

Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation but is strongly changed after hepatocyte plating

Lysiane Richert, Michael J. Liguori, Catherine Abadie, Bruno Heyd, Georges Manton,
Nermin Halkic, and Jeffrey F. Waring

Laboratoire de Biologie Cellulaire, EA 3921 Optimisation Métabolique et Cellulaire (LR, CA); KaLy-Cell, UFR des Sciences Médicales et Pharmaceutiques, Besançon, France (LR, CA); Molecular and Cellular Toxicology, Abbott Laboratories Building AP9A, Abbott Park, IL 60064, USA (MJL, JFW); Service de Chirurgie Viscérale et Digestive, Centre de Transplantation Hépatique, Hôpital Jean Minjoz, Besançon, France (BH, GM); Service de Chirurgie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland (NH);

Authors contributed equally (LR, MJL)

Running title: Genomic expression in human hepatocytes

*Corresponding author. Lysiane Richert ; Laboratoire de Biologie Cellulaire, EA3921
Optimisation Métabolique et Cellulaire, UFR des Sciences Médicales et Pharmaceutiques,
Place Saint-Jacques, 25030 Besançon cedex, FRANCE. Tel: 33 3 81 66 55 52, Fax: 33 3 81
66 56 79. E-mail: lysiane.richert@univ-fcomte.fr

Number of text pages: 25 (from Abstract to Footnotes)

Number of Figures: 5 (numbered Figures 1 –5)

Number of Tables: 3

Number of references: 40

Number of words of Abstract: 248

Number of words of Introduction: 548

Number of words of Discussion: 1642

Abbreviations:

CYP : cytochrome P450, BSA : Bovine Serum Albumin, PBS: Phosphate Buffered Saline,

FCS: Fetal Calf Serum, DMEM: Dulbecco's Modified Eagle Medium, EDTA : , UW:

University of Wisconsin solution, UGT : UDP-glucuronosyltransferase,

Abstract

Isolated primary human hepatocytes are a well-accepted system for evaluating pharmacological and toxicological effects in humans. However, questions remain regarding how culturing impacts the liver-specific functions of the hepatocytes. In addition, cryopreservation could also potentially affect the differentiation state of the hepatocytes. The first aim of the present study was to compare gene expression in freshly isolated primary hepatocytes to that of the liver of origin, and to evaluate the expression changes occurring following cryopreservation/thawing, both when maintained in suspension and after plating. The second aim of the present study was to evaluate gene expression in hepatocytes following cold storage of suspensions up to 24h, compared to freshly isolated hepatocytes in suspension. Our results show that the gene expression in freshly isolated human hepatocytes in suspension after isolation is similar to that of the liver of origin. Furthermore, gene expression in primary human hepatocytes in suspension is not affected by hepatocyte cold storage and cryopreservation. However, the gene expression is profoundly affected in monolayer cultures after plating. Specifically, gene expression changes were observed in cultured relative to suspensions of human hepatocytes that are involved in cellular processes such as phase I/II metabolism, basolateral and canicular transport systems, fatty acid and lipid metabolism, apoptosis, and proteasomal protein recycling. An oxidative stress response may be partially involved in these changes in gene expression. Taken together, these results may aid in the interpretation of data collected from human hepatocyte experiments and suggest additional utility for cold storage and cryopreservation of hepatocytes.

Introduction

The liver serves as the primary site of detoxification of exogenous and endogenous compounds in the systemic circulation. Other biological and physiological functions include the production and secretion of critical blood and bile components, such as albumin, bile salts, and cholesterol. The liver is also involved in protein, steroid, and fat metabolism, as well as vitamin, iron, and sugar storage.

One of the most complex functions specific to liver is its ability to metabolize an enormous range of xenobiotics. Many drugs present in the blood are absorbed by hepatocytes, where they can be metabolized via phase I and II biotransformation reactions. Information concerning hepatic drug uptake and metabolism, phase I and phase II induction, drug interactions affecting hepatic metabolism, as well as hepatotoxicity are essential for pharmacology and toxicology of a given drug. Due to major species differences both in the catalytic activities and regulation of enzymes involved in drug metabolism, many of these evaluations can only be accurately investigated with human tissue. Since intact cells more closely reflect the environment to which drugs are exposed in the liver, isolated primary human hepatocytes are a well-accepted system for evaluating pharmacological and toxicological effects in humans (Gomez-Lechon et al., 2004; O'Brien et al., 2004; Liguori et al., 2005) and have been described as one of the best *in vitro* models for the prediction of *in vivo* metabolism in humans (Lave et al., 1999; Li, 2001). This has prompted research groups, including ours, to optimize the isolation and culture conditions of human hepatocytes from both surgical liver resections and non-transplantable livers (Richert et al., 2004; LeCluyse et al., 2005; Donato et al., 2005).

Sparse availability of high quality human liver tissue for research purposes, the demand for standardized cell populations, and the need for proper storage of cells for future

research investigations have resulted in the development of cryopreservation techniques (Alexandre et al., 2002; Garcia et al., 2003; Roymans et al., 2005). However several problems exist with the use of thawed human hepatocytes. For instance, cryopreserved hepatocytes have displayed a substantial variability between donors both in terms of recovery of viable hepatocytes and in their capacity to attach after thawing (Alexandre et al., 2002; Blanchard et al., 2005). This has profound consequences in terms of hepatocyte differentiation rates in culture and responses to xenobiotics since it has been found that isolated hepatocytes in culture display markedly different gene expression patterns depending on attachment status (Waring et al., 2003). In addition, it has previously been shown that constitutive expression and inducibility of the cytochrome P450s (CYPs) is dependent on cell-density (Hamilton et al., 2001). Also, studies have shown that cultured thawed hepatocytes in monolayers appeared smaller than their freshly isolated hepatocyte counterparts in monolayers, suggesting a variability in the resistance to cryopreservation of the various liver hepatocyte populations (Alexandre et al., 2002).

The first aim of the present study was thus to compare gene expression in freshly isolated hepatocytes to the liver of origin, and to evaluate the changes occurring following cryopreservation/thawing, both when maintained in suspension and after plating. The second aim of the present study was to evaluate gene expression in hepatocytes following cold storage as suspensions up to 24h, compared to freshly isolated hepatocytes in suspension. A pictorial depiction of the intended study is represented in Figure 1.

Materials and methods

2.1. Materials

Bovine Serum Albumin (BSA), Phosphate Buffered Saline (PBS), Fetal Calf Serum (FCS), Dulbecco's Modified Eagle Medium (DMEM) and TriZol™ were purchased from Invitrogen-Life technologies–France). Collagenase type IV, Percoll, and EDTA were purchased from Sigma (St Louis, MO, USA).

2.2. Human hepatocyte isolation

Adult normal liver samples were obtained from 3 patients undergoing partial hepatectomy for primary or secondary tumours. All experimental procedures were done in compliance with French laws and regulations and were approved by the National Ethics Committee. **Table 1** depicts the patient's demographics. Liver resections were handled, a small portion of liver was suspended in RNALater™ and frozen at -80°C. Hepatocytes were isolated by a two-step collagenase perfusion as recently described (Richert *et al.*, 2004). After Percoll purification, cell viability was estimated by Trypan blue exclusion. Part of freshly isolated human hepatocytes were kept directly in TriZol™ (0.5mL TriZol™ for 1 million cells) and frozen at -80°C.

2.3. Human hepatocyte cold storage or cryopreservation and thawing

Part of the freshly isolated human hepatocytes was kept in UW solution at 4°C for 3 or 24 hours. Another portion of the freshly isolated human hepatocytes was immediately subjected to cryopreservation as previously described (Alexandre *et al.*, 2002).

Human hepatocytes after cold storage (3 and 24 hours timepoints) were resuspended in DMEM and washed one time before having been subjected to percoll purification. The cell

pellet obtained was suspended in TriZol™ (0.5mL TriZol™ for 1 million cells) and frozen at -80°C. (see **Figure 1**)

2.4. Human hepatocyte culture

Human hepatocytes, either directly after isolation or after cryopreservation/thawing were subjected to percoll purification and suspended in DMEM supplemented with FCS (5%), insulin (4 mg/L), hydrocortisone (10^{-6} mol/L) and gentamycin (50 mg/L). Some of the thawed hepatocyte cells were directly suspended in TriZol™ and frozen at -80°C. Freshly isolated hepatocytes and the remaining thawed hepatocytes were plated onto 60 mm dishes at a density of 3.5×10^6 cells/dish. At each time point of culture (24h, 48h and 72h for the fresh hepatocytes and only 24h for the thawed hepatocytes), hepatocyte cells were scraped in TriZol™ (0.5mL TriZol™ for 1 million cells) and frozen at -80°C. (see **Figure 1**)

RNA extraction and microarray analysis

Total RNA was isolated from the TRIzol™ extracts using the procedure from Invitrogen. Optical density at 260 nm determined RNA concentration. RNA quality was accessed using an Agilent Technologies bioanalyzer before proceeding to microarray sample preparation. Microarray analysis was performed using the standard protocol provided by Affymetrix Inc. (Santa Clara, CA) and as previously described, starting with 5 µg of total RNA (Liguori, et al. 2005). Fragmented, labeled cRNA was hybridized to an Affymetrix human genome U133A array, which contains sequences corresponding to roughly 22,200 transcripts at 45°C overnight.

