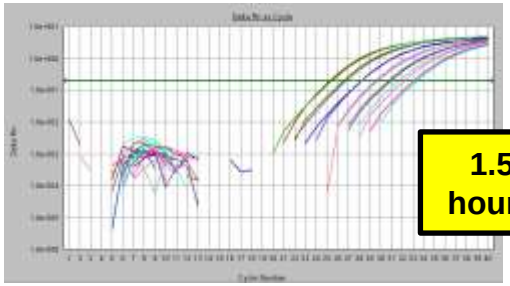
Blood Stain

Buccal swab

Sample Collection

1.5 hours

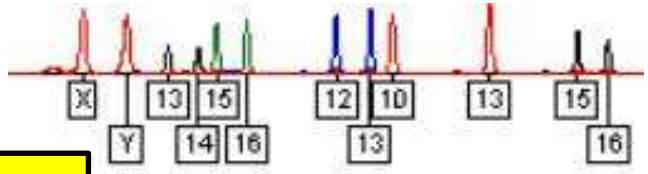
DNA Extraction



1.5 hours

DNA Quantitation

DNA separation and sizing



1.5 hours

STR Typing

Interpretation of Results

3.5 hours

Multiplex PCR Amplification

1-2 day process (a minimum of ~8 hours) with current laboratory procedures and technology

What is SRM 2372 Human DNA Quantitation Standard?

Genomic DNA prepared to be double-stranded DNA (dsDNA)

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

All solubilized in TE^{-4} buffer (10mM Tris, 0.1 mM EDTA, pH 8.0)

Certified for spectroscopic traceability in units of decadic attenuance, $D_{10.}$ The D_{10} scale is a measure of absorbance and is traceable to the unit 1.

The conventional conversion factor for aqueous DNA:
dsDNA 1.0 D_{10} at 260 nm = 50 ng/µL DNA
ssDNA 1.0 D_{10} at 260 nm = 37 ng/µL DNA

SRM 2372 Certificate of Analysis

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2372

Human DNA Quantitation Standard

Standard Reference Material (SRM) 2372 is intended primarily for use in the value assignment of human genomic deoxyribonucleic acid (DNA) forensic quantitation materials. It is not intended for any human or animal clinical diagnostic use. SRM 2372 consists of three well-characterized human genomic DNA materials solubilized in 10 mmol/L 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris HCl) and 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt (disodium EDTA) using deionized water adjusted to pH 8.0 (TE^{-4}, pH 8.0 buffer). The three component genomic DNA materials, labeled A, B, and C, are respectively derived from a single male donor, multiple female donors, and multiple male and female donors. A unit of the SRM consists of one sterile 2-milliliter vial of each component, each vial containing approximately 110 µL of DNA solution. Each of these vials is labeled and is sealed with a color-coded screw cap.

Certified Values: Table 1 lists the certified decadic attenuance, D_{10}, at five wavelengths in the ultraviolet spectral region for a cell pathlength of 1.0 cm, at a spectral bandwidth of 0.8 nm, and a temperature of 22 °C ± 1 °C. These values are calculated from measured corrected transmittances (T) as $-\log_{10}(T)$ and are indicated by the absorbance scale of the spectrophotometer when the materials are measured against TE^{-4}, pH 8.0 buffer. The D_{10} scale is of unit 1 (*unitless*). See "Primary Measurement of Decadic Attenuance" below for further information.

A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by NIST. The expanded uncertainty defines an interval within which the unknown value of attenuance can be asserted to lie with a level of confidence of approximately 95 % [1]. The expanded uncertainties of these attenuance values are dominated by the instrument dependence of spectral scatter detection and sample homogeneity. See "Determination of Expanded Uncertainties" below for further information.

Information Values: Table 2 lists information values for the DNA mass concentration of SRM 2372 components. These values are derived from the conventional assertion that a solution of double-stranded DNA with an absorbance of 1.0 at 260 nm and a pathlength of 1.0 cm has a DNA mass concentration of 50 µg/mL (50 ng/µL) [2]. An information value is considered to be a value that will be of use to the SRM user, but insufficient information is available to assess the uncertainty associated with the value.

