Utility of Pooled Cryoplateable Liverpool™ in the *In Vitro* Determination of Cytochrome P450 Metabolism and Induction

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Abstract

Primary human hepatocytes have long been the gold standard as a surrogate assessment of liver metabolism of xenobiotic compounds. Pooling of human hepatocytes has provided a model which minimizes the variation in the expression and activity of individual enzymatic properties of donor hepatocytes. Pooled human hepatocytes mitigate the risk of genetic variants and donor differences in the metabolism of test compounds. The induction of P450 enzymes by new chemical entities (NCE) has been seen as a liability for potential drug-drug interactions *in vivo* and has required delineation in *in vitro* model systems such as plated human hepatocytes by the FDA. The development of a pool of cryoplateable hepatocytes which retain metabolic and induction potential proportional to the individual donors provides a solution to the issues of individual variation in assays which require a plated long term culture format.

Pools of cryoplateable hepatocytes were formulated from individual cryoplateable hepatocyte lots into pools of 5 and 10 donors and cryopreserved. These pools and the individual constituent lots were assessed, post thaw, for the retention of cytochrome P450(CYP) metabolic capacity utilizing prototypic substrates for CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4/5 and phase II enzymes. Individual activities of donors used in each pool were averaged and compared to the corresponding values from incubation with the post thaw pooled cryoplateable hepatocytes. Deviation of the calculated predicted average for the CYP450 isoforms varied from 8-38% with an average of 17%. The individual lots and pooled cryoplateable hepatocytes were plated, showing comparable attachment, and incubated with omeprazole, phenobarbital, rifampicin and vehicle controls for 48hrs to induce CYP1A, 2B and 3A respectively. Induction was measured by CYP450 metabolic activity through incubation with prototypic substrates and by Taqman RT-PCR analysis of extracted mRNA. Variation in fold induction measured by metabolism of prototypic substrates compared to vehicle controls deviated from the calculated expected values by 20%, 4% and 26% for CYP1A, 2B and 3A respectively. Variation in fold induction measured by Taqman RT-PCR analysis of mRNA deviated by 6%, 47% and 11% for CYP1A, 2B and 3A respectively. Cryoplateable Liverpool™ showed utility in replacing individual donor hepatocytes in both metabolism and induction assays. Cryoplateable Liverpool™ provides an averaged response of multiple donors while mitigating the risk of individual variation.

Materials and Methods

Pool Construction.
Cryoplateable hepatocytes were thawed, pooled and refrozen according to patented methods at BioreclamationIVT. Hepatocyte lots were selected and pools formulated such that an equal cell number from each donor would be recovered in the finished pool. Finished pools were tested for yield, viability, ability to attach to collagen I coated culture dishes with confluency >70% at day 5 in culture and respond to prototypic inducers for CYP1A2, CYP2B6 and CYP3A4.

In order to validate the Liverpool™ method for cryoplateable hepatocytes, individual cryoplateable hepatocytes were thawed and processed with the Liverpool™ method and refrozen as individual donor "pools". These individual "pool" vials were then thawed to establish cell recovery and plating efficiency on collagen I coated plates and characterized for CYP1A and 3A induction. Comparisons were made between the original vials and the Liverpool to assess the impact of the processing and select lots suitable for pooling.

Metabolism
Donor lots and frozen pools were thawed into 49mls of *InVitro*GRO™ HT media and spun at 50xg for 5min. The cell pellet was resuspended in *InVitro*GRO™ KHB buffer and .2x10^6 cells were incubated for 1 hour with prototypic substrates for CYP1A2,2A1, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, glucuronosyltransferase and sulfotransferase activity, listed below. Metabolite production was assayed by LC/MS and results are reported in pmol/min/10^6 cells.

