Establishing standard assays for quality control and identity traceability of human embryonic stem cells

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Abstract

As the clinical use of stem cells in cell therapy and regenerative medicine moves toward transplanting cells into humans, stem cell culturing facilities need to assure the identity and quality of their cultures. It is crucial that standard quality control assays be established, since FDA approval of therapeutic cells will likely require documentation of genetic identity as well as validation of pathogen-free cultures in the near future. To establish a method of identity traceability, we used Applied Biosystems' AMPFlSTR® Identifiler® kit, which amplifies 16 different short tandem repeat (STR) loci, including the full set of FBI CODIS loci. A number of stable and genetically modified BG01V and SA02 stem cells as well as 2102 P100 human embryonal carcinoma cells and BG01V stem cells grown on different feeder layers were genotyped. All cells of the same lineage were found to have the same genotype at all 16 loci, even the cells that had been genetically modified, suggesting that gene insertion does not facilitate wide-scale genome changes. To engineer a method for pathogen detection, we tested techniques for the detection of common cell culture contaminants as well as common viruses that can be spread to cultures from researchers or stem cell donors. Real-time PCR detection of EBV, CMV, HHV-6, and *Mycoplasma* was used because the assay is fast, sensitive, and quantitative. The primers and Taq-man™ probes that we used proved to be effective in detecting 0.1 copies of EBV per genome, 10 copies of CMV per genome, 1 copy of HHV-6 per genome, and mycoplasma in contaminated cultures. In collaboration with Applied Biosystems, our results will be used to create a kit that contains pre-packaged PCR detection reactions for up to 12 pathogens. Ultimately, this kit in conjunction with the AMPFlSTR® Identifiler® assay will allow for a standard method of validation and quality control of therapeutic cells, as researchers work toward using hESCs to treat diseases and injuries such as diabetes, heart disease, and spinal cord injury.

Materials and methods

1. Genotyping human embryonic stem cells:

   Use Qiagen DNeasy® kit to extract human genomic DNA

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   Use 0.1ng genomic DNA with AMPFlSTR® Identifiler® PCR amplification kit (5-dye fluorescent system for DNA fragment analysis) to amplify 15 STRs and the Amelogenin gender determining marker (25µL reactions)

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   Generate data using ABI 3100 Genetic Analyzer

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   Analyze data using Genemapper v4.0 software

2. Pathogen detection:

   a. Run Quantitative-PCR on ABI 7500 Fast with 3 sets of primers and Taq-man™ probes designed to detect specific gene sequences of **CMV**, **EBV**, and **HHV-6**

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   Use dilution series (10-fold series ranging from 1000 copies/genome to 0.01 copies/genome with 0.2ng/µl human genomic DNA as diluent) to determine limit of detection of primers

   b. Use Sigma Aldrichs' LookOut® Mycoplasma PCR Detection kit to detect presence of *Mycoplasma*

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   Run Quantitative-PCR on ABI 7500 Fast with primers complementary to a *Mycoplasma* 16S rRNA coding region to quantify *Mycoplasma* detected with LookOut® and determine limit of detection of primers

Results

1. STR analysis consistently distinguishes human embryonic stem cell lines, even after genetic modification and in different culture conditions

Table 1: Alleles detected with the AMPFlSTR® kit for all samples tested

Marker → / Sample ID ↓	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMELOGEN	D5S818	FGA
Mit c MEF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BG01V	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
BG01V EB	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
ID1 (on iMEFs)	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
YW2	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
YW4	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
YW8	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
YA6	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
YA20	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
2102 P100	14,15	28,29	7,13	11,12	16,16	9,9.3	8,8	11,12	17,20	13,14	16,17	8,11	15,15	XX	11,11	22,22
S1.1	14,15	28,30	10,12	11,12	12,17	9,9.3	9,11	12,14	19,26	13,15	14,16	8,9	15,16	XX	12,12	24,26
S1.3	14,15	28,30	10,12	11,12	12,17	9,9.3	9,11	12,14	19,26	13,15	14,16	8,9	15,16	XX	12,12	24,26
S1.4	14,15	28,30	10,12	11,12	12,17	9,9.3	9,11	12,14	19,26	13,15	14,16	8,9	15,16	XX	12,12	24,26
S1.1 EB	14,15	28,30	10,12	11,12	12,17	9,9.3	9,11	12,14	19,26	13,15	14,16	8,9	15,16	XX	12,12	24,26
S1.3 EB	14,15	28,30	10,12	11,12	12,17	9,9.3	9,11	12,14	19,26	13,15	14,16	8,9	15,16	XX	12,12	24,26
S1.4 EB	14,15	28,30	10,12	11,12	12,17	9,9.3	9,11	12,14	19,26	13,15	14,16	8,9	15,16	XX	12,12	24,26
YW8-254-Day 7 EB	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
BG01V-A	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
BG01V-C	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22
BG01V-T	10,12	28,29	10,11	10,10	15,17	7,9.3	11,12	9,11	17,24	13,15	16,17	8,8	19,19	XY	10,12	22,22

Key: BG01V cells | Genetically modified BG01V cells | BG01V cells grown on different feeder layers | SA02 cells | human embryonal carcinoma cells