Expiration of Certification: The certification of SRM 2372 is valid, within the measurement uncertainties specified, until **31 July 2012,** provided the SRM is handled and stored in accordance with the instructions given in this certificate (see "Storage" and "Instructions for Use"). However, the certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

First released October 2007

DNA was certified in the double-stranded form for UV absorbance

Temporarily taken off of the market March 2012

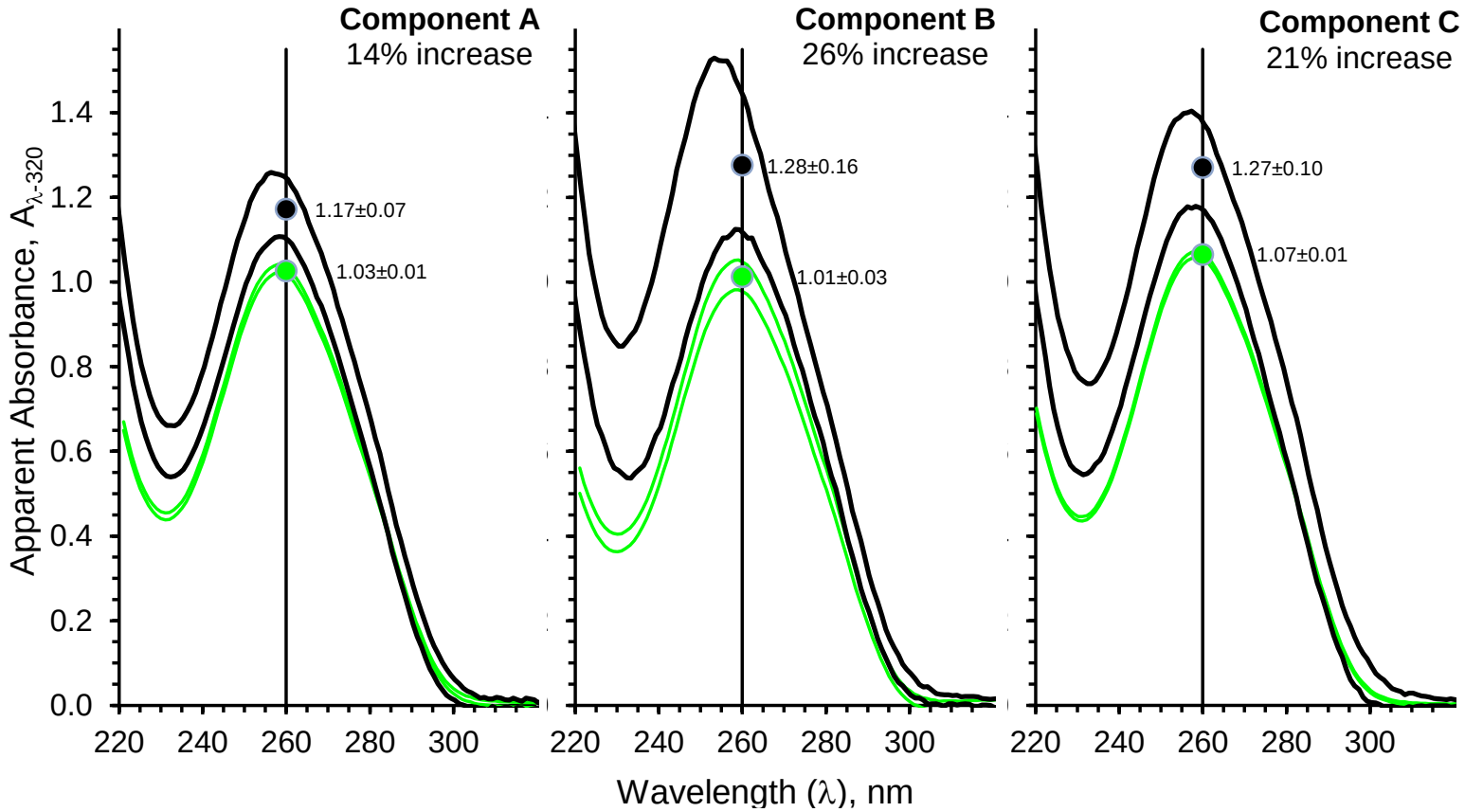
Why was SRM 2372 Taken off the Market in 2012?