Enzyme	Substrate	[µM]	Metabolites
1A2	Phenacetin	15	Acetaminophen
2A6	Coumarin	8	7-HC, 7-HCG, 7-HCS
2B6	Bupropion	250	Hydroxybupropion
2C8	Amodiaquine	20	Desethylamodiaquine
2C9	Tolbutamide	15	4-OH Tolbutamide
2C19	S-mephenytoin	20	4-OH Mephenytoin
2D6	Dextromephorphan	5	Dextrorphan
2E1	Chlorzoxazone	50	6-OH Chlorzoxazone
3A4	Testosterone	50	6β-OH Testosterone
ECOD	7-ethoxycoumarin	75	7-HC, 7-HCG, 7-HCS
SULT	7-hydroxycoumarin	30	7-HC Sulfate
UGT	7-hydroxycoumarin	30	7-HC Glucuronide

Induction
Cryoplateable hepatoctyes, both single donor and pools were thawed according to BioreclamationIVT protocol. Cells were plated on collagen I coated dishes and incubated with daily washing for two days. Inducers for CYP450 1A, 2B and 3A, omeprazole, phenobarbitol and rifampicin respectively and a vehicle control were added in serum free media to triplicate wells of the cultures for two days. Cells were incubated with specific substrates, phenacetin for 1A, buproprion for CYP2B, and testosterone for 3A for 4 hours and generation of metabolites was monitored by UPLC/MS/MS. Parallel wells were harvested with RLT buffer (Qiagen, Germantown, MD) and RNA extracted using Qiagen Rneasy mini kit. For concentration response curves, rifampicin, nifidipine, phenytoin, carbamazepine and triglitizone were exposed to hepatocytes over 2 log range for 48 hrs, and processed for mRNA as described above. Quantitation of mRNA was performed by real-time PCR utilizing TaqMan assays for the specific P450 isoforms. Fold induction values were calculated using the delta-delta Ct method with 18S rRNA utilized as the internal control.

Results

Figure 1.

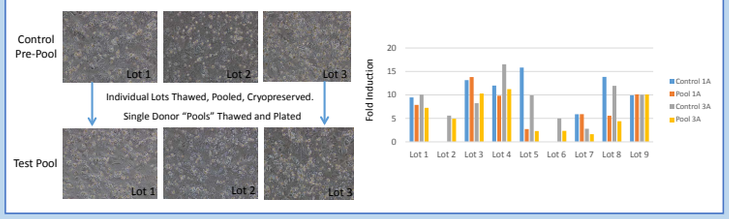

Figure 1.
The method and impact of the pooling was explored to ensure that cell performance would be retained in the cryoplateable pooled human hepatocytes. Individual lots were processed with the Liverpool™ method and cryopreserved into single donor "pools". Vials were thawed and plated according to standard BioreclamationIVT method and tested for induction of CYP1A, and 3A as described in methods. Cell monolayers were assessed for changes in confluency and induced values compared to pre-Liverpooled lots. Most lots retained similar plating efficiency and fold induction values. This methodology allowed for the exclusion of lots which did not retain appropriate characteristics from future pools.

Results

Figure 2.

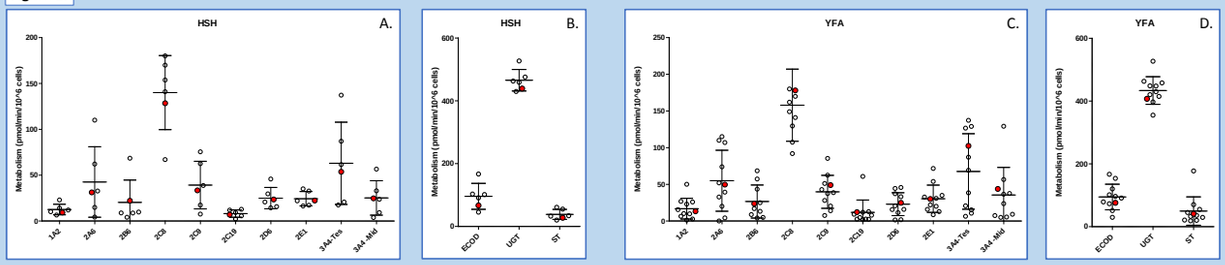

Figure 2 (A-D).
Cryoplateable Pooled Human Hepatocyte lots, both a 5-Donor lot and a 10-Donor lot (HSH and YFA) and the individual lots, from which they were formulated, were assessed for metabolic activity for a broad array of CYP isoforms and phase II conjugations. Assays were performed on hepatocytes in suspension with metabolite formation assessed by UPLC/MS/MS. Whisker plots of the metabolism data from individual donor lots are plotted (open circles) with the pooled lot(in red) expressed in pmol/min/10^6 cells. Error bars display the mean with standard deviation. Activities for the single donor lots range, for many isoforms, beyond a single standard deviation, highlighting the large diversity in CYP activity across donor human hepatocytes. The tested values for the pools for most isoforms, fall near the mean, highlighting the robustness of the procedure to retain the hepatocyte's P450 activity and the donor hepatocytes in predicted proportions.

