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Original article

A method for rapid dose–response screening of environmental chemicals using human embryonic stem cells

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ABSTRACT

Introduction*:* Human embryonic stem cells (hESC) provide an invaluable model for assessing the effects of environmental chemicals and drugs on human prenatal development. However, hESC are difficult to adapt to 96-well plate screening assays, because they survive best when plated as colonies, which are difficult to count and plate accurately. The purpose of this study is to present an experimental method and analysis procedure to accomplish reliable screening of toxicants using hESC. **Methods***:* We present a method developed to rapidly and easily determine the number of cells in small colonies of hESC spectrophotometerically and then accurately dispense equivalent numbers of cells in 96-well plates. The MTT assay was used to evaluate plating accuracy, and the method was tested using known toxicants. **Results***:* The quality of the plate set-up and analysis procedure was evaluated with NIH plate validation and assessment software. All statistical parameters measured by the software were acceptable, and no drift or edge effects were observed. The 96-well plate MTT assay with hESC was tested by performing a dose–response screen of commercial products, which contain a variety of chemicals. The screen was done using single wells/dose, and the reliability of this method was demonstrated in a subsequent screen of the same products repeated three times. The single and triple screens were in good agreement, and NOAELs and IC_{50}s could be determined from the single screen. The effects of vapor from volatile chemicals were studied, and methods to monitor and avoid vapor effects were incorporated into the assay. **Discussion***:* Our method overcomes the difficulty of using hESC for reliable quantitative 96-well plate assays. It enables rapid dose–response screening using equipment that is commonly available in laboratories that culture hESC. This method could have a broad application in studies of environmental chemicals and drugs using hESC as models of prenatal development.

1. Introduction

When evaluating the risk of exposure to environmental chemicals or drugs, it is important to consider how chemicals affect embryos and fetuses, which are usually the most sensitive stages of the human life cycle (Grandjean, Bellinger, Bergman, Cordier, et al., 2008). While direct experimentation with human embryos is not feasible, human embryonic stem cells (hESC) provide an invaluable model for evaluating the effects of environmental chemicals on prenatal development (Talbot & Lin, 2011). Several strategies exist for using hESC in toxicological studies (Adler, Pellizzer, Hareng, & Hartung, 2008; Krtolica, Ilic, Genbacev, & Miller, 2009; Talbot & Lin, 2011). Because they are pluripotent, hESC can be differentiated into any type of cell, and the effects of chemicals on differentiation or on differentiated cells can then be studied. As an alternative, hESC themselves can be used directly in toxicological assays, and the effects on various endpoints, including pluripotency, proliferation, apoptosis, survival and morphology, can be accessed (Lin, Fonteno, Weng, & Talbot, 2010). An advantage of the latter strategy is that the time to perform an assay can be greatly reduced to days or even hours, depending on the endpoint (Lin, Tran, & Talbot, 2009; Lin et al., 2010). A second advantage is that hESC model epiblast cells (Nichols & Smith, 2009; Tesar et al., 2007) which function in germ layer formation. Harm to the epiblast could result in death of the embryo or in major congenital malformations during subsequent development.

It would be highly desirable to be able to perform dose–response screens on environmental chemicals or drugs using hESC. However, hESC have proven difficult to adapt to such screens as they do not survive well when plated as single cells, which are easy to count and plate accurately. To assure survival, hESC are generally plated as small colonies or alternatively they can be plated as single cells with the inclusion of a Rho-associated kinase (ROCK) inhibitor, such as Y-27632, in the culture medium (Watanabe et al., 2007). However, both of these methods have drawbacks. Small colonies are difficult to count and plate accurately making quantitative assays difficult to perform and

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; DPBS, Dulbecco's Phosphate Buffered Saline; EDTA, Ethylenediaminetetraacetic acid; hESC, human embryonic stem cells; hPF, human pulmonary fibroblasts; mNSC, mouse neural stem cells; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROCK, Rho-associated kinase.

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the inclusion of a ROCK inhibitor can influence the outcome of the assay (Fujimura, Usuki, Kawamura, & Izumo, 2011).

To overcome the above obstacles, we have developed a spectrophotometric method to rapidly measure density of hESC in small colonies, accurately plate equal numbers of cells/well in 96-well plates, and obtain highly reproducible dose–response curves using the MTT assay. The counting, plating, and screening methods that we present are straightforward, rapid, highly reproducible, and can be done in any laboratory with basic equipment and facilities for culturing hESC.

We have applied our counting and plating method to a dose–response screen of 12 electronic cigarette refill products. Electronic cigarette refill solutions (also called e-juice) contain nicotine, flavorings, and a humectant, such as propylene glycol, and are used to replenish fluid in used electronic cigarette cartridges (Trtchounian & Talbot, 2010; Williams & Talbot, 2011). Electronic cigarette refill solutions were chosen for screening as they represent environmental chemical products that have not yet been subjected to cytotoxicity testing; however, we know that some solutions contain chemicals that are toxic to hESC and other cell types (unpublished data).