- During measurement of the DNA samples to verify stability of certified values the UV absorbance values for the sample increased
  - Not due to degradation of the DNA
  - Due to unraveling or opening up of the DNA strands in the TE^{-4} Buffer
    - Single-stranded DNA absorbs more UV light than double-stranded DNA
  - **SRM 2372 is certified for UV absorbance**

The changes over time which impacted the UV absorbance, did not affect the qPCR performance

Why did SRM 2372 need to be re-certified?

Six years after production the D_{10} absorbance of these dsDNA solutions had *increased* significantly, suggesting partial conversion to single-stranded DNA (ssDNA)



Green Traces 2006 low/high absorbance spectra
Black Traces 2012 low/high absorbance spectra

How did we re-certify SRM 2372?

- Material was forced to all ssDNA conformation
- Measurements were made using a modification of ISO 21571 Annex B “Methods for the quantitation of the extracted DNA”
  - Combine equal volumes of the DNA extract and freshly prepared 0.4 mol/L NaOH
  - Measure against equal volumes of TE^{-4} buffer and the 0.4 mol/L NaOH
- Apparent Absorbance is $D_{10\ (260\ nm)} – D_{10\ (320\ nm)}$

Component A	Component B	Component C
0.777 (0.725 – 0.829)	**0.821** (0.739 – 0.903)	**0.804** (0.753 – 0.855)

Conversion of Apparent Absorbance to ng/µL

- Assertion that a solution of ssDNA with an absorbance of 1.0 at 260 nm and a pathlength of 1.0 cm has a DNA mass concentration of 37 µg/mL (37 ng/µL)

Parameter	A	B	C
2012 DNA Mass Concentration	57	61	59
2007 DNA Mass Concentration	52.4	53.6	54.3
Theoretical difference, %	9 %	14 %	9 %
Theoretical difference, Ct	0.12 cycle	0.19 cycle	0.12 cycle

The difference between the original value and re-certified values is within the noise of the assay.

SRM 2372 went back on sale December 31, 2012

Why use SRM 2372?

Standard DNA within Quant Kits

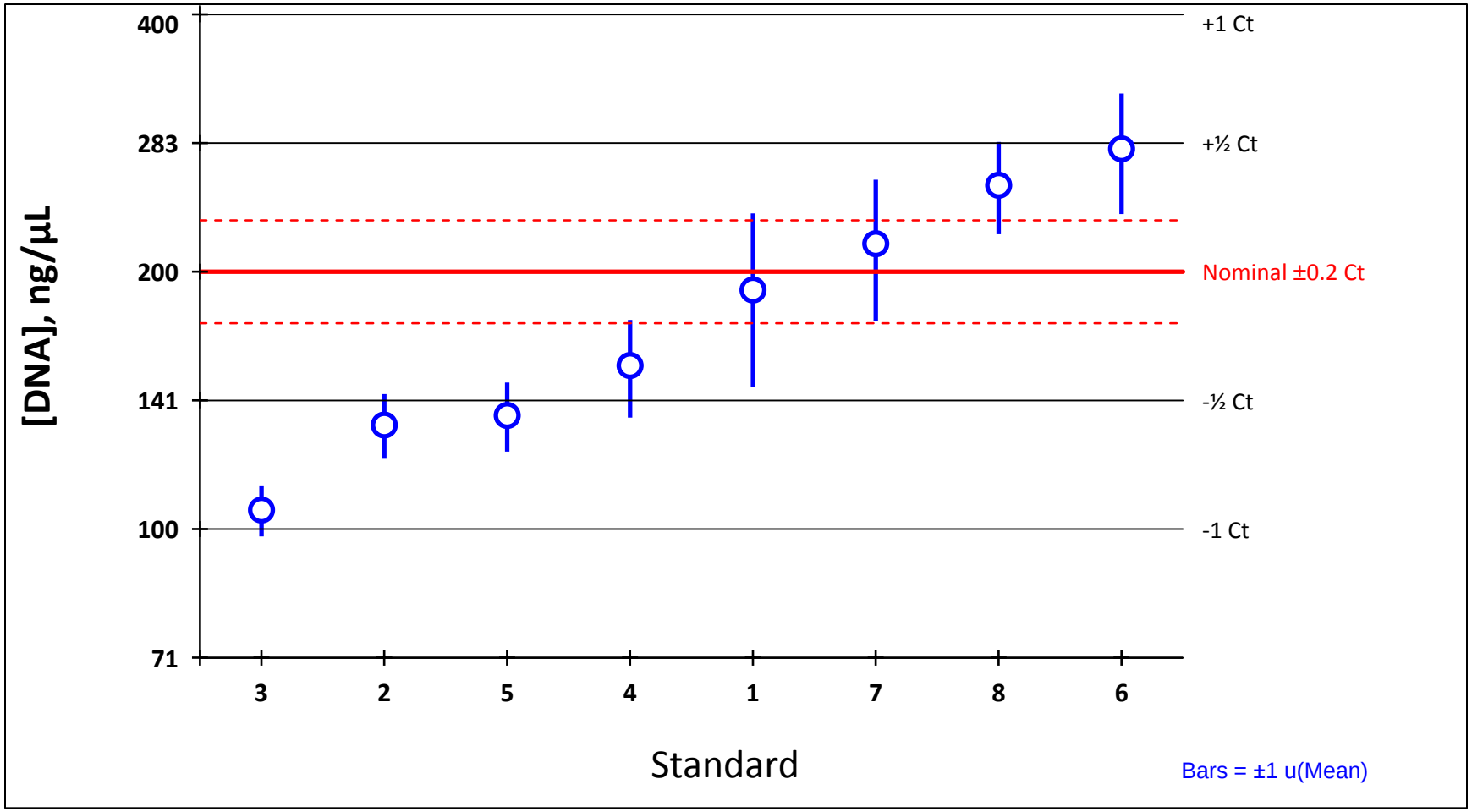
- Standard DNA is provided within every commercial quantitation kit
- Manufacturer recommended protocols use this sample to assign the standard curve
  - Which is then applied to unknown samples to derive a concentration

