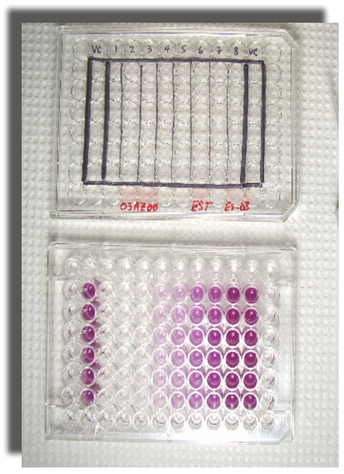
Critical Factors Impacting Interlaboratory Transferability of the Mouse Embryonic Stem Cell Test

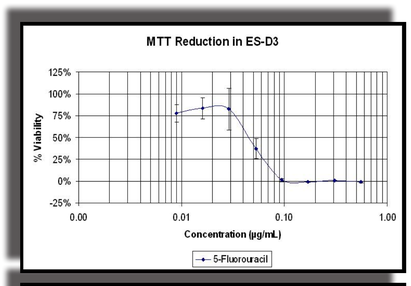
Hans A. Raabe[1], Angela M. Sizemore[1], Erica L. Dahl[1] and Daniel M. Bagley[2]
[1]Institute for In Vitro Sciences, Gaithersburg, MD USA
[2]Colgate-Palmolive, Piscataway, NJ, USA

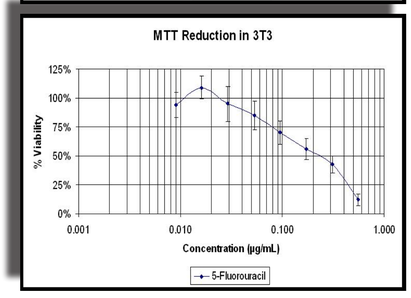
Background: Mouse Embryonic Stem Cell Test

The Embryonic Stem Cell Test classifies chemicals as nonembryotoxins, moderate embryotoxins, or strong embryotoxins, based on three endpoints in two cell lines. Cytotoxicity (IC_{50}) and inhibition of differentiation (ID_{50}) are determined in parallel.

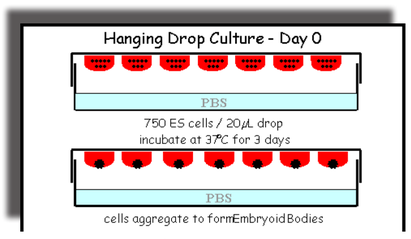
Cytotoxicity
The IC_{50} for each test article is determined in 10 day cytotoxicity assays using an adult mouse cell line (3T3) and a mouse embryonic stem cell line (D3). The cytotoxicity assays are performed in a 96 well plate format and measure the cells' ability to reduce MTT to a blue formazan precipitate as an endpoint.



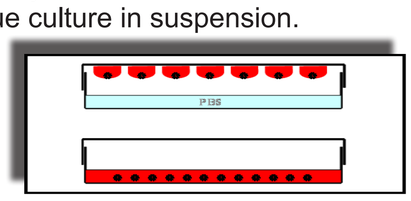




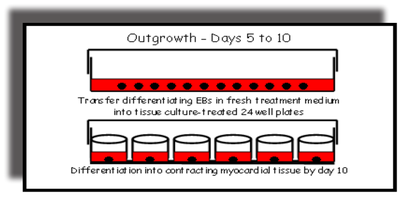
Inhibition of differentiation
The ID_{50} is the concentration at which differentiation of the D3 cells into contracting myocardiocytes is inhibited. These cells are kept in an undifferentiated state by mouse Leukemia Inhibitory Factor (mLIF) in the culture medium. Upon removal of mLIF and suspension in a hanging drop culture, the cells will form embryoid bodies over the course of several days...



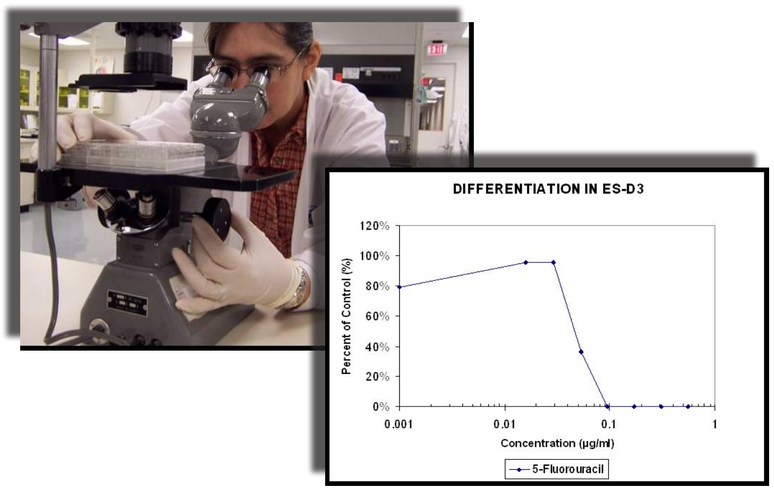
...after which they are transferred to an untreated bacterial petri dish to continue culture in suspension.