Table 1.

Lot	ECOD	UGT	ST	1A2	2A6	2B6	2C8	2C9	2C19	2D6	2E1	3A4-Tes	3A4 -Mid
HSH	34.3%	6.7%	31.6%	29.4%	30.3%	10.7%	9.8%	17.2%	16.7%	6.8%	9.5%	17.1%	2.2%
YFA	21.1%	6.6%	18.8%	2.2%	10.5%	12.1%	14.4%	26.0%	3.4%	9.2%	1.2%	59.8%	26.8%

Table 1.
The percentage variation from the mean for the metabolic analysis performed on the individual and pooled lots is show in Table 1. It shows variation of less than 20% for the majority of the metabolites assessed. This retention of activities and close approximation to the theoretical expected value is consistent with expected recovery and representation of donor lots in the Cryoplateable Pooled Human Hepatocyte lots.

Figure 3.

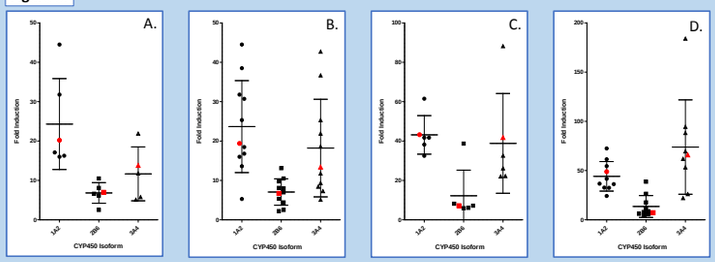

Figure 3 (A-D).
Cryoplateable Pooled Human Hepatocyte lots and the individual lots, from which they were formulated, were assessed for inducibility with prototypic inducers as described in material and methods. Fold induction calculated using metabolite formation data for the 5-Donor Lot (HSH) and the 10 Donor Lot (YFA) (Panels A and B), as well using mRNA analysis (Panels C and D) are shown. Error bars in the whisker plots indicate mean and standard deviation (1SD) from the mean. Pools (marked Red) fall near to the mean with single donor values falling over a large range for most isoforms and analysis methods. The percentage variation from the mean fell within 30% for all combinations with the exclusion of 2B6 measured by mRNA which was .

Figure 4.

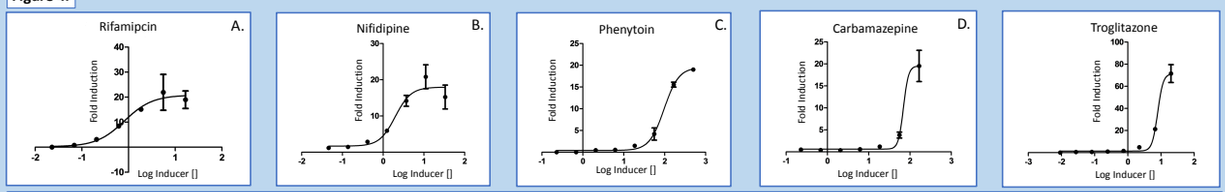

Figure 4..
Prototypic CYP450 3A inducers were used over an appropriate wide range of concentrations to generate dose dependency curves. Fold induction data was generated using an mRNA readout as described in materials and methods. Curves were fitted by non-linear regression using a 4-parameter fit with maximal induction constrained where no plateau was obtained for solubility or toxicity reasons using Graphpad Prizm (version 5.0).

Conclusions:

- Cryoplateable Human Hepatocytes can be used in the Liverpool™ process to generate multi-donor pooled cryoplateable reagents.
- The Cryoplateable Pooled Human Hepatocytes generate cultures of good confluence and representation of starting donors.
- These pools retain metabolic activity and show good predictability in their formulation.
- Induction characteristics are retained in the Cryoplateable Pooled Human Hepatocytes and are an average representation of the donors. They show good predictability of fold induction base on donor characteristics.
- Cryoplateable Pooled Human Hepatocytes show robust dose dependent induction with CYP3A4 inducers. This may allow for their use in concentration response curve induction screening methodologies.