- Question: Are all lots of a commercial DNA standard the same concentration?

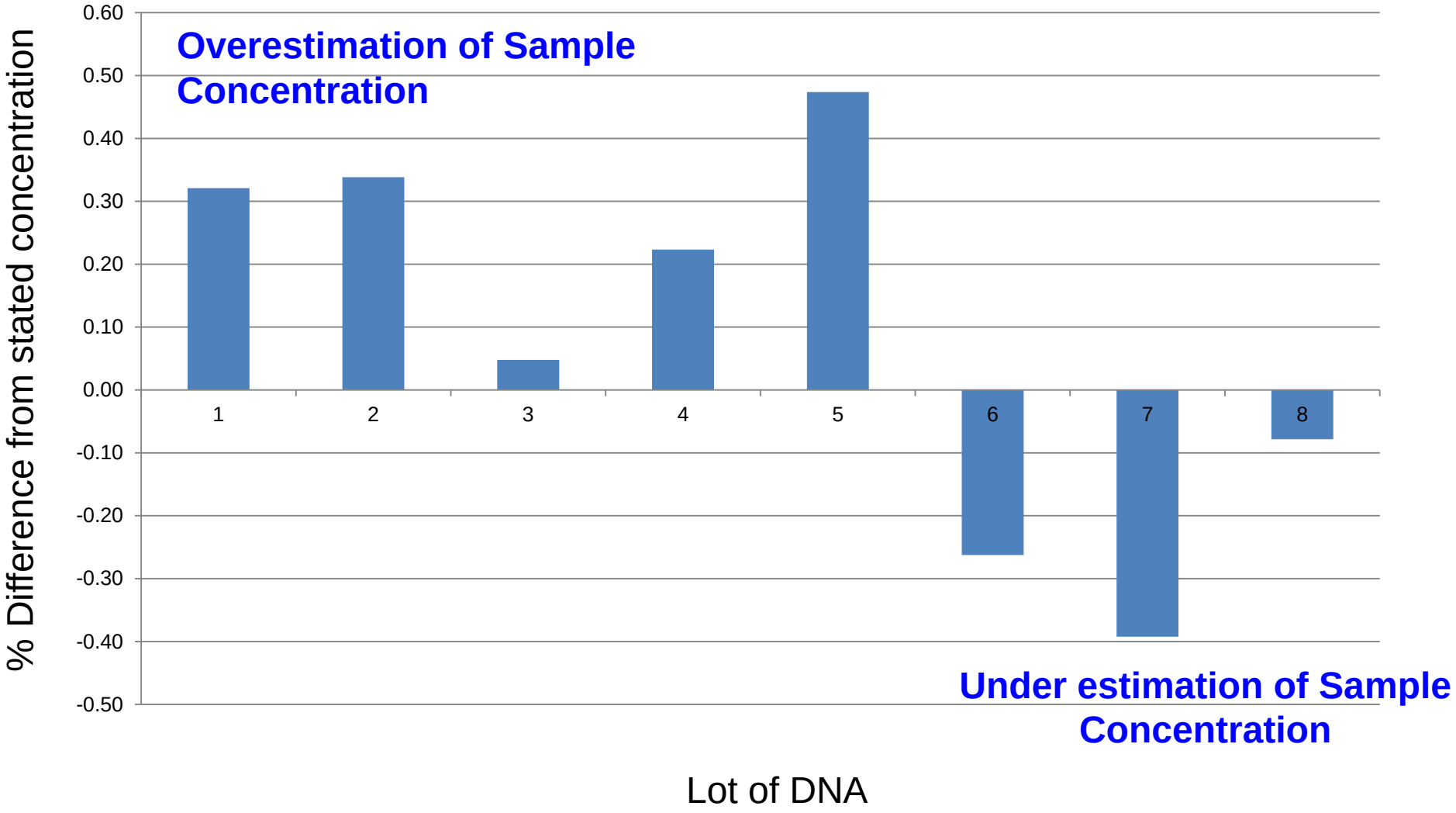
Standard DNA within Quant Kits

- 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 Independent Lots of DNA Quant Standard



Effect of Commercial Standard Variation on Unknowns



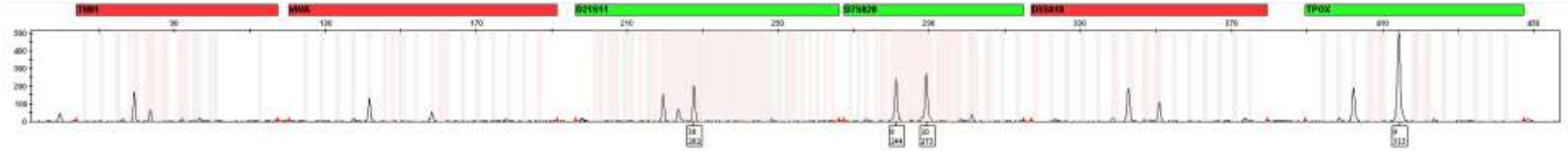
What does this mean?

- If assuming the concentration provided by the manufacturer you could possibly over/under estimate your [DNA] by up to 50%

Unknown Sample		Quant Results