Microarray data analysis

The microarray scanned image and intensity files were imported in Rosetta Resolver™ gene expression analysis software version 5.0 (Rosetta Inpharmatics, Kirkland, WA). Resolver's

Affymetrix error model was applied and ratios were built for each step in the study using freshly isolated human hepatocytes as the baseline. Using Rosetta Resolver, a p-value is calculated for every fold change, using the Rosetta Resolver error model. For all of the gene changes shown, the p-value is displayed in the figure legend. If the p-value is greater than the listed value, the fold change is shown as 1.0 (or black on a heatmap). Gene ontologies were deciphered using NETAFFX™ or GenMAPP (Liu et al 2003; Dahlquist et al 2002).

Results

Study Design

Gene expression analysis was conducted for three distinct human donors according to the study design as revealed in **Figure 1**. Briefly, microarray analysis was performed on human liver and freshly isolated primary human hepatocytes as well as hepatocyte monolayers from 24-72 hrs. Additionally, a portion of the primary hepatocytes was cryopreserved, and these cells underwent genomic analysis immediately after thaw and again after culture for 24 hrs. Some of the hepatocytes were stored in a cell suspension of UW solution, and subsequent gene expression analysis was conducted at the 3 hour and 24 hour timepoints. For data analysis purposes, the freshly isolated primary human hepatocytes were considered a control and were used as a baseline for ratio builds.

Primary Human Hepatocyte Morphology, Viability, and Attachment

Figure 2 displays light micrographs of different stages of the study for hepatocytes from donor 2. **Table 2** exhibits the percent recovery of viable primary human hepatocytes subsequent to cold storage and cryopreservation. For all donors, viability declined upon continued cold preservation in UW solution up to 24 hrs, the loss of cells being more evident for donor 1 than for donor 2 and donor 3. Also, viability was decreased upon cryopreservation (liquid nitrogen), with recovery being equivalent for donor 2 and donor 3, about 50 %, higher than donor 1 (10%). **Table 3** illustrates that primary human hepatocyte attachment efficiency declined subsequent to cryopreservation. Again, attachment rate of plated viable thawed hepatocytes was lower for donor 1 than for donor 2 and 3. As also seen in **Table 3**, when considering total cell protein level of attached thawed hepatocytes, the values were very similar for the 3 donors, being respectively 0.85, 0.99 and 0.83 mg protein/dish. After thawing, this total cell protein level was lower, respectively 53% (0.45

mg/dish; donor 1), 60% (0.59 mg/dish; donor 2) and 82% (0.68 mg/dish; donor 3) from that of the respective freshly isolated attached total protein level.

Gene Expression Analysis

Using freshly isolated primary human hepatocytes as the baseline, microarray analysis on human liver and primary human hepatocytes at various stages of preparation revealed relatively few gene expression changes for intact human liver, hepatocytes from 3 and 24 hr cold storage, and thawed primary hepatocytes post-cryopreservation (**Figure 3**). However, the gene expression profiles from primary human hepatocytes after plating for 24-72 hrs both from fresh and cryopreserved preparations result in a markedly increased number of differentially regulated transcripts relative to freshly isolated primary hepatocytes. The gene expression changes in plated hepatocytes relative to freshly isolated hepatocytes were, in general, consistent over time for all donors (**Figure 3**). A supplemental MS Excel table is included with this communication detailing all gene expression changes (relative to freshly isolated human hepatocytes) with at least +/- 1.5-fold change and p-value of 0.05 or less. The fold changes for probe sets with p-values greater than 0.05 are represented as "1."

Hepatic phase I and II biotransformation reactions are critical to xenobiotic metabolism. Examination of CYP gene expression reveals a down-regulation of most transcripts upon plating of the primary hepatocytes relative to freshly isolated primary hepatocytes, whereas the CYP expression level is predominately unchanged in samples from intact liver, cold storage, and immediately post-cryopreservation (**Figure 4A**). Most of the major CYP isoforms (CYP1, 2, 3, 4) have a reduced expression level upon plating of the primary hepatocytes, with the exception of CYP2C18, which was slightly up-regulated upon plating for all donors. Additionally, there are some cytochrome P-450 genes (CYP1B1, CYP20A1, CYP51A1, CYP2C18, CYP21A2, and CYP26A1) that had elevated levels of

expression upon primary hepatocyte plating, especially for donor 2 (**Figure 4B**). However, many of these described CYPs vary from donor to donor and are not induced in every donor nor at every plating timepoint. Many gene products for the phase II conjugation system, including transcripts encoding several UDP-glucuronosyltransferases (UGTs) initially exhibited reduced levels of expression upon plating, while remaining unchanged in samples from liver, cold storage, and post-cryopreservation (**Figure 4C, 4D**). Most of the Phase II genes that were regulated for the plated human hepatocytes were decreased quite consistently across all three donors evaluated.

Hepatic transport is a critical function for the uptake and excretion of xenobiotic compounds. Microarray analysis revealed that the greatest effect on transporter mRNA levels was seen in plated hepatocytes. **Figure 4E** shows that many of the solute carrier family of transporters have reduced levels of transcripts with respect to freshly isolated hepatocytes. For instance, SLC22A7 (solute carrier family 22), an organic anion transporter, was down-regulated after plating for all donors. Alternatively, there are transcripts such as SLC38A1, which is responsible for transport of glutamine (Gu et al. 2001), that have elevated levels upon primary hepatocyte plating. Most transporter mRNA levels remained unchanged for human hepatocytes in cold storage and immediately after cryopreservation, suggesting that the transporter gene expression changes result from plating of the primary hepatocytes. Furthermore, many transporter genes, such as ABCG5, SLCO2B1, and ABCB11, were consistently regulated across all three donors, while the expression of others, including ABCC3, SLC38A2, and SLC4A4, varied quite extensively from donor to donor.

Another important function of the liver pertains to its role in lipid and fatty acid metabolism. In particular, several genes involved with fatty acid degradation exhibited a trend of down-regulation. For the 24 hr. primary hepatocyte monolayer for donor 1, genes encoding enzymes such as carnitine palmitoyltransferase II, carnitine/acylcarnitine

translocase, acyl-CoA dehydrogenases, and long chain fatty acid CoA ligase 5 were all down-regulated relative to freshly isolated primary hepatocytes (**Figure 5A**). Additionally, a gene for short chain 3-hydroxy-acyl-CoA dehydrogenase (HADHSC, probe ID 201035_at), an essential enzyme for mitochondrial β -oxidation catalyzing the conversion of 3-hydroxyacyl-CoAs to 3-ketoacylCoAs, was strongly reduced. Furthermore, for many transcripts, a trend of down-regulation is apparent with genes involved in lipid metabolism (as defined by gene ontology) for primary hepatocyte monolayers (**Figure 5B**). Intact human liver, primary hepatocytes in cold storage, and post-cryopreservation primary hepatocytes show the fewest gene expression changes for lipid metabolism.

The proteasome is the cellular machinery responsible for protein regulation, degradation, and recycling, and it serves a vital function in maintaining homeostasis. It is a multi-subunit ubiquitin dependent holoenzyme with a core complex responsible for hydrolytic activity (20S core) and a regulatory complex with ATPase activity (19S regulator) that mediates identification and proper trafficking of peptide substrates into the active site (Meiners, et al. 2003). There was a significant up-regulation in the expression level of genes that encode for proteasome subunits upon plating of primary hepatocytes for all donors (**Figure 5C**). More specifically, genes such as PSMD12 (a non-ATPase regulatory subunit), PSMD8 (a non-ATPase regulatory subunit), and PSMC6 (a subunit with ATPase activity), were all significantly overexpressed upon plating of the human hepatocytes (data not shown). This effect was largely not observed in samples from intact liver, primary hepatocyte cold storage, and post-cryopreservation, which suggests the possibility that some aspect of the plating process may play a role in this enhancement of expression.