2. Methods

2.1. Cell culture

H9 hESC from WiCell (Madison, WI) were maintained on Matrigel (Fisher Scientific, Bedford, MA) coated 6-well plates (Falcon, Fisher Scientific, Chino, CA) in mTeSR®1 medium (Stem Cell Technologies, Vancouver, BC, Canada) using methods previously described in detail (Lin & Talbot, 2011). Medium was changed daily, and cells were used when 60–80% confluent. To subculture or prepare hESC for experiments, wells were washed with Dulbecco's Phosphate Buffered Saline (DPBS) containing magnesium and calcium (GIBCO, Invitrogen, Carlsbad, CA), then colonies were enzymatically detached using Accutase (eBioscience, San Diego, CA) and sterile glass beads. For MTT experiments, cell concentration was adjusted spectrophotometrically to produce 30,000 cells/well.

mNSC, obtained from Dr. Evan Snyder, were cultured in Nunc T-25 tissue culture flasks (Fisher Scientific, Tustin, CA) in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Walkersville, MD) containing 10% fetal bovine serum, 5% horse serum, 1% sodium pyruvate (Lonza, Walkersville, MD) and 1% penicillin–streptomycin (GIBCO, Invitrogen, Carlsbad, CA). Medium was replaced on alternate days, and cells were used in experiments when about 80% confluent. For screening, the flasks were washed with DPBS, then treated with 0.05% trypsin EDTA/DPBS (GIBCO, Invitrogen, Carlsbad, CA) for 1 min at 37 °C. For the MTT assay, cells were plated at 2500 cells/well in 96-well plates.

Human pulmonary fibroblasts (hPF) (ScienCell, Carlsbad, CA) were cultured in complete fibroblast medium containing 2% fetal bovine serum, 1% fibroblast growth serum, and 1% penicillin/streptomycin using the supplier's protocol. hPF were grown on poly-L-lysine (15 µl/10 ml) coated T-25 flasks, and medium was changed every other day. hPF were used for screening when about 80% confluent. For subculturing and experimental setup, cells were washed with DPBS and detached with 0.01% trypsin diluted in DPBS for 1 min at 37 °C. For the MTT assay, plating was done at 20,000 cells/well in 96-well plates.

2.2. Generating a standard curve to count cell numbers in hESC colonies

After following the basic protocol for passaging two confluent wells of hESC, the hESC pellet was suspended with 1 mL of mTeSR medium then gently broken to generate small colonies of 2–10 hESC/colony. After being blanked with DPBS, the % transmittance was read at 560 nm in a BioMate 3S spectrophotometer (Thermo Scientific). 500 µL of the sample were diluted with 500 µL of DPBS. A reading was taken and the same procedure

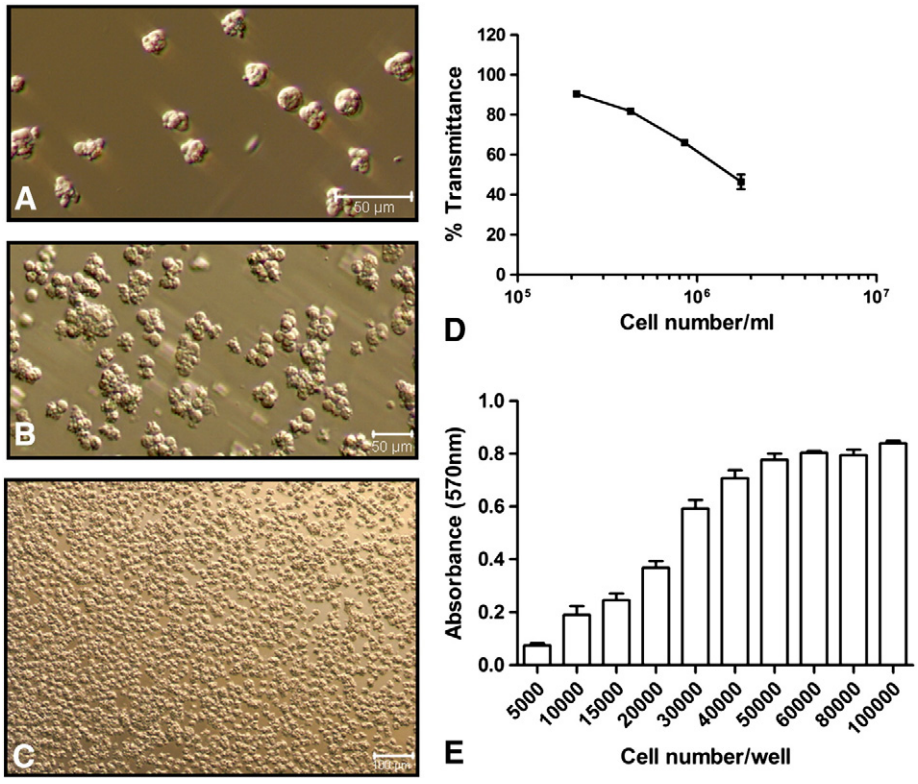

Fig. 1. Accurately counting and plating hESC colonies. (A) Single hESC which do not survive well unless plated with a ROCK inhibitor. (B) Small colonies of hESC that can survive plating without ROCK inhibitor. (C) Example of a dispersion of small hESC colonies that can be measured spectrophotometerically and plated with excellent survival. (D) A standard curve for rapidly determining hESC concentration in a small colony suspension as shown in C. (E) A graph used to determine the number of hESC to plate in 96-well plates in order to have about 70–80% confluency after 72 h. Various concentrations of cells were plated and the MTT assay was performed after 72 h. In panels D and E, each point is the mean ± standard deviation of three experiments.

