Cautionary Considerations When Exploring Cell Lines as Potential Reference Materials

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Poster # OTH05 :
Association for Molecular Pathology 2010 Annual Meeting.
San Jose, CA, November 17-20, 2010

Disclosure of Potential Competing Interests: *All authors are employed by the National Institute of Standards and Technology which produces and sells Standard Reference Materials*

Introduction: Cell lines can be a source of large quantities of genomic material (e.g. DNA) that can be used as reference materials. Reference materials are important tools in realizing a number of aspects of measurement quality and can be/are used for: method validation, calibration, estimation of measurement uncertainty, training, internal quality control (QC), and external quality assurance (QA) purposes (proficiency testing). Useful reference materials must be homogeneous, stable, and commutable with routine sample materials. Therefore cell lines selected to serve as reference materials must be thoroughly characterized to ensure that they have the properties needed for their intended use. For example: cell lines immortalized with Human Telomerase Reverse Transcriptase (hTERT) may not quantify "correctly" with one of the commercial qPCR methods; sequence mutations and aneuploidy may result in PCR amplification imbalances that are not expected by the end user including tri-allelic patterns and complete drop-out. These and other properties of 30 cell lines have been investigated during the research and production phase of several Standard Reference Materials® (SRMs) developed by the Applied Genetics Group at the National Institute of Standards and Technology (NIST) for use by the forensic DNA identity, and clinical genetic testing communities.

Methods: Cell lines were purchased from Coriell and ATCC cell repositories as extracted DNA or cells to be grown in-house. Extracted DNA from all cell lines was quantified with several commercially available qPCR kits, in-house qPCR assays and measured with a micro-volume spectrophotometer. These materials were genotyped with several commercially available STR typing kits and in-house STR typing assays. Table 1 lists the quantitation methods and whether they use multicopy targets. Tables 2 and 3 list the qPCR conditions for the in-house methods.

Table 1. DNA Quantitation Methods

Method #	Locus	Chromosome	Primers	Product-Probe size	Method Type
1	**D4S2364**	4	F_TGTTGTCTGTAGGAGCTGAGAA R_GGTGTTTGGAGATGGCTGTT	258 bp	In-house
2	**hTERT [1]**	5	Quantifiler Human	63 bp	Commercial
3	**D10S1435**	10	F_AGTGAGCCCTCGAAGAGGTT R_GTGGTGGTGTGCACCTGTAGT	355 bp	In-house
4	**TH01**	11	F_TGAAAAGCTCCCGATTATCCA R_CACTCGGAAGCCCTGTGTACA	62 bp	In-house
5	**RPPH1/SRY [2]**	14/Y	Quantifiler Duo	140/130 bp	Commercial
6	**Plexor HY [3]**	17/Y	Multicopy Autosomal/Y chromosome	99/133 bp	Commercial
7	**Alu [4]**	na	F_GTCAGGAGATCGAGACCATCCC R_TCCTGCCTCAGCCTCCCAAG Multicopy Autosomal	124 bp	In-house
8	**UV_spec. [5]**	na	na	na	Commercial

Table 2. qPCR conditions for D4S2364 and D10S1435

PCR: 20 µL reaction volume containing 0.4 µM of each primer, 10 µL of Power SYBR Green PCR Master Mix Applied Biosystems, 7.6 µL DI Water, and 2 µL test DNA.

Instrument: 7500 Real Time PCR System Applied Biosystems in 9600-emulation mode (ramp speeds of 1 °C/s)

Thermal cycling: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 45 s.

Data Collection: During the 72°C for 45 s step.

Table 3. qPCR conditions for TH01

PCR: 20 µL reaction volume containing 0.6 µM of each primer, 10 µL of Power SYBR Green PCR Master Mix Applied Biosystems, 3.5 µL DI Water, and 2 µL test DNA.

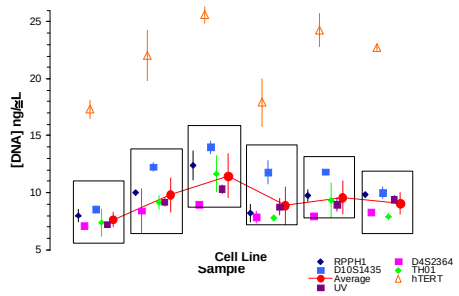
Instrument: 7500 Real Time PCR System Applied Biosystems in 9600-emulation mode (ramp speeds of 1 °C/s)

Thermal cycling: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s

Data Collection: During the 60 °C for 60 s step.

Results: Figure 1 displays DNA concentration values, [DNA], for six cell lines determined with a number of methods chosen to evaluate targets located on different chromosomes. Five of the qPCR methods and the UV micro-spectrophotometer (Table 1, methods 1-5 & 8) agreed with each other within an average of 15 %. The commercial qPCR method using an hTERT target (Table 1, method 2) gave 2-fold larger [DNA]. These cell lines are not suitable for use as forensic standards.

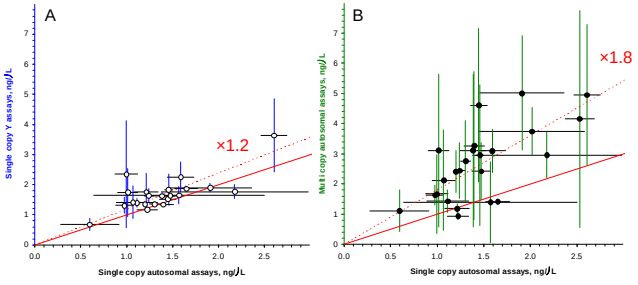
Figure 1. DNA Quantitation Results for 6 cell lines



These six cell lines had the highest difference of the average [DNA] compared to the hTERT assay of the 30 cell lines tested.
Error bars in the graph are ±1 standard deviation of multiple replicates of the samples.