Table 2: Specific descriptions of all cells tested

Sample ID	Description
Mit c MEF	Feeder cells for hESC (Mitomycin C inactivated MEFs)
BG01V	Variant hESC line with abnormal karyotype from BresaGen, Inc. on feeders
BG01V EB	Differentiated BG01V hESCs
ID1 (on iMEFs)	hOct4-GFP expressing stable BG01V clone on feeders
YW2	BG01V stable clone
YW4	BG01V stable clone
YW8	BG01V stable clone
YA6	BG01V stable clone
YA20	BG01V stable clone
2102 P100	human embryonal carcinoma cells
S1.1	SA02 (Sahlgrenska 2 hESC line) stable clone
S1.3	SA02 stable clone
S1.4	SA02 stable clone
S1.1 EB	SA02 stable clone
S1.3 EB	SA02 stable clone
S1.4 EB	SA02 stable clone
YW8-254-Day 7 EB	BG01V stable clone - Targeted twice for gene cassette insertion
BG01V-A	Variant hESC on feeders harvested using Accutase
BG01V-C	Variant hESC on feeders harvested using Collagenase
BG01V-T	Variant hESC on feeders harvested using TrypLE

- **All samples of the BG01V stem cell line had the same genotype**
- **Our BG01V genotype matched the STR profile for all 8 STR loci and the Amelogenin gender determining marker provided by the ATCC**
- **All samples of the SA02 stem cell line had the same genotype**
- **Mouse embryonic feeder cells were not amplified, since AMPFlSTR® kit targets the amplification of human STRs**

2. Q-PCR detects 1 viral copy per genome or less for CMV, EBV, and HHV-6

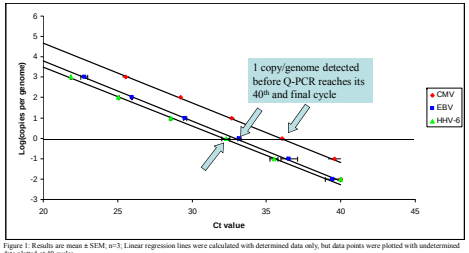


Figure 1: Results are mean ± SEM, n=3; Linear regression lines were calculated with determined data only, but data points were plotted with undetermined data plotted at 40 cycles.

Table 3: Regression Line Analysis

Primers	Linear Regression Line	Standard Curve Slope	Q-PCR Efficiency
CMV	y = -0.292x + 10.5	-3.41	96.6%
EBV	y = -0.295x + 9.70	-3.36	98.6%
HHV-6	y = -0.289x + 9.29	-3.45	95.0%

Table 4: Limit of Detection for each primer/probe set

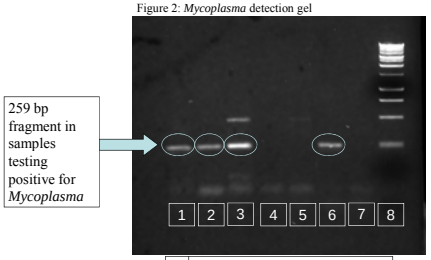
Primers	Log(copies of virus per genome)	Copies of virus per genome
CMV	-1.18	0.0655
EBV	-2.10	0.00800
HHV-6	-2.28	0.00522

- **Primer cross reactivity analysis:**

CMV, EBV, and HHV-6 primers and probes showed no cross reactivity with the other control plasmids at 10 copies per genome

3. Mycoplasma detected with Sigma Aldrich LookOut® Mycoplasma kit

Figure 2: *Mycoplasma* detection gel



Lane	Description
1	10ng of B104 (neuroblastoma cell line) rat DNA
2	media from B104 rat cell culture
3	media from B104 rat cell culture (boiled before PCR run)
4	bottled media not exposed to cells
5	bottled media not exposed to cells (boiled before PCR run)
6	positive control
7	negative control
8	DNA 1kb ladder - Promega

- ***Mycoplasma* detected in B104 rat cell (neuroblastoma cell line) media and DNA**
- **Confirmation that bottled media was not contaminated**

Conclusions

1. AMPFlSTR® Identifiler® PCR Amplification kit showed 100% efficiency in genotyping cells of the same line (Table 1)

The genotypes of cell lines grown on different feeder layers and cell lines with gene cassettes inserted were not affected by culture conditions, co-culturing with other cells, or minor genome modifications (Table 1)

2. CMV, EBV, and HHV-6 primers and probes can detect at or below 1 copy of viral DNA per genome with no cross reactivity at 10 copies per genome (Figure 1)

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The 3 primers and probes can adequately detect the worst case scenario, where there is only 1 copy of the virus

3. The Sigma Aldrich LookOut® Mycoplasma kit can be used to detect *Mycoplasma*, but cannot quantify the infection (Figure 2)

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Future studies will use *Mycoplasma*-specific 16S rRNA sequence primers and Taq-man™ probes to quantify the *Mycoplasma* infection in the media that tested positive and determine the limit of detection of the primers and probes

Literature cited

Wada, K., N. Kubota, Y. Ito, H Yagasaki, K. Kato, T. Yoshikawa, Y. Ono, H. Ando, Y. Fujimoto, T. Kiuchi, S. Kojima, Y. Nishiyama, and H. Kimura. (2007). Simultaneous quantification of Epstein-Barr virus, cytomegalovirus, and human herpesvirus 6 DNA in samples from transplant recipients by multiplex real-time PCR assay. *Journal of Clinical Microbiology*, 45(5): 1426-32.

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