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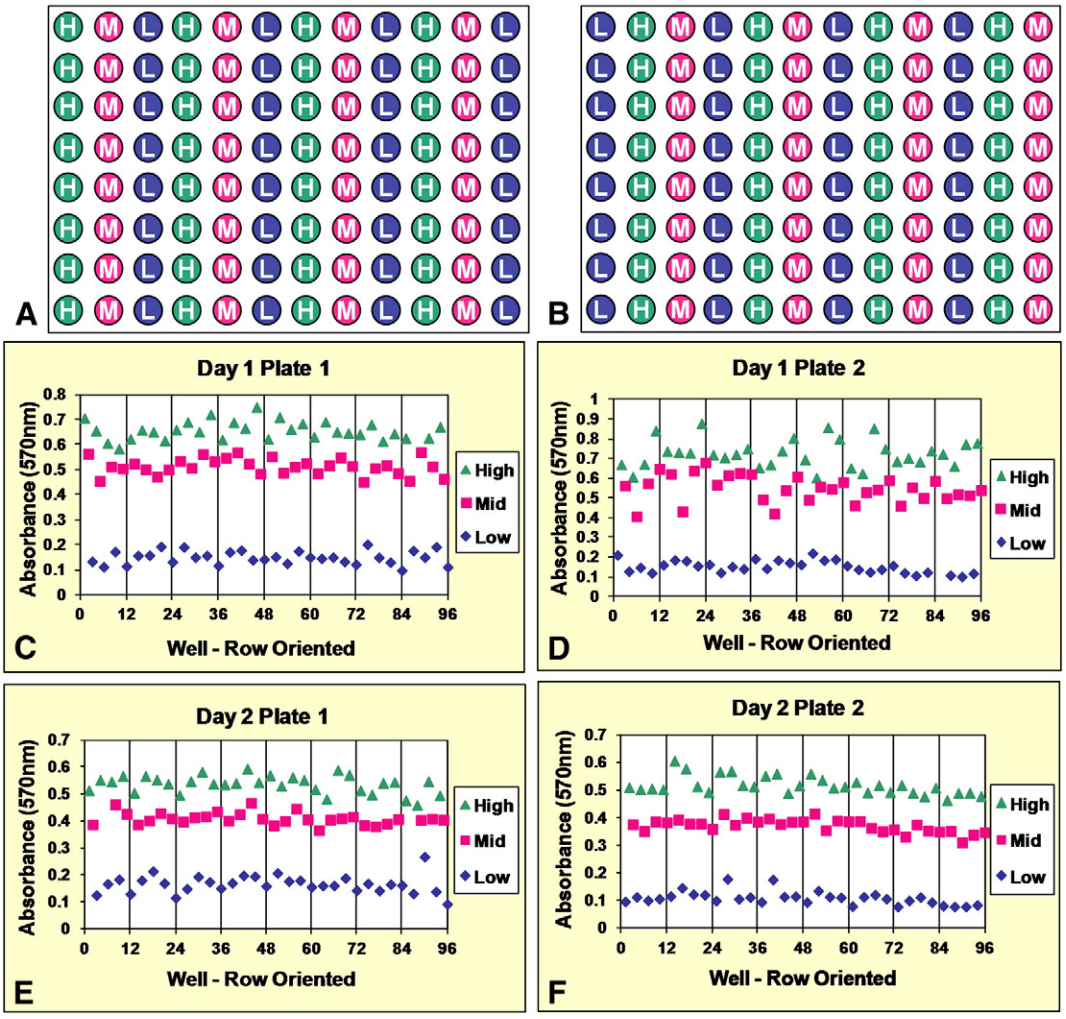

Fig. 2. Data from the NIH plate assessment and validity test. (A–B) The two patterns used for plating various concentrations of 5-fluorouracil in the MTT assay. H = high signal (low dose), M = mid signal (middle dose), L = low signal (high dose). (C–F) Data from duplicate plates done on two different days showing the distribution of the high, mid, and low signals on each plate. Drift and edge effects were not observed.

was used to obtain readings for two-fold serial dilutions up to 1:128. Cell counts for 1:4, 1:8, and 1:16 dilutions were made using a hemocytometer to obtain the total cell number/mL. The % transmittance for each dilution was then recorded and appropriate calculations were made to determine cell concentration from the hemocytometer readings. Data were plotted on semi-log graph paper to produce a standard curve of cell concentration as a function of % transmittance. This procedure was repeated on 3 days with different batches of cells and averaged to produce the final standard curve.

2.3. Method for determining the optimal number of cells to plate

To determine the optimal cell number to plate for a 72 h experiment, hESC were plated at 5000–100,000 cells/well in a 96-well plate. After 72 h of incubation, the MTT assay was performed and the number of cells that gave 80% confluency was determined.

2.4. Performance of the MTT assay with hESC in 96-well plates

The performance of the 96-well plate MTT assay with hESC colonies was evaluated using the NIH Assay Guidance Wiki software tool (http://assay.nih.gov/assay/index.php/Assay_Validation_2011). The tool checks if there is sufficient separation in signals obtained at low, mid and high doses, and if there is signal variability due to edge effects or drift. To evaluate performance using the MTT assay with hESC, a dose–response curve was first generated using 5-fluorouracil. High (5×10^{-6} M), mid (2.5×10^{-6} M), and low (1×10^{-6} M) doses were chosen from the curve and used in the performance experiment. To collect performance data, two sets of MTT experiments were prepared with 5-fluorouracil on two different days with 2 plates/day. The plate design for each set of experiments can be found at the NIH Chemical Genomics Center (NCGC) website given above. The MTT data obtained from both plates was entered into the NIH Assay Guidance Wiki software tool, which computes the coefficient of variation for each dose and for signal separation. In addition, it also checks for edge effects and drift.