Figures 2A and 2B display [DNA] for 24 cell lines that did not show the above imbalance when quantified with qPCR Table 1, methods 2,4-7. Figure 2A displays results of the single copy Y-chromosome assays plotted against results from single copy autosomal assays. There is good agreement between these single copy assays for most of the cell lines. Figure 2B displays results of the multicopy autosomal assays likewise plotted against results from single copy autosomal assays. On average the multicopy assay [DNA] results are 1.8-fold larger than the single copy.

Figure 2. DNA qPCR Results for 24 cell lines



X Error bars in the graphs are ±1 standard deviation of multiple replicates of the samples evaluated by single copy methods (2,4 & 5).
Y Error bars in the graphs are ±1 standard deviation of multiple replicates of the samples evaluated by single copy Y methods 5 & 6 (A) or Multicopy autosomal methods 6 & 7 (B).
Solid Red line = theoretically where the results of the compared assays are equivalent.
Dotted Red line = calculated multiplicative standard deviation: in 2A is a factor of 1.2; in 2B is a factor of 1.8

References:
[1] 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.
[2] M. Barbisin, R. Fang, C.E. O'Shea, L.M. Calandro, M.R. Furtado, J.G. Shewale, Developmental validation of the Quantifiler Duo DNA Quantification kit for simultaneous quantification of total human and human male DNA and detection of PCR inhibitors in biological samples. J Forensic Sci. 2009;54(2):305-19.
[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.
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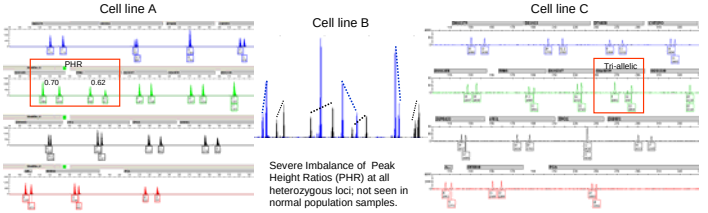
[5] Sambrook, J. and Russell D.W. (2001) Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.
[6] ATCC Standards Development Organization Workgroup ASN-0002 (2010);Cell line misidentification: the beginning of the end. Nature Reviews Cancer 10:441-448.
[7] Capes-Davis, A., et al.(2010) Check your cultures! A list of cross-contaminated or misidentified cell lines. Int. J. Cancer: 127: 1-8.

Figure 3 displays electropherographic results for two cell lines (lines A and B) giving unusually large within-locus peak height ratio (PHR) imbalances and a third with an unusual tri-allelic pattern (C). Only loci D3S1358 (chromosome 3) and TH01 (chromosome 11) are much affected in cell line A, suggesting localized mutations in the PCR binding regions. All heterozygous loci are affected in cell line B, suggesting aneuploidy. These atypical patterns render these materials unfit for use as for reference materials for applications requiring the interpretation of mixed-source samples.

Figure 3. STR profiles of three different cell lines.



Misidentification of cell lines has been a known problem since the late 1950's with periodic reminders published throughout the years [6]. There are lists of known cross-contaminated or misidentified cell lines [7]. Table 4 lists a several potential additional identification issues that we found while reviewing cell line web-available STR profiles from four different cell line repositories.

Table 4. STR Profiles of cell lines from different cell repositories

Cell name	Locus names								
	D5S818	D13S317	D7S820	D16S539	VWA	TH01	AM	TPOX	CSF1PO
HEPM	11,12	8,11	10,12	10,12	17,17	7,8	X,X	8,11	11,12
HEPM	11,13	8,12	8,10	11,12	17,18	6,9.3	X,X	8,11	10,11
KCL-22	10,11	8,12	11,12	12,12	14,14	7,9	X,X	8,8	12,12
KCL-22	10,12	8,11	12,12	12,12	14,14	7,9	X,X	8,8	12,12
HSC-2	10,12	11,12	9,12	12,12	16,18	6,7	X,X	8,8	12,13
HSC-2	10,12	11,12	9,12	12,12	16,18	6,7	X,Y	8,8	12,13
BeWo	10,11	9,11	10,12	13,14	16,16	9,9.3	X,Y	8,8	11,12
JEG-3	10,11	9,11	10,12	13,14	16,16	9,9.3	X,Y	8,8	11,12

The two HEPM cell lines do not seem to be related; KCL-22 cell lines have three differences at three loci; One of the HSC-2 has a loss of the Y-amelogenin marker; BeWo STR typing is an exact match for JEG-3, these names would not seem to indicate that these cell lines are related.

Cell line authentication measures must be taken by everyone requiring repeatable, reproducible results. We are currently working with the American Type Culture Collection (ATCC) Standards Development Organization Workgroup ASN-0002 on the authentication of cell lines using STR loci. The draft document "ASN-0002 Authentication of Human Cell Lines: Standardization of Short Tandem Repeat (STR) Profiling" is currently in ANSC public review. To request a copy for review please contact:
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Conclusions: A thorough characterization of cell lines beyond the properties being certified is essential for a reference material to be fit for purpose. While a genotype may be certified, the sample must still be able to be appropriately quantified by conventional means. As new analytical methods of analysis are developed existing reference materials can be used to validate the methods. Producers of these existing reference materials need to be notified of anomalous behavior of their products. When warranted, the reference can be re-evaluated and a new Certificate of Analysis issued to describe apparent issues. Such changes/improvements to the Certificate are not immediate but when implemented result in the product being more useful to the end user.

Acknowledgements: Many thanks to Erica Butts and Becky Hill of the NIST Applied Genetics group for their assistance in data acquisition, and Dave Duewer of the NIST Analytical Chemistry Division for assistance with data analysis.