Apoptosis is a type of cell death that has distinct morphological and biochemical characteristics and that is under strict regulation. As displayed in **Figure 5D**, the expression level of several key transcripts associated with induction of apoptosis were increased in

primary hepatocyte monolayers relative to freshly isolated hepatocytes. For instance, caspases, which are enzymes involved in the execution phase of programmed cell death, such as CASP7 (all donors) and CASP3 (donor 1 in 24 hr monolayer and donor 3) were significantly overexpressed in the plated human hepatocytes. Furthermore, other prominent players in apoptotic cell death exhibited enhanced expression in plated human hepatocytes, such as PARP, an activator of cellular proliferation and of various nuclear proteins involved with DNA repair mechanisms. Also, BID, involved in apoptosis activation via mitochondrial cytochrome c release, and *c-myc*, a transcription factor that assists with cell cycle regulation, were induced in samples of plated human hepatocytes for most donors and timepoints. The expression of these pro-apoptotic genes was changed upon cultivation of the primary hepatocyte monolayer, but was largely unchanged in samples from intact liver, cold storage, and post-cryopreservation.

Discussion

Gene expression in human hepatocytes in suspension after isolation is similar to that of the liver of origin

The relatively few genes that were regulated in the intact livers versus freshly isolated hepatocytes tended to be down-regulated, suggesting that some of these expression changes may result from the fact that the liver is composed of a heterogeneous cell population while primary hepatocyte cultures are a more homogenous population. These small changes in gene expression in hepatocytes following their isolation are consistent with our recent observation that CYP-dependent and UGT-dependent activities are equivalent or only slightly decreased in microsomes prepared from human hepatocytes after their isolation compared to liver microsomes from the liver of origin (data not shown).

Gene expression in human hepatocytes in suspension is not affected by hepatocyte cold storage and cryopreservation

The equivalent gene expression profile observed in suspension of freshly isolated human hepatocytes, after cryopreservation/thawing and after cold-storage, are also in line with our recent observations that metabolic capacity of human hepatocytes in suspension after cold storage or after cryopreservation was intact and clearance predictions were equivalent to suspensions of freshly isolated hepatocytes from the same donor (Blanchard et al., 2005). Shitara et al. (2003) also observed that the transporter systems in human hepatocytes were functional after cryopreservation. Interestingly, gene expression for primary human hepatocytes in cold storage and cryopreservation is similar to that of the liver of origin, which could have implications for hepatocyte model systems being translated to intact liver.

The recovery of viable hepatocytes after a 24h cold storage is at least equivalent to that after cryopreservation (see **Table 2**), suggesting that cold storage could be an alternative for human hepatocyte short-term shipping and storage. The data also suggest that CYP expression levels remain relatively stable at baseline levels upon cold storage of human hepatocytes. In addition, few gene expression changes were evident for critical hepatocellular processes including transport, lipid metabolism, phase II metabolism, and protein processing, degradation, and recycling (proteasome subunits). Furthermore, human hepatocytes after cryopreservation and in cold storage may be less prone to initiating and undergoing apoptosis as evidenced by few expression changes for genes involved in apoptosis induction for these samples.

Gene expression in human hepatocytes is strongly changed after plating

Significant gene expression changes occur upon plating of hepatocytes. It has been previously shown that major cytochrome P450 dependent enzyme activities decline in culture, and this is associated with decreases in both gene and protein expression, symptomatic of a dedifferentiated state (Madan et al., 2003). The data of the present communication thus confirm these observations and highlight that some other CYP isoforms are, in contrast, up-regulated following plating. We (Richert et al., 2002; Binda et al., 2003) have previously suggested that the decline in CYP expression could be related to oxidative stress occurring during time in culture. Several genes associated with an cellular oxidative stress response, such as superoxide dismutase 2, glutathione reductase, p53, and peroxiredoxin, are over-expressed upon plating of human hepatocytes (data not shown). The present study therefore presents further evidence that oxidative stress occurs during plating, and also further highlights that pathways leading to apoptosis are activated as well, most probably, at least in part oxidative stress related. In support of this, it has been reported that reactive oxygen species mediate apoptosis in hepatocytes (Rauen et al., 1999; Ishihara et al, 2005).

UDP-glucuronosyltransferases (UGTs) are a family of Phase II enzymes critical for xenobiotic biotransformation reactions. The findings from this study indicate that the mRNA levels of several members of this class are reduced upon primary hepatocyte plating at the 24 hour timepoint. At 72 hours, there appears to be a slight trend toward increasing levels of mRNA relative to the 24 or 48 hour timepoint for some UGT genes, especially for donor 2. This reflects a similar tendency observed in other reports with primary rat hepatocytes (Li et al., 1999; Zurlo et al., 1996). Furthermore, in activity measurements, UGTs retain basal metabolizing capabilities upon primary rat hepatocyte plating compared to whole liver (Richert et al., 2002). Additionally, cryopreservation did not affect the gene expression of UGTs in this study, in concordance with other studies (Li et al., 1999).

Hepatocellular transporter systems are essential regulators of homeostasis, are involved in drug disposition and bile formation, and may be critical for toxicological and pharmacological studies (Chandra and Brouwer 2004). The data indicate that the expression of many transporter systems, especially those in the solute carrier family series, is decreased upon plating of the primary human hepatocytes. Messenger RNA from major basolateral transporters, such as OATP2 (SLC22A7) and NTCP (SLC10A1), have been previously shown to be markedly reduced upon plating of primary rat hepatocyte cultures (Rippin et al. 2001; Luttringer, et al, 2002). Our results also support this trend, and furthermore offer evidence of down-regulation of additional basolateral transporters, such as OCT1 (SLC22A1) and MRP6 (ABCC6) upon plating of primary human hepatocytes, in agreement with studies from other laboratories (Jigorel et al., 2005).

Hepatic canicular transport proteins incorporate a variety of ATP-dependent membrane associated pumps that encompass the ATP binding cassette superfamily, including the well studied MDR1 or p-glycoprotein (Rippin, et al. 2001; Chandra and Brouwer 2004). In primary rat hepatocytes, MDR1b (ABCB1) and MRP3 (ABCC3) transcription products have been observed to increase upon plating and cultivation (Luttringer et al., 2002). Similarly, in plated primary human hepatocytes, we observe over-expression of ABCB1 mRNA relative to freshly isolated hepatocytes, which could result in more informed interpretation for studies involving xenobiotic metabolism of ABCB1 substrates. Other canicular pumps, such as ABCB11 and ABCB4, tend to be under-expressed in plated primary human hepatocytes for these evaluated donors.

To our knowledge, the present study is the first report on drastic changes in the expression of genes involved with fatty acid and lipid metabolism in primary human hepatocytes after plating as determined by microarray analysis. A large number of lipid metabolism transcripts are under-expressed in monolayers of primary human hepatocytes

relative to their freshly isolated counterparts. This, in part, could be related to an oxidative stress response since both arachidonic acid mobilization and phospholipase A2 activity were found affected by oxidative stress (Babenko et al., 1998). Even more relevant to the present results, a direct link between oxidative stress and ApoB100 degradation and VLDL secretion has been recently reported (Pan et al., 2004).

It has been well documented that free fatty acids can have dramatic direct or indirect effects on expression of genes involved in lipid metabolism via regulation of nuclear transcription factors such as the PPARs, SREBPs, LXRs, or HNF (Jump, 2002; Swagell, et al., 2005). Although no significant gene expression regulation of PPARs (alpha, beta, or gamma) or HNF1 was detected, LXRA (203920_at) was under-expressed for all donors, and SREBP1 (202308_at) was under-expressed for donor 1 and 3 only in the monolayer cultures relative to freshly isolated human hepatocytes (data not shown). Perhaps changes in the abundance and variety of fatty acids from intact liver to cell culture may be at least partially responsible for the observed changes in expression.

Interestingly, many proteasomal subunits, the proteolytic machinery responsible for degradation and recycling of ubiquitin-protein complexes, are over-expressed in primary human hepatocyte monolayers compared to freshly isolated hepatocytes. Increased expression of these subunits may be associated with an increased proteasome presence, and ultimately potentially elevated proteolytic activity relative to the native state (Hobler, et al., 1999; Fang et al., 2000). A recent communication highlights that mild oxidative stress may augment proteasome activity and expression in response to a heightened level of oxidatively damaged proteins (Elkon, et al., 2004), whereas severe oxidative stress may result in proteasome failure and in abolishment of proteasome activity. Therefore, it is possible that some degree of oxidative stress in plated human hepatocytes may drive, in part, the over-expression of these proteasomal subunits. Awareness of this potential increased proteolytic

activity and turnover may assist in some types of data interpretation for primary human hepatocyte studies.