2.5. Screening electronic cigarette refill products using hESC

To evaluate the usefulness of the hESC MTT assay in screening environmental chemicals, 10 different samples of electronic cigarette refill fluid plus single bottles of propylene glycol (PG) and vegetable glycerin (VG) were purchased from Freedom Smoke USA, Johnson Creek, or Red Oak (a constituent of Johnson Creek) and used to perform a dose–response screen with hESC. The initial screen was performed with one well per dose for each refill product over six doses (0.001%, 0.01%, 0.03%, 0.1%, 0.3%, and 1%). Plates were set up and evaluated using the MTT assay as described above, and dose–response curves were created for each product. To validate this screening strategy, the

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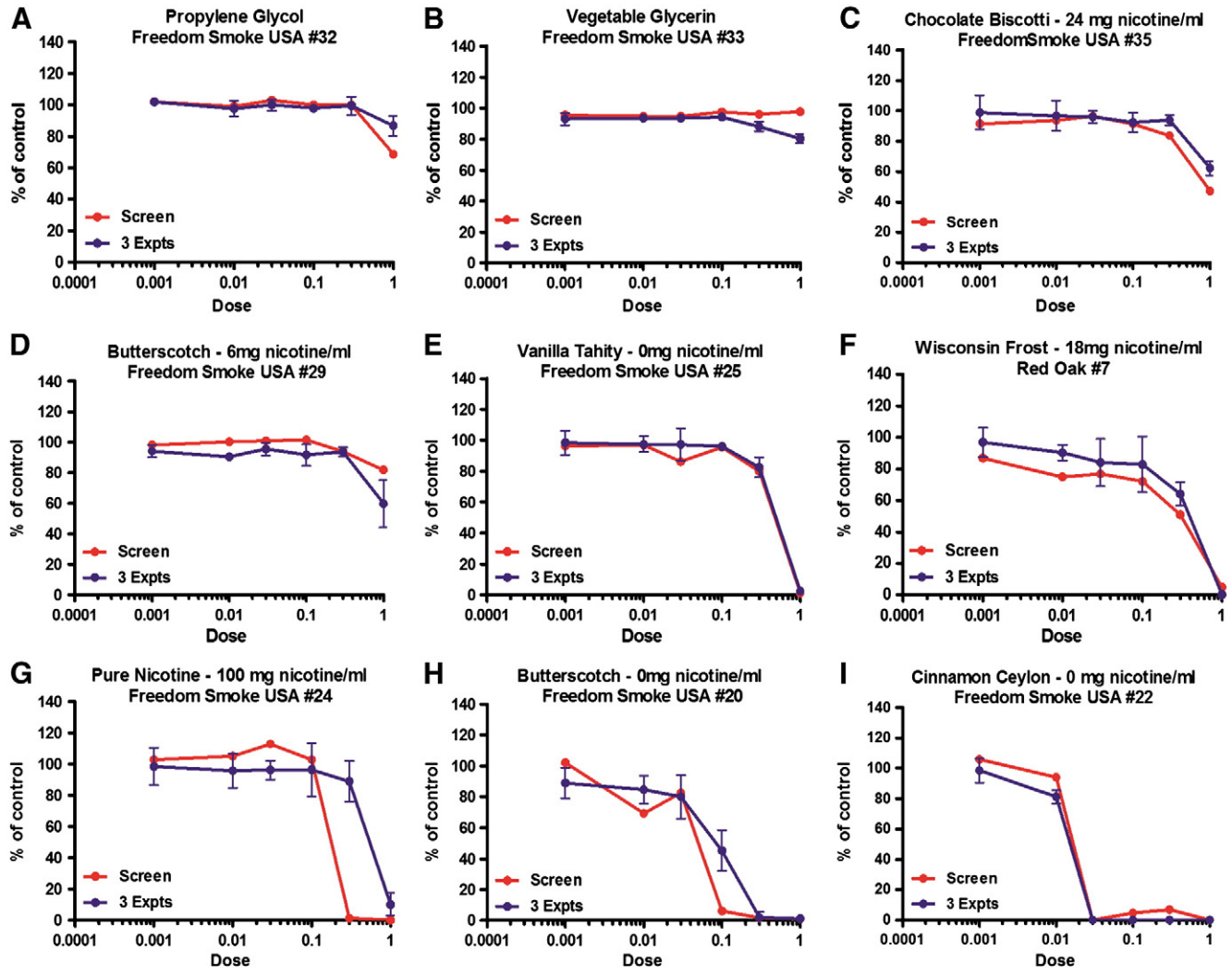

Fig. 3. Dose–response screen of electronic cigarette refill solutions comparing single well/dose and triplicate experiments. Two humectants (A, B) and seven refill samples (C–I) were screened using hESC. The single well and triplicate experiments are in good agreement. Means and standard deviations are shown for the triplicate experiment. Doses are 3.19×10^{-X} M.

same products were screened again in experiments that were repeated three times (triplicate experiments). Duplicate wells were averaged for each dose in each experiment. Means and standard deviations were computed for the three experiments for each sample of refill solution. To determine if this screening strategy worked well with other cell types, hPF and mNSC were used in a similar dose–response screen using the same refill samples. Single wells were used for each dose and two screens were run for each sample to compare reproducibility.

2.6. Evaluation of vapor effects in 96-well plates

Because vapors from volatile chemicals could affect the results in a 96-well plate screen, vapor effects were first evaluated in dose–response experiments that used high doses of phenol with hESC and mNSC. To demonstrate that chemical vapors were carried between wells of a 96-well plate, doses of various chemicals (10^{-6} M to 10^{-2} M) in water were added to 96-well plates with control wells containing water only at each end of the dose range. To detect chemicals in each well, absorbances were read at 280 nm at time 0 and again 48 h after the plate had incubated at 37 °C and 95% relative humidity in 5% CO_2. The experiment was then repeated using 10^{-3} M solutions as the highest dose.

3. Results

3.1. Establishing a rapid method to count and plate hESC in suspensions of small colonies

hESC do not attach and grow well when plated as single cells (Fig. 1A); however, excellent plating efficiencies can be obtained when hESC are plated as small colonies (Fig. 1B). Fig. 1C shows a field of small hESC that was used to generate the standard curve in Fig. 1D, as described in the Methods section. Percent transmittance readings were taken three times for the same sample in a spectrophotometer and produced a coefficient of variation of 1.5%, demonstrating the reproducibility of this method. To plate a similar number of cells in each well of 96-well plates, a suspension of hESC in a 15 ml conical vial was flicked gently three times before pipetting. The coefficient of variation for multiple control wells on the same plate ranged from 0.7% to 8.5% for five experiments, showing that pipetting of small colonies was adequately uniform.