1 ng		0.5 ng to 2 ng

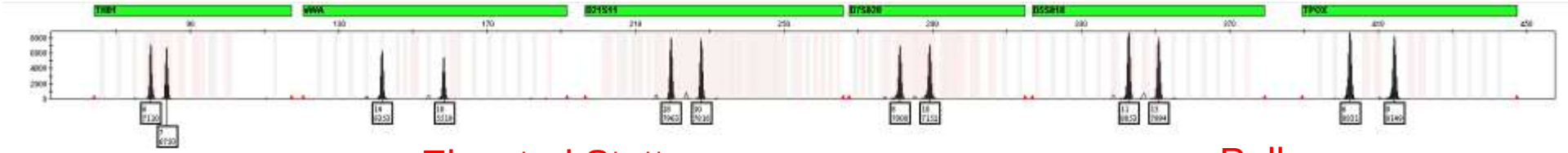
Effects on STR Typing

Overestimation of sample concentration: Not enough sample added into PCR



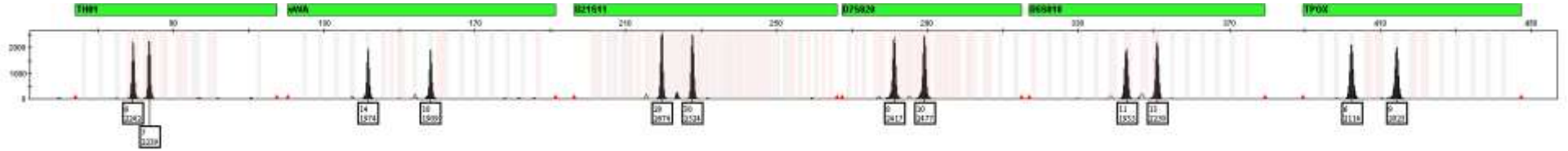
Dropout Elevated Stutter Imbalance

Underestimation of sample concentration: Too much sample added into PCR



Elevated Stutter Pullup

Use of SRM 2372 as a calibrant: Correct amount of DNA added into PCR



Well balanced profile with minimal stochastic effects

How will you use SRM 2372?