Gene expression after plating is not affected by cold storage or cryopreservation

In the present study, we found that the gene expression pattern for the most of the major genes evaluated in plated cryopreserved/thawed human hepatocytes was similar to that of their respective plated fresh monolayers (24 hr timepoint) for all donors. One should be aware however that in the present study, the plating density of thawed hepatocytes was 50-80% of the plating density of their respective freshly isolated hepatocytes, and this could contribute to the similar pattern of gene expression for plated thawed human hepatocytes. Indeed, in a previous study, we found that human hepatocytes in culture displayed markedly different gene expression patterns depending on attachment status (Waring et al., 2003), in accordance with the observations of Hamilton et al. (2001), who described a decrease in constitutive levels of CYP3A4 in human hepatocytes that correlated with the plating density. Nonetheless, it is noteworthy that in the latter work, the fold induction of CYP3A4 observed following exposure to rifampicin was equivalent in cultures as long as the cell density was not reduced to more than 50% of standard density. Taken together, these results suggest that plated cryopreserved hepatocytes are a valid tool for drug metabolism and toxicological evaluations as long as the plating density is at least 50% of that of a confluent monolayer.

It is possible that the expression profiles for some genes in other human donors may behave differently than observed in this study due to the large degree of primary human hepatocyte donor to donor heterogeneity (Liguori et al., 2005). However, it is clear from these

results that plating of primary human hepatocytes leads to a vigorous change in gene expression from the native state, which may be a critical factor for some studies involving human hepatocytes. Taken together, these results may aid in the interpretation of data collected from human hepatocyte experiments and suggest additional utility for cold storage and cryopreservation of hepatocytes.

REFERENCES

- Alexandre E, Viollon-Abadie C, David P, Coassolo P, Heyd B, Manton G, Wolf P, Bachellier P, Jaeck D and Richert L (2002). Cryopreservation of adult human hepatocytes obtained from resected liver biopsies. *Cryobiology* **44**: 103-113.
- Babenko NA, Ruiz-Larrea MB, Martinez R, Martin C, Lacort M. (1998). Inhibition by estrogens of the oxidant-mediated mobilization of arachidonic acid in hepatocytes. *J Physiol Biochem.* **54**:77-84.
- Binda D, Lasserre-Bigot D, Bonet A, Thomassin M, Come MP, Guinchard C, Bars R, Jacqueson A, Richert L. (2003) Time course of cytochromes P450 decline during rat hepatocyte isolation and culture: effect of L-NAME. *Toxicol In Vitro.* **17**:59-67.
- Blanchard N, Alexandre E, Abadie C, Lave T, Heyd B, Manton G, Jaeck D, Richert L and Coassolo P (2005). Comparison of clearance predictions using primary cultures and suspensions of human hepatocytes. *Xenobiotica* **35**: 1-15.
- Chandra P, Brouwer KL (2004) The complexities of hepatic drug transport: current knowledge and emerging concepts. *Pharmaceutical Res* **21**: 719-735.
- Dahlquist K, Salomonis, Vranizan, Lawlor, and Conklin. (2002) GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nature Genetics* **31**:19-20.

Donato MT, Serralta A, Jimenez N, Perez G, Castell JV, Mir J and Gomez-Lechon MJ. (2005) Liver grafts preserved in Celsior solution as source of hepatocytes for drug metabolism studies: comparison with surgical liver biopsies. *Drug Metab Dispos.***33**:108-114.

Elkon H, Melamed E, Offen D. (2004). Oxidative stress, induced by 6-hydroxydopamine, reduces proteasome activities in PC12 cells: implications for the pathogenesis of Parkinson's disease. *J Mol Neurosci.* **24**:387-400.

Fang CH, Li BG, Fischer DR, Wang JJ, Runnels HA, Monaco JJ, Hasselgren PO (2000). Burn injury upregulates the activity and gene expression of the 20 S proteasome in rat skeletal muscle. *Clin Sci (Lond).* **99**:181-7.

Garcia M, Rager J, Wang Q, Strab R, Hidalgo IJ, Owen A and Li J (2003) Cryopreserved human hepatocytes as alternative in vitro model for cytochrome p450 induction studies. *In Vitro Cell Dev Biol Anim.***39**:283-7.

Gomez-Lechon MJ, Donato MT, Castell JV and Jover R (2004). Human hepatocytes in primary culture: the choice to investigate drug metabolism in man. *Curr Drug Metab* **5**:443-462.

Gu S, Roderick HL, Camacho P, Jiang JX. (2001). Characterization of an N-system amino acid transporter expressed in retina and its involvement in glutamine transport. *J Biol Chem.* **276**:24137-24144.

Hamilton GA, Jolley SL, Gilbert D, Coon DJ, Barros S and LeCluyse EL (2001). Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. *Cell Tissue Res* **306**:85-99.

Hobler SC, Williams A, Fischer D, Wang JJ, Sun X, Fischer JE, Monaco JJ, Hasselgren PO (1999). Activity and expression of the 20S proteasome are increased in skeletal muscle during sepsis. *Am J Physiol*. **277**:R434-440.

Ishihara Y, Shiba D, Shimamoto N. (2005). Primary hepatocyte apoptosis is unlikely to relate to caspase-3 activity under sustained endogenous oxidative stress. *Free Radic Res*. **39**:163-73.

Jigorel E, Le Vee M, Boursier-Neyret C, Bertrand M, Fardel O. (2005). Functional expression of sinusoidal drug transporters in primary human and rat hepatocytes. *Drug Metab Dispos*. E-publication ahead of print.

Jump DB. (2002). Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr Opin Lipidol*. **13**:155-64.

Lave T, Coassolo P, Reigner B (1999). Prediction of hepatic metabolic clearance based on interspecies allometric scaling techniques and in vitro-in vivo correlations. *Clin Pharmacokinet* **36**:211-231.

LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S and Richert L (2005). Isolation and culture of primary human hepatocytes. *Methods Mol Biol*, **290**:207-229.

Li AP (2001). Screening for human ADME/Tox drug properties in drug discovery. *Drug Discov Today* **6**:357-366.

Li, Y.Q., Prentice, D.A., Howard, M. L., Mashford, M. L., Desmond, P.V. (1999) The effect of hormones on the expression of five isoforms of UDP-glucuronosyltransferase in primary cultures of rat hepatocytes. *Pharmaceutical Research* **16**:191-197.

Liguori MJ, Anderson LM, Bukofzer S, McKim J, Pregenzer JF, Retief J, Spear BB, Waring JF (2005) Microarray analysis in human hepatocytes suggests a mechanism for hepatotoxicity induced by trovafloxacin. *Hepatology* **41**:177-86.

Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmeekam V, Sun S, Kulp D, Siani-Rose MA (2003). NetAffx: Affymetrix probesets and annotations. *Nucleic Acids Res.* **31**:82-86.

Luttringer O, Theil FP, Lave T, Wernli-Kuratli K, Guentert TW, de Saizieu A. (2002) Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. *Biochem Pharmacol.* **64**:1637-50.

Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, Downey AD, Czerwinski M, Forster J, Ribadeneira MD, Gan LS, LeCluyse EL, Zech K, Robertson P, Koch P, Antonian L, Wagner G, Yu L, Parkinson A (2003). Effects of prototypical microsomal

enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab Dispos* **31**: 421-431.

Meiners S, Heyken D, Weller A, Ludwig A, Stangl K, Kloetzel PM, Kruger E (2003).
Inhibition of Proteasome Activity Induces Concerted Expression of Proteasome Genes and de
Novo Formation of Mammalian Proteasomes. *J. Biol. Chem.* **278**: 21517-21525.

O'Brien PJ, Chan K, Silber PM (2004). Human and animal hepatocytes in vitro with
extrapolation in vivo. *Chem Biol Interact.* **150**:97-114.

Pan M, Cederbaum AI, Zhang YL, Ginsberg HN, Williams KJ, Fisher EA. (2004). Lipid
peroxidation and oxidant stress regulate hepatic apolipoprotein B degradation and VLDL
production. *J Clin Invest.* **113**:1277-87.

Rauen U, Polzar B, Stephan H, Mannherz HG, de Groot H. (1999) Cold-induced apoptosis in
cultured hepatocytes and liver endothelial cells: mediation by reactive oxygen species.
FASEB J. **13**:155-68.

L. Richert, D. Binda, G. Hamilton, C. Viollon-Abadie, E. Alexandre, D. Bigot-Lasserre, R.
Bars, P.Coassolo and E. LeCluyse. (2002) Evaluation of the effect of culture configuration on
morphology, survival time, antioxidant status and metabolic capacities of cultured rat
hepatocytes. *Toxicology in Vitro* **16**, 89-99.

Richert L, Alexandre E, Lloyd T, Orr S, Viollon-Abadie C, Patel R, Kingston S, Berry D,
Dennison A, Heyd B, Manton G and Jaeck D (2004) Tissue collection, transport and isolation

procedures required to optimise human hepatocyte isolation from waste liver surgical resections. A multi-laboratory study. *Liver Int.* **24** : 371-378.