3.2. Determining the optimal number of cells to plate

Small hESC colonies were plated at various concentrations, allowed to grow for 72 h, then subjected to the MTT assay (Fig. 1E). Plating

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20,000 to 30,000 cells/well gave about 80% of the maximum absorbance and therefore this concentration range was used for plating in subsequent experiments.

3.3. Evaluation of drift, edge effects, and other 96-well plate assay parameters

The NIH Assay Guidance Wiki software tool (http://assay.nih.gov/assay/index.php/AssayValidation_2011) was used to evaluate plate uniformity and signal variability of the MTT assay with hESC in 96-well plates. This assay involved plating high, medium, and low concentrations of a test chemical (5-fluorouracil) in patterns prescribed by the wiki (Fig. 2A, B) and monitoring various parameters that establish the performance of the assay. The evaluation was done on two different days with two plates used each day. All statistical parameters measured by the software were within the acceptable range. There were no drift or edge effects in any of the plates (Fig. 2C–F). The coefficients of variation for the high, mid and low signal were less than 20% for all plates. Both the signal window and Z′ factor, which evaluate the signal separation of the assay, exceeded the minimum acceptable value for all plates, indicating sufficient spread between the doses. Finally, the inter-plate and inter-day tests were acceptable (<two-fold shift) for all four plates. We concluded that our method for counting and plating hESC in small colonies was reproducible and useful in a 96-well plate format.

3.4. Results of a dose–response screen of 12 electronic cigarette refill products and validation of results with triplicate experiments

We performed a dose–response screen of 12 refill products using the method described above with hESC (Fig. 3). Each refill sample was evaluated at doses of 0.001, 0.01, 0.03, 0.1, 0.3, and 1%, and each dose was tested one time for each sample in the screen. We reasoned that the doses themselves provide some replicate information and that a single series of doses would give sufficient information to estimate the cytotoxicity of each sample. Results for the screen show that cytotoxicity of the refill products was variable. The two humectants, propylene glycol and vegetable glycerol, were not cytotoxic to hESC over the range of concentrations that were tested (Fig. 3A, B). Several samples, Chocolate Biscotti and Butterscotch, showed some cytotoxicity at the 1% dose (Fig. 3C, D). Two samples, Vanilla Tahity and Wisconsin Frost, showed strong cytotoxicity at the 1% dose. Two samples were strongly cytotoxic at 0.3% (pure nicotine) and 0.1% (Butterscotch) (Fig. 3G, I), and one sample, Cinnamon Ceylon, was strongly cytotoxic at 0.03% (Fig. 3I).

To evaluate the reliability of the screen, three additional similar screens were done using different passages of hESC on different days, and the data from these screens were combined (Fig. 3). Standard deviations were in general small, which is consistent with the findings of the plate validation that showed good reproducibility between plates and between days. Curves obtained from the single screen were in good agreement with the mean curves obtained by averaging three experiments. For the 9 samples shown in Fig. 3 plus 3 additional samples (not shown), the IC_{50}s in the single well screen and the IC_{50}s in the averaged three experiment screen were always within a factor of 3, which would be the expected resolution for a three-fold dose range.

Duplicate screens were done for two additional cell types, hPF and mNSC (Fig. 1 Supplement). Duplicates were generally in very good agreement.

3.5. Detection and elimination of vapor effects in 96-well plates

Although vapor emitted by volatile chemicals can factor into 96-well plate assays (Blein, Ronot, & Adolphe, 1991), volatility is not often considered when performing such assays. To determine how volatility affects the position of the dose–response curve, further tests were performed in which control wells without phenol were plated on either side of the wells containing varying doses of phenol (Fig. 4A, B). In the absence of a vapor effect, all control wells should be approximately equal. When 10^{-2} M phenol was the highest dose used, control-2 (CN2) to the right of the high dose had a low absorbance, indicating that a vapor effect had occurred; however, this effect was not observed in control-1 which was positioned further from the high dose (Fig. 4A). Reducing the highest concentration of phenol to 10^{-3} M eliminated the vapor effect with hESC (Fig. 4B). The vapor effect was not as strong when mNSC were used (Fig. 4C), indicating a species or cell type difference in sensitivity to phenol.

To determine if phenol actually passed through the air into adjacent wells, various doses of phenol in water were placed in a 96-well plate. Control wells on either end of the row of wells with phenol had water only (CN1 and CN2 in Fig. 5). Absorbances of each well were read in a spectrophotometer before and after the plate incubated at 37 °C for 48 h (Fig. 5). All control wells had no absorbances at the start of the experiment; however, control well absorbances increased substantially by 48 h in the wells next to the high dose (10^{-2} M) of phenol (Fig. 5A). The controls adjacent to the lowest dose had a negligible increase in absorbance. Reducing the concentration of phenol to 10^{-3} M, eliminated the vapor effect (Fig. 5A).

