Using SRM 2372 Human DNA Quantitation Standard
Are there differences between qPCR assays?

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Modern highly-multiplexed short tandem repeat (STR) assays used by the forensic human identity community require tight control of the initial amount of sample DNA amplified in the polymerase chain reaction (PCR) process. This in turn requires the ability to reproducibly measure the concentration of human DNA, [DNA], in a sample extract. Quantitative PCR (qPCR) techniques can assay the number of intact stretches of DNA of specified nucleotide sequence in an extremely small sample; however, these assays must be calibrated with DNA extracts of well characterized and stable composition. Because of the variability in the targeted sequences of various qPCR assays, some variability in the quantitation results are sometimes seen. We have been studying these differences between qPCR assays.

Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard was prepared to serve as a way to assign a [DNA] to qPCR "kit" calibrants. Interlaboratory data from 32 laboratories (6 different qPCR assays) as well as five qPCR methods performed at NIST were used in evaluating the suitability of the materials used to prepare SRM 2372. Additionally we have evaluated two newer qPCR kits with SRM 2372 components. The data from the new studies as well as detailed instructions for the proper use of SRM 2372 are presented.

Characteristics SRM 2372 Components

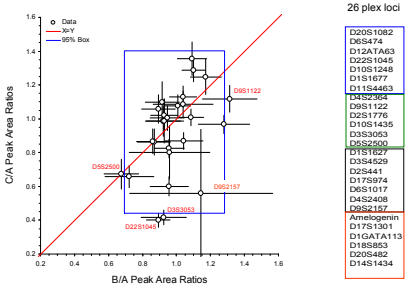
	A	B	C
Extraction	"salt out" & RNased	"salt out"	unknown RNased
Purification	EtOH ppt	EtOH ppt	Cesium chloride Ultracentrifugation
[DNA] Abs^{260}	52.4 ng/µL	53.6 ng/µL	54.3 ng/µL
Sample type	Blood	Blood	Tissue
Gender	Single Male	Multiple Female	Multiple Male & Female
# mtDNA copies/ng gDNA	A ≈ B	B ≈ A	**C ≈ ½ quantity of A or B**

Commercial qPCR assays examined

Probe qPCR Assays	Human	Y	Product size
Quantifiler Duo	RPPH1 (14p11.2)	SRY (Yp11.3)	140 bp/130 bp
Quantifiler Human	hTERT (5p15.33)		63 bp
Quantifiler Y		SRY (Yp11.3)	64 bp or 61 bp
Plexor HY	Chr 17 multicopy	Multicopy	99 bp /133 bp

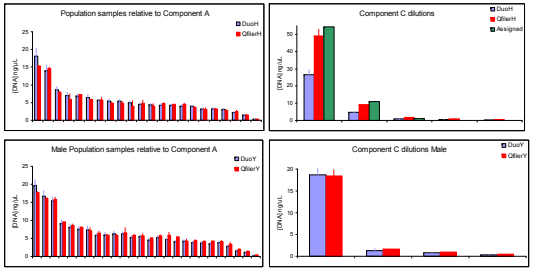
Anytime a new qPCR assay is developed and a new target locus is chosen, the material used to establish the calibration curve must be validated as "fit for purpose".

We demonstrate this with the NIST 26plex; there is considerable locus-to-locus variability among the loci with the SRM 2372 components. In the graph below, peak areas were normalized by the total peak area within a dye color.



The graph displays the ratios of the locus to color peak areas for C/A and B/A with associated approximate 95% confidence intervals. Data that fall on the x=y line indicate that both the multi-donor Components B and C are performing the same relative to Component A. Shifts off the x=y line indicate bias for components B or C. Loci D22S1045 and D3S3053 are outside the 95% confidence box and indicate Component C did not amplify as well as Component B. While D9S2157 is within the 95% confidence box, this larger-sized locus did not amplify well in the multi-source samples and too few data were available for reliable analysis.

Quantifiler Duo, Quantifiler Human & Quantifiler Y

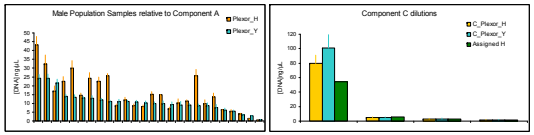


	Population Samples				Component C dilutions			
Ratio	DH/QH	DY/QY	Duo Y/H	Qfiler Y/H	DH/QH	DY/QY	Duo Y/H	Qfiler Y/H
Max	1.25	1.11	1.28	1.66	0.71	1.01	0.86	-
Min	0.87	0.76	1.00	1.08	0.41	0.78	0.56	-
Mean	1.04	0.93	1.10	1.3	**0.55**	0.87	**0.7**	**0.4**

DH – Duo Human, QH – Quantifiler Human, DY – Duo Male, QY – Quantifiler Y

The qPCR results for the Quantifiler Duo Human assay are biased (factor of 2) with respect to Component C of SRM 2372, while Components A and B have the expected results. However, as expected, the Y quantifications are similar between Duo male – Quantifiler Y since these kits have the same targets just different amplified product sizes.
In the table above a ratio of 1 indicates the assays being compared give equivalent results.