Richert L, Lambole C, Viollon-Abadie C, Grass P, Hartmann N, Laurent S, Heyd B, Manton G, Chibout SD and Staedtler F (2003). Effects of clofibric acid on mRNA expression profiles in primary cultures of rat, mouse and human hepatocytes. *Toxicol. Appl. Pharmacol.* **191**:130-146.

Rippin SJ, Hagenbuch B, Meier PJ, Stieger B. (2001) Cholestatic expression pattern of sinusoidal and canalicular organic anion transport systems in primary cultured rat hepatocytes. *Hepatology* **4**:776-82.

Roymans D, Annaert P, Van Houdt J, Weygers A, Noukens J, Sensenhauser C, Silva J, Van Looveren C, Hendrickx J, Mannens G, Meuldermans W. (2005) Expression and induction potential of cytochromes P450 in human cryopreserved hepatocytes. *Drug Metab Dispos.* **33**: 1004-1006

Shitara Y, Li AP, Kato Y, Lu C, Ito K, Itoh T, Sugiyama Y. (2003) Function of uptake transporters for taurocholate and estradiol 17beta-D-glucuronide in cryopreserved human hepatocytes. *Drug Metab Pharmacokinet.* **18**: 33-41.

Swagell CD, Henly DC, Morris CP (2005). Expression analysis of a human hepatic cell line in response to palmitate. *Biochem Biophys Res Commun.* **328**:432-41.

Wang H, LeCluyse EL (2003). Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin Pharmacokinet.* **42**:1331-1357

Waring JF, Ciurlionis R, Jolly RA, Heindel M, Gagne G, Fagerland JA, Ulrich RG (2003). Isolated human hepatocytes in culture display markedly different gene expression patterns depending on attachment status. *Toxicol In Vitro* **17**:693-701.

Waring JF, Jolly RA, Ciurlionis R, Lum PY, Praestgaard JT, Morfitt DC, Buratto B, et al. (2001) Clustering of hepatotoxins based on mechanism of toxicity using gene expression profiles. *Toxicol. Appl. Pharmacol.* **175**:28-42.

Zurlo J, Arterburn LM. (1996) Characterization of a primary hepatocyte culture system for toxicological studies. *In Vitro Cell Dev Biol Anim.* **32**:211-20.

Footnotes:

Address reprint requests to: Dr. Lysiane Richert; KaLy-Cell, Laboratoire de Biologie Cellulaire, EA3921 Optimisation Métabolique et Cellulaire, UFR des Sciences Médicales et Pharmaceutiques, Place Saint-Jacques, 25030 Besançon cedex, France ; e-mail : lysiane.richert@univ-fcomte.fr

Authors contributed equally to this work: Lysiane Richert, Michael J. Liguori

LEGENDS TO FIGURES

Figure 1. Study design is illustrated. Three distinct human liver donors were evaluated using gene expression analysis along multiple steps of the human hepatocyte preparations. Gene expression changes were monitored using freshly isolated primary human hepatocytes (t=0hr.) as the baseline.

Figure 2.

Hepatocytes in suspension after isolation from donor 2 (A), in primary culture, 24h after seeding (B), 48h after seeding (C), 72h after seeding (D), and in primary culture 24h after thawing (E). All panels are at 40X magnification.

Figure 3. Global gene expression profiles for all of the primary human hepatocyte donors at various stages of preparation. Each horizontal lane in the heat map represents a different stage of human hepatocyte preparation for its corresponding donor. Each vertical lane represents a single Affymetrix probe set. For the heat maps in all the figures, sequence agglomerative cluster analysis, using average link heuristic criteria and Euclidean distance for similarity measure, was performed to group together genes with similarities in gene expression. Genes represented were regulated at least +/-2.0-fold with a p-value of 0.01 or less. Increases in mRNA level are represented as shades of red with decreases in shades of green. If the p-value for a particular gene expression change is greater than 0.01 (or other value as indicated for subsequent figures), the log(ratio) is represented as zero or black on a heat map.

Figure 4. A. Gene expression changes for the cytochrome P-450 transcripts present on the array. **B.** Observed gene expression changes for the major CYP isoforms (right hand panel)

and for those CYPs that were induced (left hand panel). The majority of CYPs were down-regulated upon plating the primary human hepatocytes, while cells in suspension or after cryopreservation showed few changes. **C.** Under-expression of phase II transcripts upon plating the hepatocytes. **D.** Gene expression changes for several UDP-glucuronosyltransferases (UGTs) during various stages of human hepatocyte preparation **E.** Regulation of transcripts associated with canicular and basolateral transporter systems in primary human hepatocytes. The greatest number of changes in transporter expression is observed in plated primary human hepatocytes. For all panels, genes were filtered for p-value of 0.05 or less.

Figure 5. A. Regulation of genes involved in the cellular fatty acid degradation pathway for 24 hour monolayer human hepatocytes from donor 1 using GenMAPP visualization software (Dahlquist et al., 2002). An over-expressed gene is colored in red and an under-expressed gene is colored in green. The quantitative fold change for each gene is shown as a number to the right of the gene box. The statistical cutoffs for a gene being designated as regulated are at least +/-2.0-fold change with a p-value of 0.01 or less. **B.** A global view of the gene expression changed for fatty acid and lipid metabolism transcripts (as defined by gene ontology designation) for the various stages of human hepatocyte preparation. For many of these probe sets, transcription is down-regulated. **C.** Expression profiles for genes that code for proteasomal subunits for the various stages of human hepatocyte preparation. These genes are overwhelmingly induced upon plating of human hepatocytes. For the heatmaps, genes were filtered for a p-value of 0.05 or less. **D.** A heat map for mRNAs which are associated with positive regulation of apoptosis (as defined by gene ontology). Many transcripts that code for proteins associated with apoptosis induction were induced upon hepatocyte plating.

Table 1. Characteristics of donors and yield of viable isolated hepatocytes.

	Age	Gender	Disease	Size of resection (g)	Yield (Viable hepatocytes /g liver)
Donor No. 1 Date: 3/16/04	54	F	Metastasis (colorectal)	83	3.86×10^6
Donor No. 2 Date: 2/24/04	32	F	Metastasis (breast)	34	7.41×10^6
Donor No. 3 Date: 2/18/04	52	F	Metastasis (ovary cancer)	70	19.8×10^6

Table 2. Recovery of viable hepatocytes after cold preservation and cryopreservation/thawing

	Donor No. 1	Donor No. 2	Donor No. 3
Recovery of viable cells after 3h in UW solution (%)	55%	97%	66%
Recovery of viable cells after 24h in UW solution (%)	35%	56%	61%
Recovery of viable cells after cryopreservation (%)	10%	54%	49%

Hepatocyte viability was assessed by trypan blue exclusion before and after cold- or cryopreservation. From this, recovery of viable cells was calculated and expressed as percentage of viable cells before preservation.

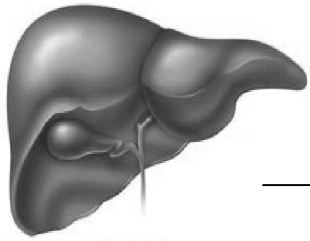
Table 3. Extent of attachment, 24 hours after seeding, of hepatocytes freshly isolated and after cryopreservation / thawing

	Donor No. 1	Donor No. 2	Donor No. 3
Extent of attachment of viable freshly isolated hepatocytes (%)	92%	92%	87%
Cell proteins of attached freshly isolated hepatocytes (mg/dish)	0.85 mg/dish	0.99 mg/dish	0.83 mg/dish
Extent of attachment of viable thawed hepatocytes (%)	35%	55%	65%
Cell proteins of attached thawed isolated hepatocytes (mg/dish)	0.45 mg/dish	0.59 mg/dish	0.68 mg/dish

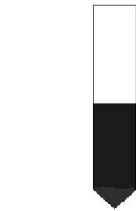
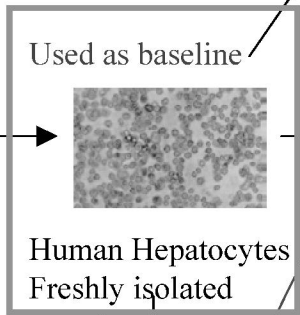
Hepatocytes were plated at a density of 3.5×10^6 viable cells/dish (see Materials and Methods section). After a 24 hour attachment period, monolayers were washed, cells were detached with trypsin, and counted. From this, the extent of attachment of viable seeded hepatocytes was calculated. In addition, the protein content of the cell monolayers was determined.

Figure 1 Experimental Design

Donation Dates
March 16, 2004
February 24, 2004
February 18, 2004



Human Liver Intact



Human Hepatocyte Cell
Suspension (2-8°C)
UW 3 Hr.