Four additional chemicals were tested for transfer to adjacent wells (Fig. 5B–E). All chemicals that had high volatility (pyrazine, 4-methylpyridine, and 3-ethlypyridine) produced measurable absorbance readings in the control wells adjacent to the high dose at 48 h indicating significant transfer of the test chemicals between wells (Fig. 5B–D). Reducing the highest dose of the volatile chemicals to 10^{-3} M resulted in absorbance readings of zero in the controls adjacent to the high dose of 3-ethylpyridine and readings less than 0.1

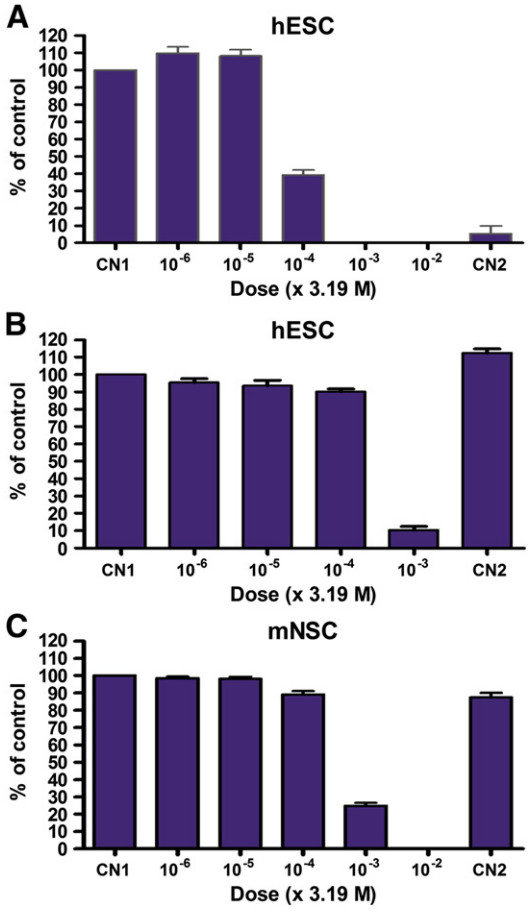

Fig. 4. Demonstration of a vapor effect using phenol. (A) Dose–response data showing that the control (CN2) near the high dose (10^{-2} M) decreased significantly for hESC and (B) that this effect did not occur when the highest dose was 10^{-3} M. (C) In contrast to hESC, mNSC did not show a vapor effect in the CN2 control when the high dose was 10^{-2} M.

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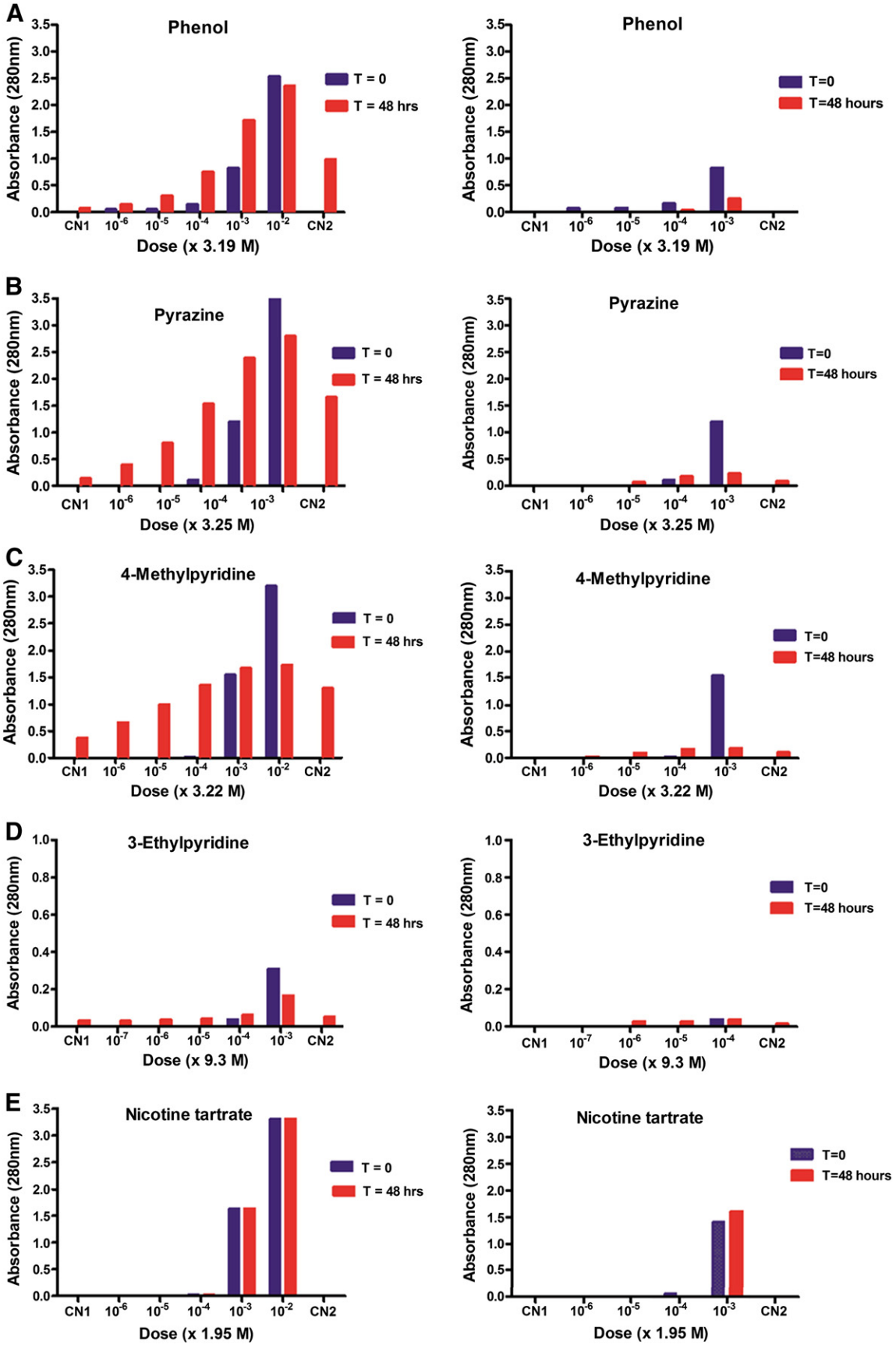

Fig. 5. Demonstration that volatile chemicals from high dose wells can pass into fluid in adjacent wells. (A–D) When the high dose of the volatile chemicals was 10^{-2} M, absorbance in the CN2 controls increased after 48 h of incubation indicating transfer of the volatile chemicals to these controls. Lowering the high dose to 10^{-3} M, significantly reduced or eliminated absorbance in the CN2 control. (E) Nicotine, which is not volatile, did not transfer in measurable amounts, even at the 10^{-2} M dose.