SRM 2372

- SRM 2372 is to be used as the standard curve to assign quant values to your every day use calibrant
- Dilute the material you are going to use as your daily use calibrant to be a [DNA] within the linear range of your qPCR assay
  - These will be your “unknowns” within your qPCR plate
- Factoring in the dilutions, assign a [DNA] to your daily use material.

The newly assigned concentration may be different than what was originally labeled on the tube from the manufacturer, but this newly assigned concentration is now NIST traceable.

Making your material NIST Traceable

- Analyze **your materials** (eg. a standard DNA provided within a commercial quant kit) with **your DNA Quantification Methods** (eg. Quantifiler, Plexor, Quantiplex, etc)
- Assign a [DNA] of **your material** based on the values obtained using SRM 2372 materials to generate your standard curve
  - **Your material** will be the unknowns on the qPCR plate

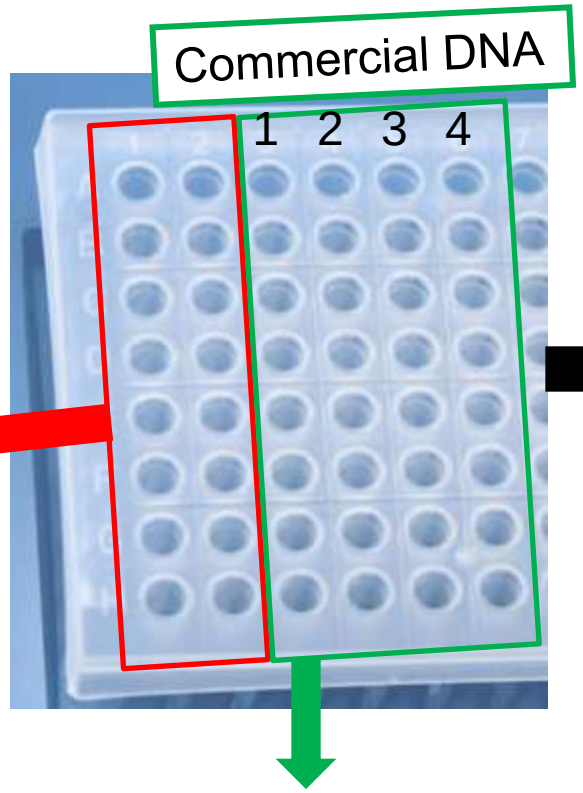
Serial Dilution Scheme for SRM 2372 Components

Components

Dilution	µL TE^{-4}	µL DNA	**A** [DNA] ng/µL	**B** [DNA] ng/µL	**C** [DNA] ng/µL
neat	0	10	57	61	59
1→5	16	4	11.4	12.2	11.8
1→5	16	4	2.28	2.44	2.36
1→2	10	10	1.14	1.22	1.18
1→2	10	10	0.57	0.61	0.59
1→2	10	10	0.28	0.3	0.29
1→2	10	10	0.14	0.15	0.15

These newly recertified values are slightly increased from the original certified values.

How to use SRM 2372



Dilution Curve of SRM 2372 in duplicate

Every day use calibrant dilutions (this is the material you will make your future standard curves from)

Dilutions (4) of your every day use calibrant make up the unknown dilution columns.

Assign a quant value to your every day use calibrant.

This value is now the permanent quant value for this current lot of material.

Dilutions for standard curves are to be made from this material with the newly assigned quant value.

Any new lots of every day use calibrant material must be re-assigned a quant value against the SRM 2372 materials.

Assigning a value to your material

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

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 <u>regardless</u> of what is labeled by the manufacturer on the tube

Conclusions

- NIST SRM 2372 has been re-certified through forcing dsDNA to become ssDNA in order to improve the UV absorbance measurements
- qPCR measurements **have not been** significantly impacted by the new certified (and DNA concentration) values
- SRM 2372 should be used to make an outside material NIST Traceable for everyday use within a laboratory
- 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 to generate standard curves may not accurately reflect ability of new, more sensitive STR kits to obtain results

Acknowledgments

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