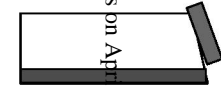
Human Hepatocyte Cell
Suspension (2-8°C)
UW 24 Hr.



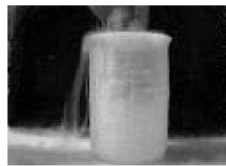
Human Hepatocyte
Monolayer 24 Hr.



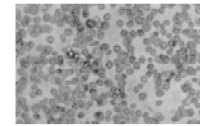
Human Hepatocyte
Monolayer 48 Hr.



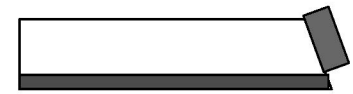
Human Hepatocyte
Monolayer 72 Hr.



Human Hepatocytes
Frozen



Human Hepatocytes
Freshly Thawed



Human Hepatocytes
24 Hour Monolayer
postfreeze

Microarray Analysis
HG-U133A Chip (~22,200 Transcripts)

Figure 2

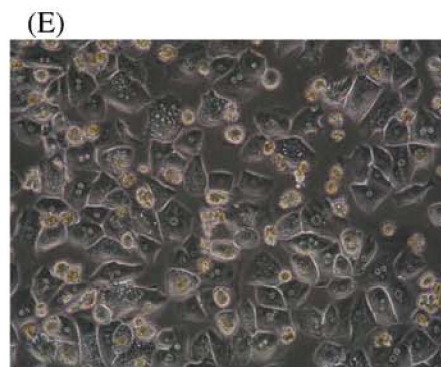
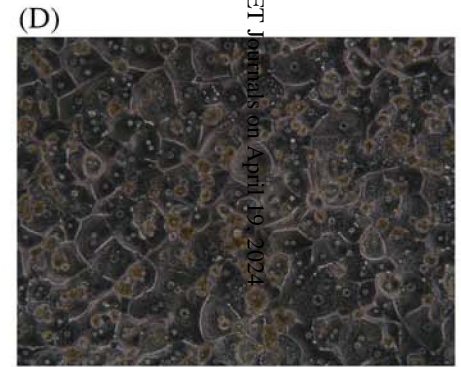
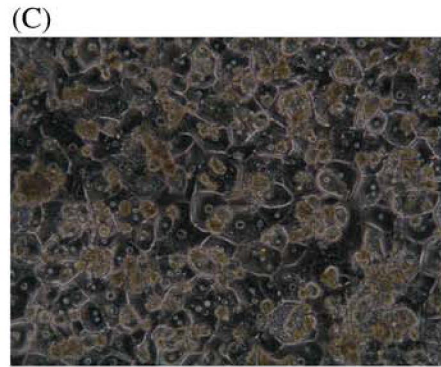
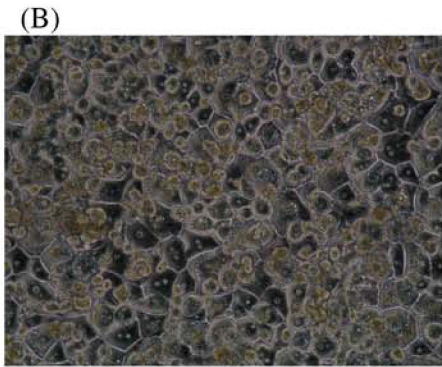
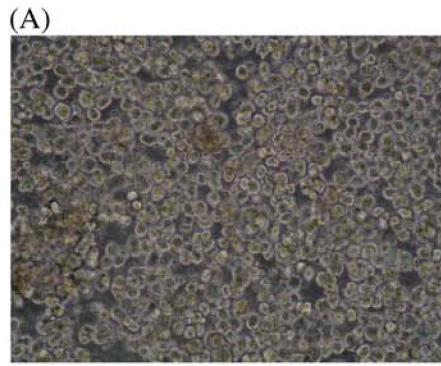
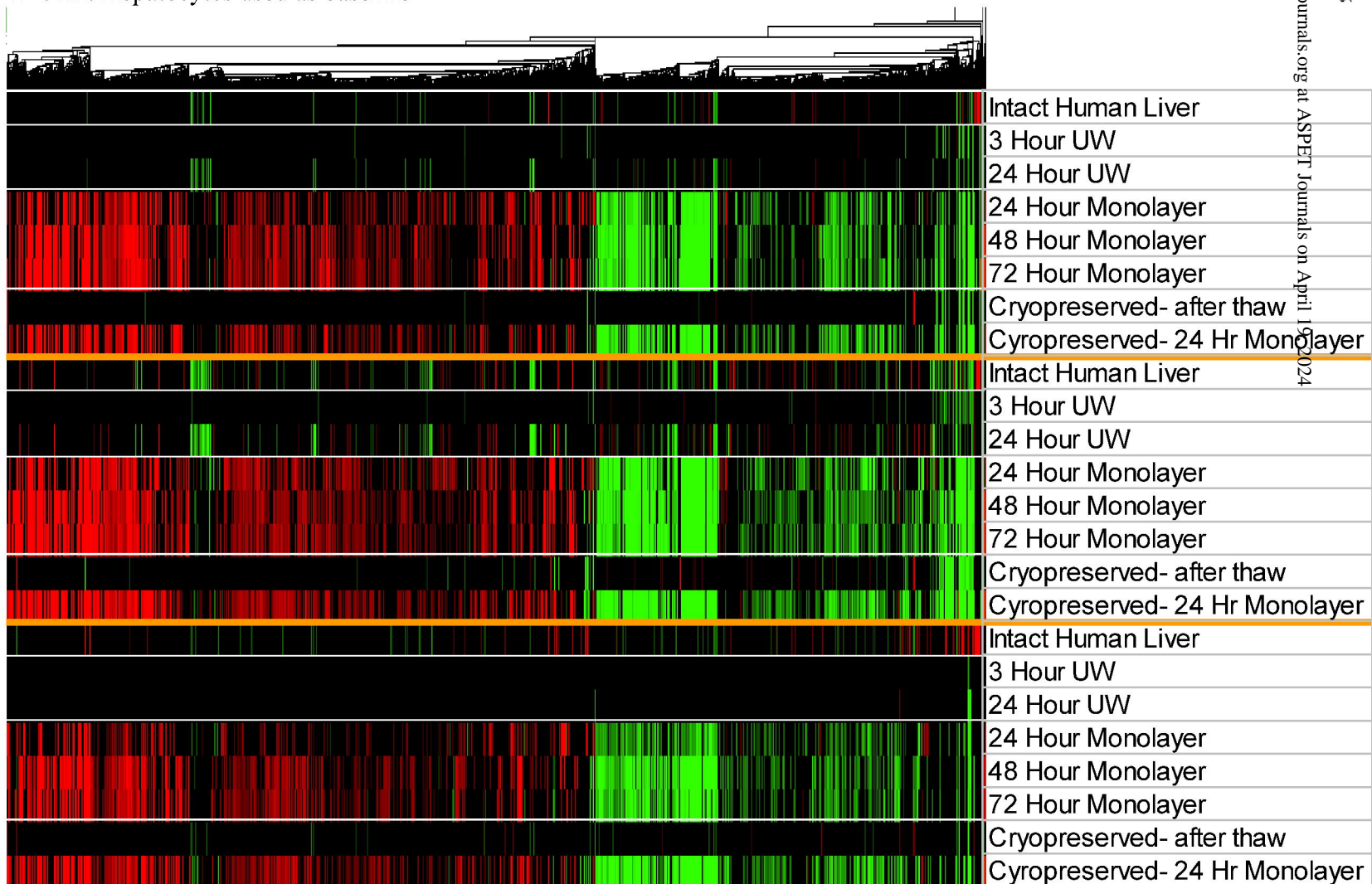