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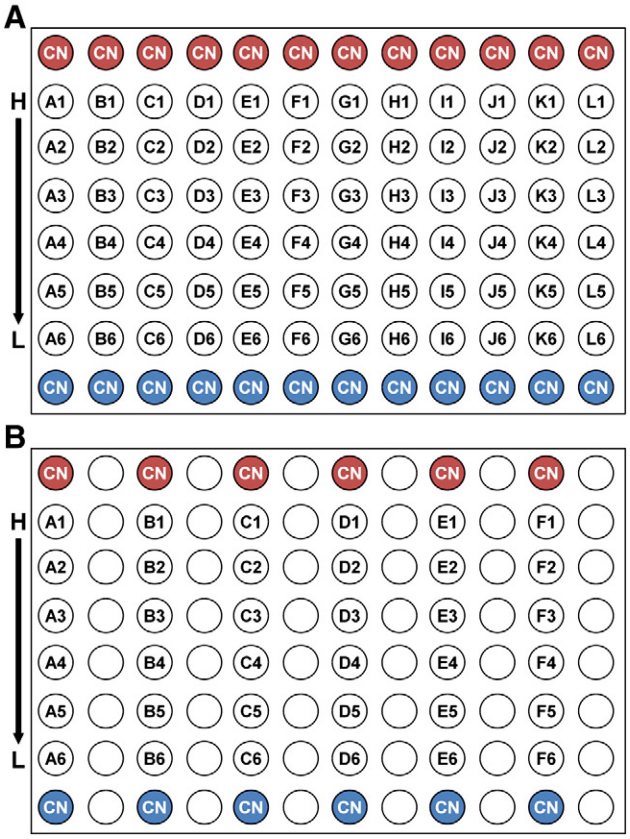

Fig. 6. 96-well plate designs for screening with hESC. (A) Control wells are included at the top and at the bottom of each row to provide a coefficient of variation (wells near low dose) and to detect a vapor effect (controls near high dose). (B) If the chemicals being screened are known to be volatile, alternate rows can be left empty to avoid vapor effects across rows.

in the 4-methylpyridine and pyrazine groups (Fig. 5A–D). Nicotine, which is not volatile, did not show a measurable transfer to the controls adjacent to the 10^{-2} M dose (Fig. 5E). These data demonstrate transfer of volatile chemicals between wells and can explain the results observed in Fig. 4A.

4. Discussion

A method is presented that allows hESC to be reproducibly plated in 96-well plates with uniform confluency from well to well and from day to day. By spectrophotometerically determining cell concentration in suspensions of hESC colonies and by swirling and resuspending colonies between each pipetting, we were able to obtain cell concentration data, make dilutions, and plate colonies rapidly and accurately. The rapidity with which these steps can be performed enhanced cell survival and plating efficiency. The reliability of obtaining uniformity in 96-well plates was confirmed by the high inter-plate and the inter-day reproducibility. Edge effects and drift were not observed in 96-well plates, which were always placed on the same shelf in the same orientation and not stacked during incubation. When using this method, care should be taken to get a uniform suspension of hESC colonies of about the same size used to create the standard curve each time concentration is determined. Other labs adopting this procedure should create their own standard curve for cell concentration, as the curve could be affected by the spectrophotometer that is used. To obtain similar numbers of cells per well during plating, it is important to gently flick the tube containing hESC colonies between each pipetting step. Flicking resuspends the colonies without breaking them into single cells.

We reasoned that it should be possible to screen chemicals and obtain reliable dose–response curves using single wells for each dose. The doses themselves provide a type of duplication which allows outliers to be recognized. When a single well screen of refill products was compared to a traditional dose–response experiment using means and standard deviations from three experiments, we found excellent agreement between the dose–response curves obtained in the single well screen and the triplicate experiment. Screens are often done using a single concentration. This approach can work well, particularly in high throughput screens, but does not acquire valuable dose–response data. Moreover, the use of single doses is prone to false positive and negative results (Bibette, 2012). Robust high throughput microfluidic platforms have been used recently in dose–response screens (Miller et al., 2012). However this method may not be readily adaptable to most labs, while our screening protocol could be used in any lab with standard equipment and facilities for culturing hESC. Another advantage of hESC MTT screen we have presented is the relatively short time it takes to perform. Assays can be completed in 48 or 72 h, depending on the experimental design.

Our data show that 96-well plate assays are affected by volatile chemicals that can produce vapor effects and shift dose–response curves to the left, making a test chemical appear more potent than it may actually be. Similar effects have been reported previously for ethanol and butanol (Blein et al., 1991). Wells, including control wells, near high doses can pick up significant levels of the test chemical during incubation. Moreover, the dose in each well changes over time when volatile chemicals are tested and may decrease to negligible levels if exposure continues long enough without replacing the medium. Vapor effects can be reduced by keeping the highest dose as low as possible and by using single rather than multiple wells of the high dose when screening. Ideally when performing a screen, vapor effects would not occur.

To determine if vapor effects have influenced the results of a screen, a plate with the design shown in Fig. 6A can be used. In this layout, control wells with cells but no test chemical are placed adjacent to the highest and lowest doses. In our experience, the control adjacent to the lowest dose is not affected by vapor, even when 10^{-2} M concentrations were used. The absorbances of the controls at the high and the low end of the dose range can be compared to determine if vapor from the high dose affected the outcome. If a vapor effect is observed for any test chemical, the experiment can be rerun iteratively with a lower high dose until the vapor effect is not observed. Depending on the purpose of the screen, this iteration may not be necessary. When working with chemicals that are known to be highly volatile, the design can be modified to leave alternate rows empty (Fig. 6B). This minimizes the possibility of interference between chemicals in adjacent columns, but it reduces the number of chemicals that can be tested per plate by half.