Once they reach the appropriate size they are transferred to a 24 well cell culture treated dish and allowed to form adherent cultures.



After several days the cultures differentiate into contracting myocytes that can be observed microscopically.



ID_{50} is calculated relative to negative control cultures. Once the values for the ID_{50}, the IC_{50} 3T3 and the IC_{50} D3 are obtained, they are plugged into the prediction model, which classifies the test article according to predicted embryotoxicity.

Prediction model

Fn I : 5.916 log (IC_{50} 3T3) + 3.500 log (IC_{50} D3) – 5.307 [(IC_{50} 3T3–ID_{50}) / IC_{50} 3T3] –15.27
Fn II : 3.651 log (IC_{50} 3T3) + 2.394 log (IC_{50} D3) – 2.033 [(IC_{50} 3T3–ID_{50}) / IC_{50} 3T3] – 6.85
Fn III : –0.125 log (IC_{50} 3T3) – 1.917 log (IC_{50} D3) + 1.500 [(IC_{50} 3T3–ID_{50}) / IC_{50} 3T3] – 2.67

If Function I > Function II and III --> non-embryotoxic
If Function II > Function I and III --> weak embryotoxic
If Function III > Function I and II --> strong embryotoxic

Introduction:

The EST has been formally validated by the European Centre for the Validation of Alternative Methods (ECVAM) as an acceptable *in vitro* embryotoxicity assay. During the prevalidation trials, the technology was easily transferred between laboratories in the European Union. However, transferring the assay to the Institute for In Vitro Sciences (IIVS) was problematic. Though we were able to provide good quality data, we experienced significant difficulty consistently running assays that passed our quality control criteria, which specify that at least 87.5% of the negative control cultures in the differentiation assay must contain well differentiated, beating myocytes. We began a research program to examine the parameters and technical factors which may have impacted our ability to consistently run this assay.

Summary of differentiation control culture outcomes for EST experiments run during prevalidation.

Experiment number	% of contracting myocytes (87.5% required)	Observations
1	*	Embryoid bodies lost
2	*	Embryoid bodies lost
3	*	Small embryoid bodies
4	83.3%	
5	83.3%	
6	*	Poor differentiation
7	**95.6%**	**Valid**
8	*	Poor differentiation
9	83.3%	
10	16.6%	
11	**95.6%**	**Valid**
12	62.5%	
13	75.0%	
14	25.0%	
15	20.8%	
16	**90.9%**	**Valid**

* Contracting myocytes were not evaluated.

Approach

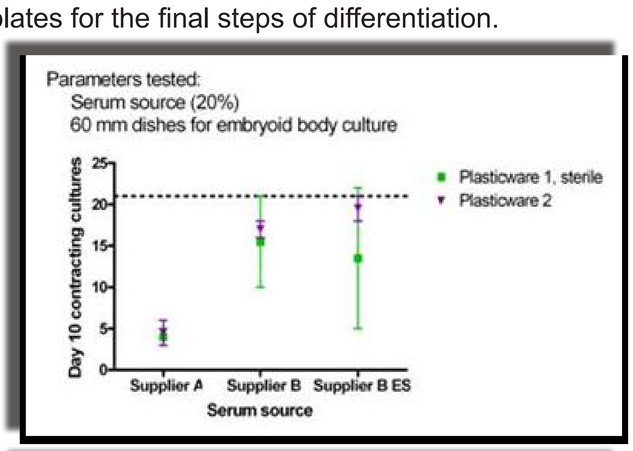
The detailed protocol, which has been evaluated and validated by ECVAM is publicly available through the ECVAM website at http://ecvam.jrc.ec.europa.eu/. We reviewed this protocol and identified a number of individual parameters which could have impacted the consistency of the differentiation assay, which are listed in the table below.

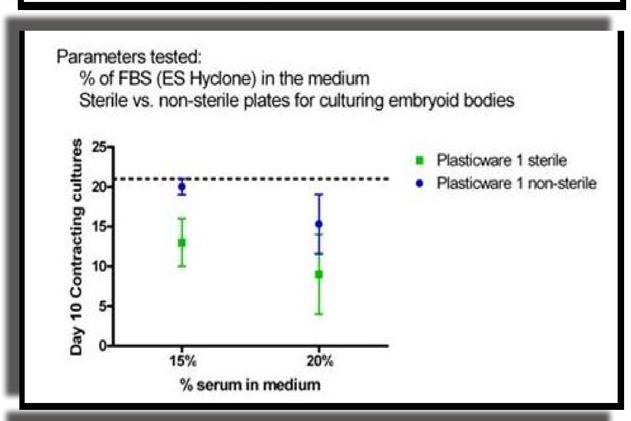
Parameters identified through protocol reviews which may have affected the EST differentiation assay.