Figure 3

Fold Change ≥ 2.0 and $p \leq 0.01$
*T=0 Hr. Hepatocytes used as baseline

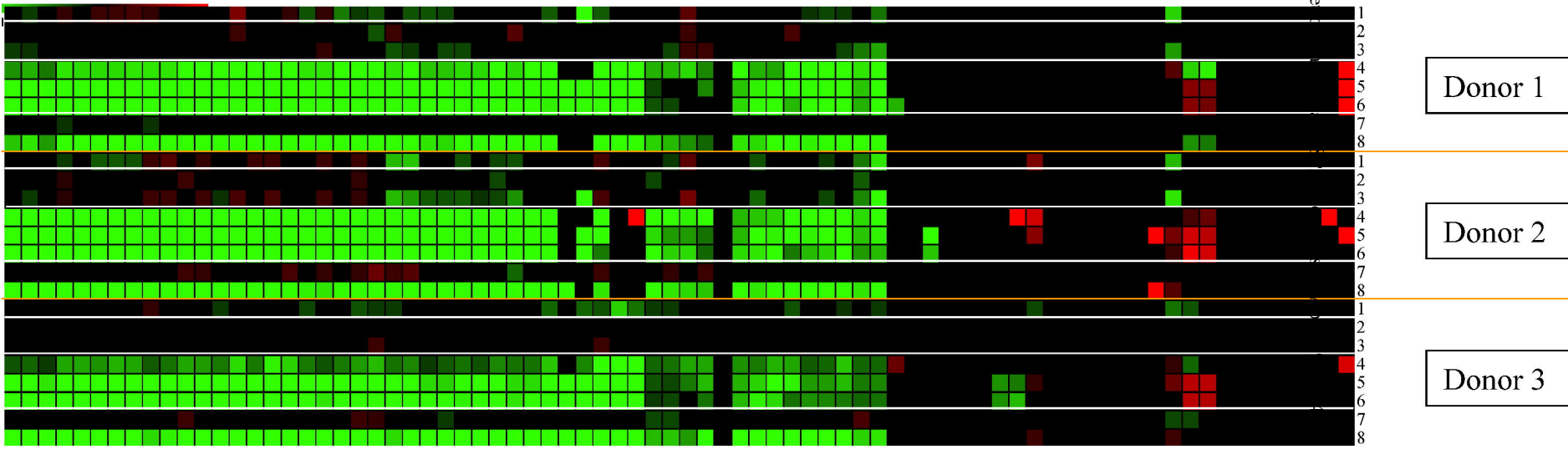
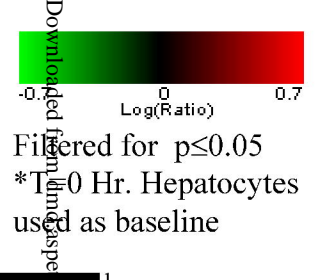


Donor 1

Donor 2

Donor 3

Figure 4A



All Cytochrome P450s on Array

- 1) Intact Human Liver
- 2) 3 Hour UW
- 3) 24 Hour UW
- 4) 24 Hour Monolayer
- 5) 48 Hour Monolayer
- 6) 72 Hour Monolayer
- 7) Cryopreserved- immediately after thaw
- 8) Cryopreserved- 24 Hr Monolayer

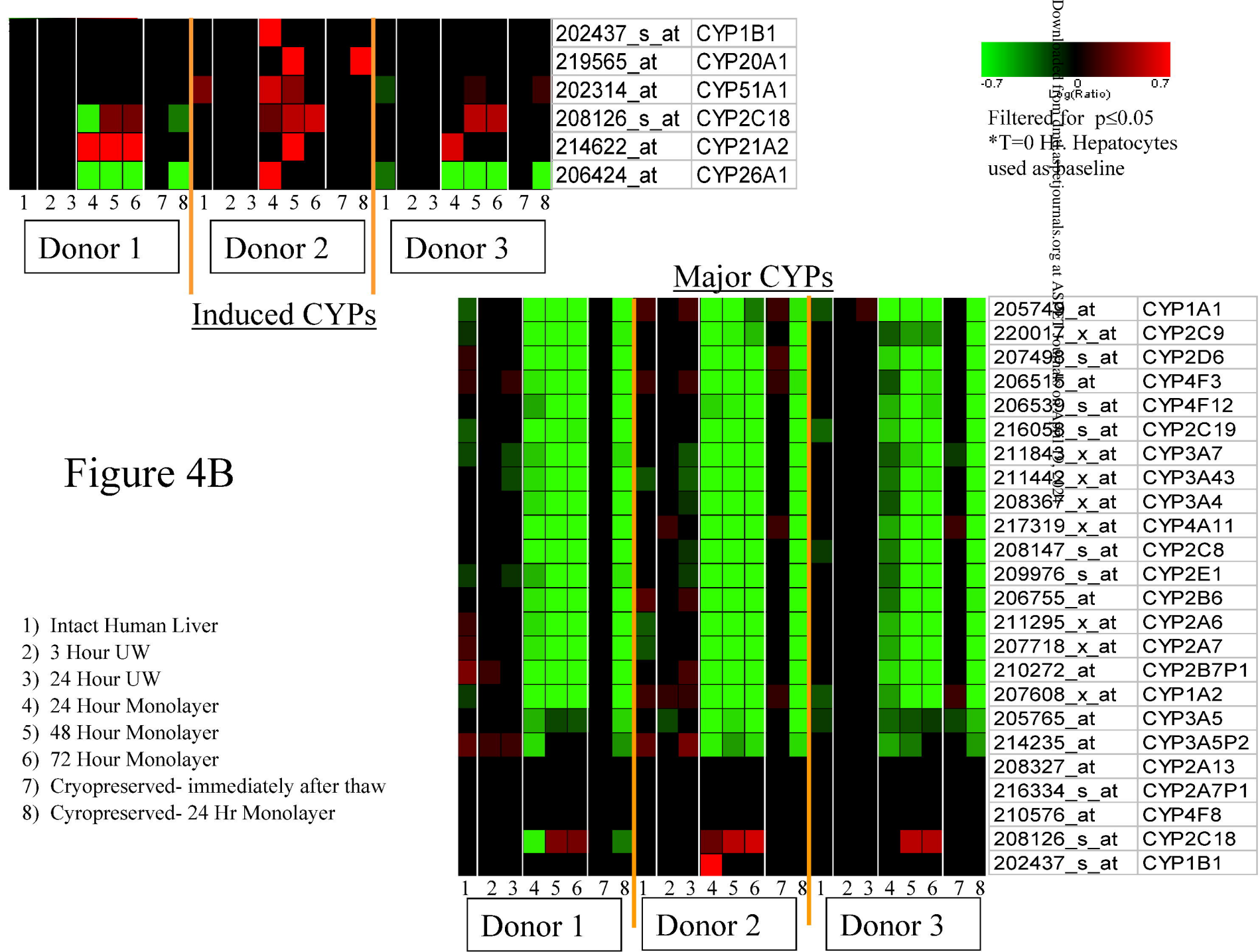
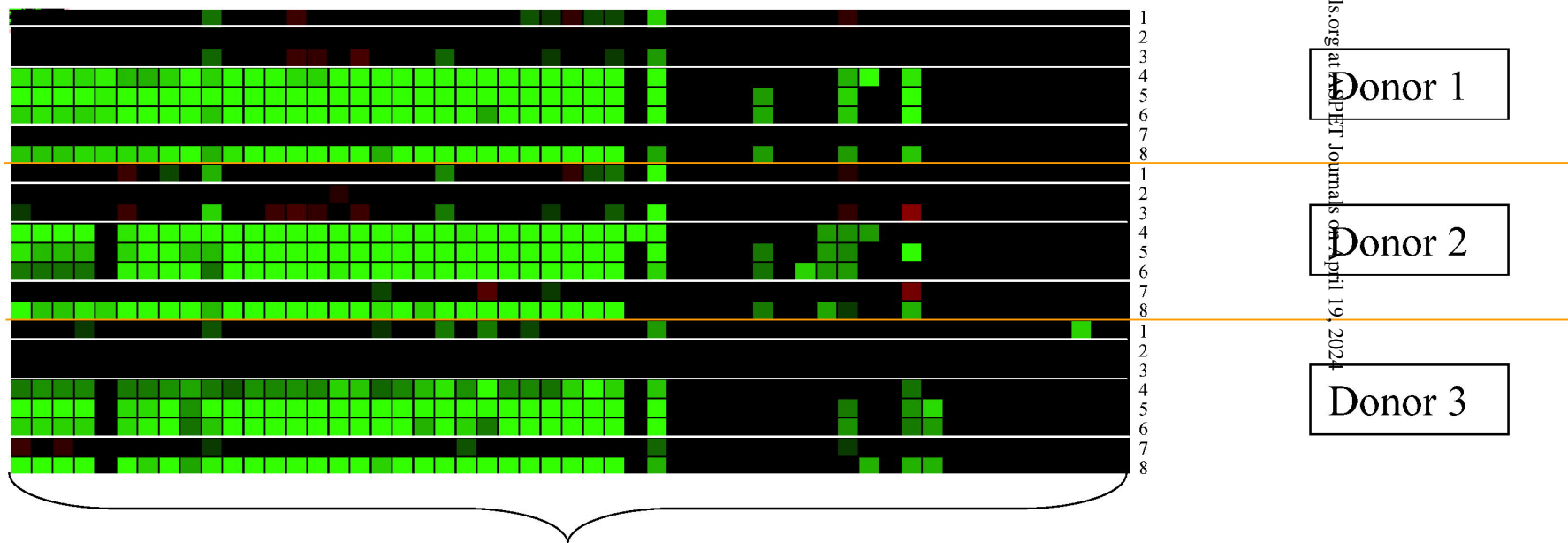