The hESC screening method used in this study demonstrated that electronic cigarette refill products vary in their cytotoxicity. This information may help electronic cigarette users identify products that are the least likely to produce adverse health effects. The screening protocol could be applied to any group of chemicals using 96-well plates. For example, screening 1000 chemicals at 5 doses would require 83 plates if the plate layout shown in Fig. 6A is used. This type of screen could be very valuable as part of the series of assays that are evolving with hESC (Adler et al., 2008; Krtolica et al., 2009; Lin et al., 2010) and could be a useful first step to identify potentially harmful environmental chemicals that may be cytotoxic to early post-implantation stages of human development.

The hESC 96-well assay and screen can be performed using equipment that is generally available in laboratories, and the screen can be scaled to low or medium throughput evaluation of environmental or other chemicals. While we worked out the demonstration data for this screen using the MTT assay, it could be applied to variations of the MTT assay (Hamid, Rotshteyn, Rabadi, Parikh, & Bullock, 2004) or to other 96-well plate assays. We have used the MTT in two protocols, one in which cells and chemicals are plated together and one in which cells are plated first and after 24 h chemicals are added. These

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assays monitor somewhat different parameters, but we have found both useful. Plating cells and chemicals together reduces the total time of the assay to 48 h.

Adaptation of pluripotent hESC to a toxicology assay and screen provides a means to monitor effects of chemicals on cells that model the epiblast, an early and important stage of pre-implantation development. This strategy has the advantage of requiring relatively little time to reach an endpoint and provides a complement to existing assays with embryonic stem cells. Our data showed that small colonies of hESC can be plated uniformly and accurately without using ROCK inhibitors. The assays can be extended to low and medium throughput screens in 96-well plates using equipment commonly available in laboratories. By performing dose–response screens, NOAELS and IC_{50}s can be estimated. When using cells in 96-well plate screens, users should be aware that vapor can travel between wells and may produce effects that shift the dose–response curve. The ability to use hESC without ROCK inhibitor in 96-well plate assays opens up many opportunities for evaluating stem cells and performing toxicological screens.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.vascn.2012.07.003.

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References

Adler, S., Pellizzer, C., Hareng, L., & Hartung, T. (2008). First steps in establishing a developmental toxicity test method based in human embryonic stem cells. *Toxicology In Vitro*, *22*, 200–211.

Bibette, J. (2012). Gaining confidence in high-throughput screening. *Proceedings of the National Academy of Sciences*, *109*, 649–650.

Blein, O., Ronot, X., & Adolphe, M. (1991). Cross contamination associated with the use of multiwell culture plates for cytotoxicity assessment of volatile chemicals. *Cytotechnology*, *6*, 79–82.

Fujimura, M., Usuki, F., Kawamura, M., & Izumo, S. (2011). Inhibition of the Rho/ROCK pathway prevents neuronal degeneration in vitro and in vivo following methylmercury exposure. *Toxicology and Applied Pharmacology*, *250*, 1–9.

Grandjean, P., Bellinger, D., Bergman, A., Cordier, S., Davey-Smith, G., Eskenazi, B., et al. (2008). The Faroes statement: Human health effects of developmental exposure to chemicals in our environment. *Basic & Clinical Pharmacology & Toxicology*, *102*, 73–75.

Hamid, R., Rotshteyn, Y., Rabadi, L., Parikh, R., & Bullock, P. (2004). Comparison of alamar blue and MTT assays for high throughput screening. *Toxicology In Vitro*, *18*, 703–710.

Krtolica, A., Ilic, D., Genbacev, O., & Miller, R. K. (2009). Human embryonic stem cells as a model for embryotoxicity screening. *Regenerative Medicine*, *4*, 449–459.

Lin, S., Fonteno, S., Weng, J. H., & Talbot, P. (2010). Comparison of the toxicity of smoke from conventional and harm reduction cigarettes using human embryonic stem cells. *Toxicological Sciences*, *118*, 202–212.

Lin, S., & Talbot, P. (2011). Methods for culturing mouse and human embryonic stem cells. *Methods in Molecular Biology*, *690*, 31–56.

Lin, S., Tran, V., & Talbot, P. (2009). Comparison of toxicity of smoke from traditional and harm reduction cigarettes using embryonic stem cells as a novel model for pre-implantation development. *Human Reproduction*, *24*, 386–397.

Miller, O. J., Harrak, A. E., Mangeat, T., Baret, J. -C., Frenz, L., Debs, B. E., et al. (2012). High-resolution dose–response screening using droplet-based microfluidics. *Proceedings of the National Academy of Sciences*, *109*, 378–383.

Nichols, J., & Smith, A. (2009). Naive and primed pluripotent states. *Cell Stem Cell*, *4*, 487–492.

Talbot, P., & Lin, S. (2011). Mouse and human embryonic stem cells: Can they improve human health by preventing disease? *Current Topics in Medicinal Chemistry*, *11*, 1638–1652.

Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., et al. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature*, *448*, 196–199.

Trtchounian, A., & Talbot, P. (2010). Electronic nicotine delivery systems — Is there a need for regulation? *Tobacco Control*, *20*, 47–52.

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., et al. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology*, *25*, 681–686.

Williams, M., & Talbot, P. (2011). Variability among electronic cigarettes in pressure drop, airflow rate and aerosol production. *Nicotine & Tobacco Research*, *13*, 1276–1283.

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