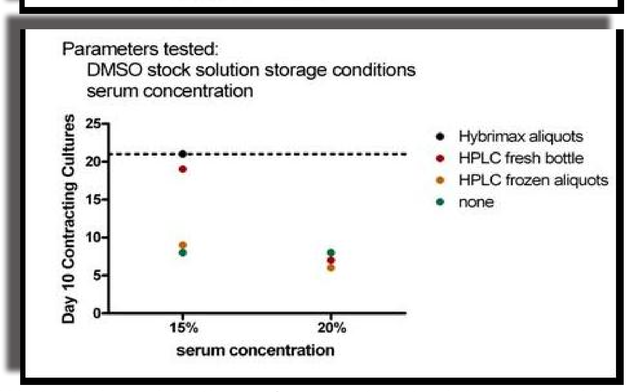
Parameter	Tested?	Comments
D3-ES cells		
Cell stocks	Yes	No significant impact
mLIF stocks	No	
Passage number	Yes	Loss of differentiation beyond passage 15
Technical considerations		
Individual technicians	Yes	No significant impact
Seeding density	Yes	Inconclusive (no improvements noted)
Length of assay	Yes	Ongoing; occasionally beating observed on day 8 or 9 diminishes by day 10.
Equipment		
Incubators	Yes	Ongoing; vibration may have an impact
Culture medium		
Serum supplier/lot	Yes	Major impact
Serum concentration	Yes	Major impact
Antibiotics	Yes	Inconclusive (no improvements noted)
Nonessential AAs	No	
Plasticware		
Dilution tubes	No	
Tissue culture flasks	No	
60 mm petri dishes, sterile	No	
60 mm petri dishes, non-sterile	Yes	Major impact
24 well tissue culture plates	No	
Other reagents		
DMSO storage	Yes	Possible impact
Trypsin-EDTA solution	No	

Results

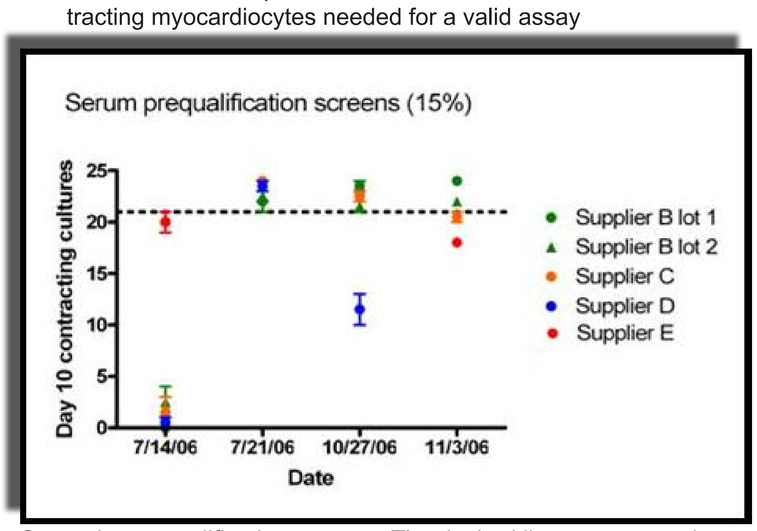
We found that changing only three parameters greatly increased the reliability of the differentiation assay. The source and specific lots of the serum used to prepare the assay medium had a major impact. In addition, assays using medium containing 15% serum performed better than those using 20% serum. Finally, the untreated bacterial petri dishes from Greiner were generally better than the radiation sterilized dishes, since the embryoid bodies tended to adhere to the surface of the sterilized dishes, preventing them from being transferred to 24 well plates for the final steps of differentiation.







We tested combinations of parameters to determine which were having the greatest effects on the differentiation assay. The dashed line represents the minimum number of contracting myocardiocytes needed for a valid assay



Serum lot prequalification screens. The dashed line represents the number of contracting myocardiocytes needed for a valid assay

We have developed an internal serum prequalification screening procedure that tests each lot of serum in the differentiation assay, scoring a total of 24 untreated embryoid bodies for their ability to form contacting myocytes. Qualified lots of serum, heat inactivated by the supplier, are stored at -20°C and thawed just before use. We have altered our protocol to specify using serum at a concentration of 15%, and the nonsterile 60 mm petri dishes from Greiner for the suspension cultures. Since implimenting these changes in 2006 we have run a number of EST assays that have passed our validation criteria.

Conclusions and Recommendations

We have optimized the EST to perform reliably. We recommend clarifying the protocol available through ECVAM to specify the use of untreated, non-sterile 60 mm dishes for the suspension culture, since the catalog number for these dishes differs between the US and EU. The protocol should also be modified to specify that serum should be used at a 15% concentration rather than 20%.

We found that the supplier and lot of the serum had a significant effect on the reliability of the assay. This is a more complicated issue that will require the development of a standardized qualification procedure to ensure that laboratories are using satisfactory serum before undertaking this assay.

We recommend establishing a public forum for researchers who are working with the EST so we can communicate about serum qualification efforts, as well as other technical difficulties laboratories may encounter while setting up the EST. The "Communities of Practice" available through the AltTox website (http://www.alttox.org/forums/) offers an excellent opportunity for such a forum.