Plexor HY



Plexor	Population	Component C
Ratio	Y/H	Y/H
Max	2.00	1.39
Min	0.34	1.04
Mean	0.90	1.16

With Plexor, samples at 50 ng/µL appear to be outside the linear dynamic range of the assay. Samples at the lower concentrations, including Component C, are more consistent with the expected Y and autosomal [DNA]. There is a large sample-to-sample variability with the male/human ratios. This may be due to the multicopy loci used. More work is needed to verify these results.

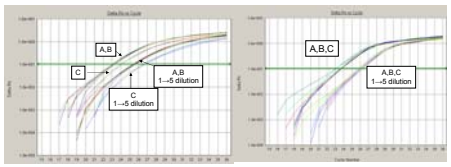
Sequencing

We further investigated and independently reproduced the Component C bias for the RPPH1 locus by designing SybrGreen qPCR assays and sequencing the RPPH1 locus. The 340 bp RPPH1 sequence was obtained from Genbank and two sets of primers were selected that amplified a 295 bp fragment for sequencing purposes and a 232 bp fragment for the qPCR assay.

The sequencing results obtained for the RPPH1 locus for Components A, B, and C were identical with the reference sequence.

Sybr Assay	Ct Difference		
Locus	$C^{Ct} - A^{Ct}$	$B^{Ct} - A^{Ct}$	Target size
D4S2364	-0.24	0.18	258 bp
D10S1435	-0.17	0.01	325 bp
D9S2157	0.42	0.08	497 bp
D22S1045	**0.7**	**-0.04**	**342 bp**
D3S3053	**1.2**	**0.07**	**245 bp**
RPPH1	**0.9**	**-0.10**	**232 bp**

RPPH1b SybrGreen assay | D4S2364 SybrGreen assay



The SybrGreen qPCR assay for RPPH1 had the same bias for Component C seen with Quantifiler Duo Human.
Several of the 26 plex loci were amplified in the SybrGreen qPCR using different primers than the ones used in the multiplex (large PCR products). The table expressing the difference in Ct values for Component C – A and Component B – A demonstrate the same bias seen in the other assays

Summary

Based on the information presented here, SRM 2372 components will perform differently depending on the chromosomal region (locus) targeted in the qPCR assay. There appears to be an "effective copy number" bias for component C. Therefore, it is strongly suggested that all three SRM 2372 components be used to evaluate new qPCR assays. **For Quantifiler Duo, we suggest using component A or B for assignment of [DNA] of the "standard".** For Plexor HY, more work is needed.

Poster available for download from STRBase: http://www.cstl.nist.gov/biotech/strbase/pub_pres/Promega2009poster.pdf

References

[1] Kline MC, Duewer DL, Travis JC, Smith MV, Redman JW, Vallone PM, Decker AE, Butler JM. (2009) Production and certification of NIST Standard Reference Material 2372 Human Quantitation Standard. Anal Bioanal Chem, 394: 1183-1192.

[2] Applied Biosystems (2003) Quantifiler human DNA quantitation kit and Quantifiler Y human male DNA quantitation kit user's manual, Part Number 4344790 Rev A. Foster City, CA.

[3] Krenke BE, Nassif N, Sprecher CJ, Knox C, Schwandt M, Storts DR. (2008) Developmental validation of a real-time PCR assay for the simultaneous quantification of total human and male DNA. FSI-Genetics, 3(1): 14-21.

[4] Hill, C.R., Butler, J.M., Vallone, P.M. (2009) A 26plex autosomal STR assay to aid human identity testing. J. Forensic Sci. 54(5): 1008-1015. -- see also Http://www.cstl.nist.gov/biotech/strbase/str26plex.htm.

NIST SRM 2372
See http://www.cstl.nist.gov/biotech/strbase/srm2372.htm

How to Use SRM 2372

(Assigning a DNA concentration to a daily use calibrant)

SRM → Assign value to → Everyday use calibrant

- Run SRM as the standard in a calibration curve and your everyday use calibrant as an unknown.
- Write the new – SRM assigned – value on the everyday use calibrant tube
- Use this new – SRM assigned – value for your everyday quantifications
- When changing lots of everyday use calibrant, repeat the above procedure to assign it a value

Serial Dilution Scheme for SRM 2372 Components

Dilution	µL TE^{-4}	µL DNA	A [DNA] ng/µL	B [DNA] ng/µL	C [DNA] ng/µL
neat	0	10	52.4	53.6	54.3
1→5	16	4	10.48	10.72	10.86
1→5	16	4	2.10	2.14	2.17
1→2	10	10	1.05	1.07	1.09
1→2	10	10	0.52	0.54	0.54
1→2	10	10	0.26	0.27	0.27
1→2	10	10	0.13	0.13	0.14
1→2	10	10	0.07	0.07	0.07

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. Run several dilutions of the daily use calibrant as "unknowns" along with the SRM 2372 diluted components that are used to establish the calibration curve.

Factoring in the dilutions, assign a [DNA] to your daily use material. This may be a different [DNA] than what was originally on the tube but this [DNA] is NIST traceable.

Diluted sample	Serial Dilution	qPCR Result	sd	Dilution Factor	[DNA] ng/µL	sd
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

Use the SRM material to assign the [DNA] to your daily use material. ***Dilution scheme presented is only a suggestion and may be modified.***