Figure 4C



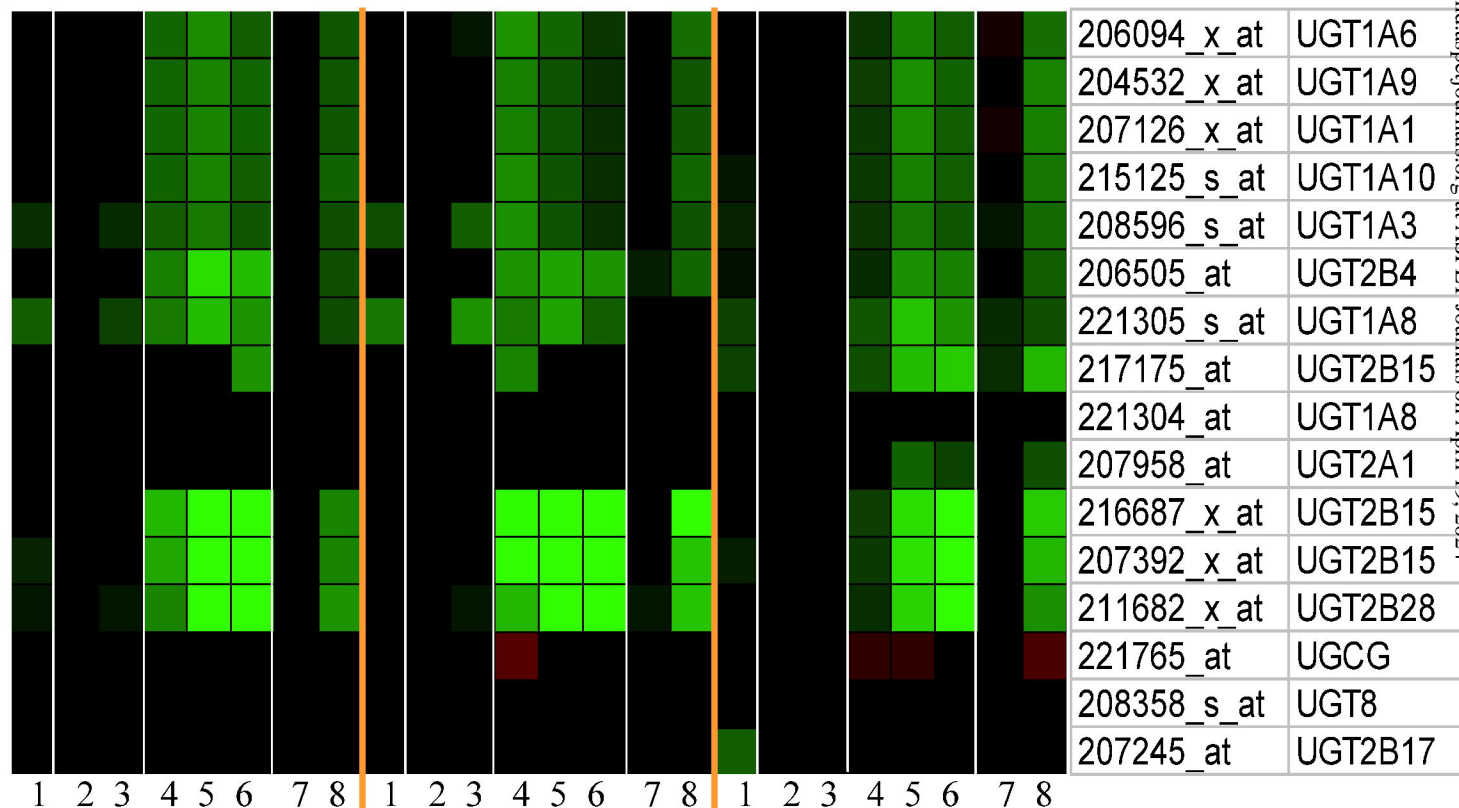
- 1) Intact Human Liver
- 2) 3 Hour UW
- 3) 24 Hour UW
- 4) 24 Hour Monolayer
- 5) 48 Hour Monolayer
- 6) 72 Hour Monolayer
- 7) Cryopreserved- immediately after thaw
- 8) Cryopreserved- 24 Hr Monolayer

Phase II Metabolizing Enzymes



Filtered for $p \leq 0.05$
*T=0 Hr. Hepatocytes used as baseline

Figure 4D



Donor 1

Donor 2

Donor 3

- 1) Intact Human Liver
- 2) 3 Hour UW
- 3) 24 Hour UW
- 4) 24 Hour Monolayer
- 5) 48 Hour Monolayer
- 6) 72 Hour Monolayer
- 7) Cryopreserved- immediately after thaw
- 8) Cryopreserved- 24 Hr Monolayer

Figure 4E

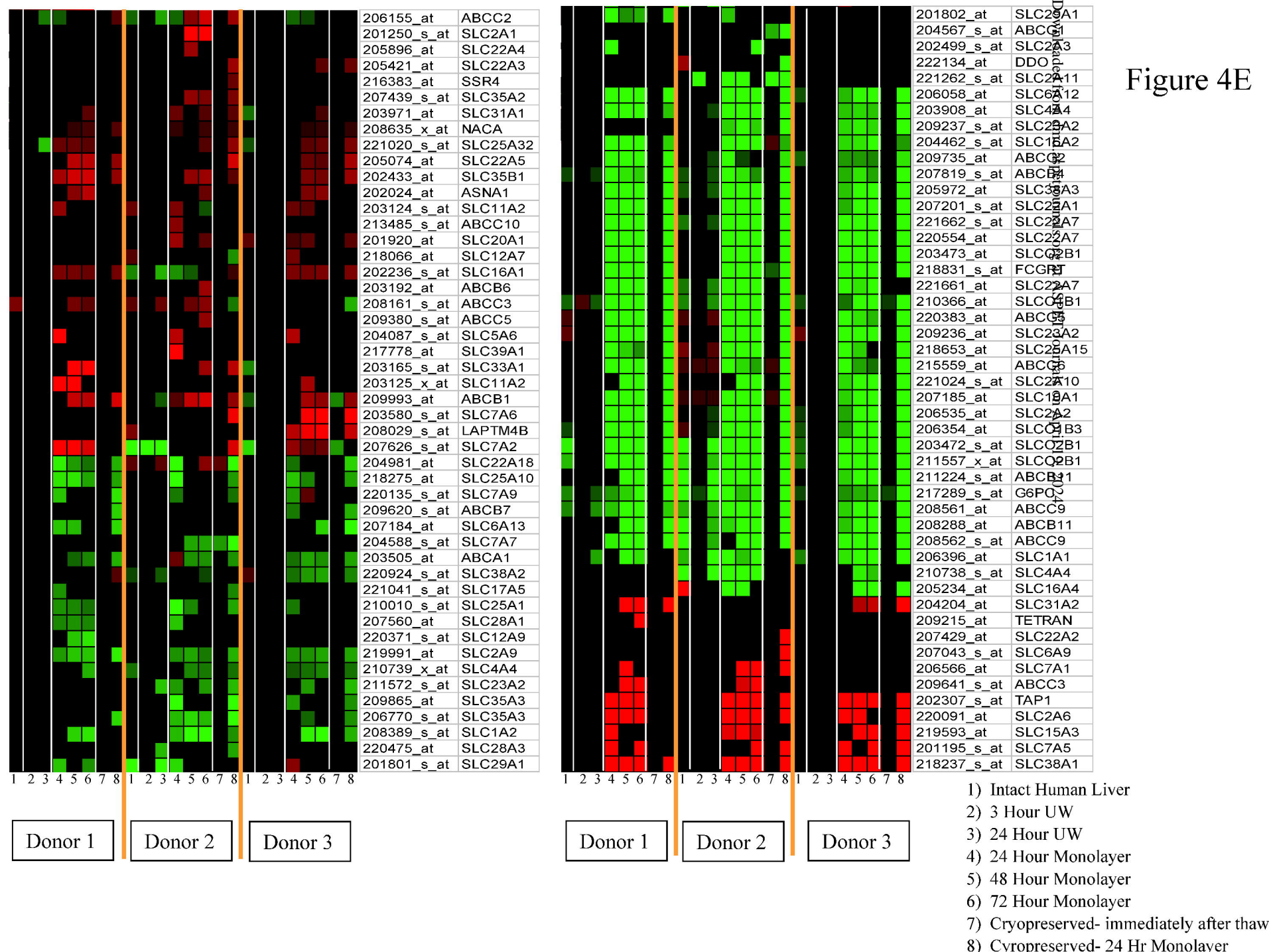
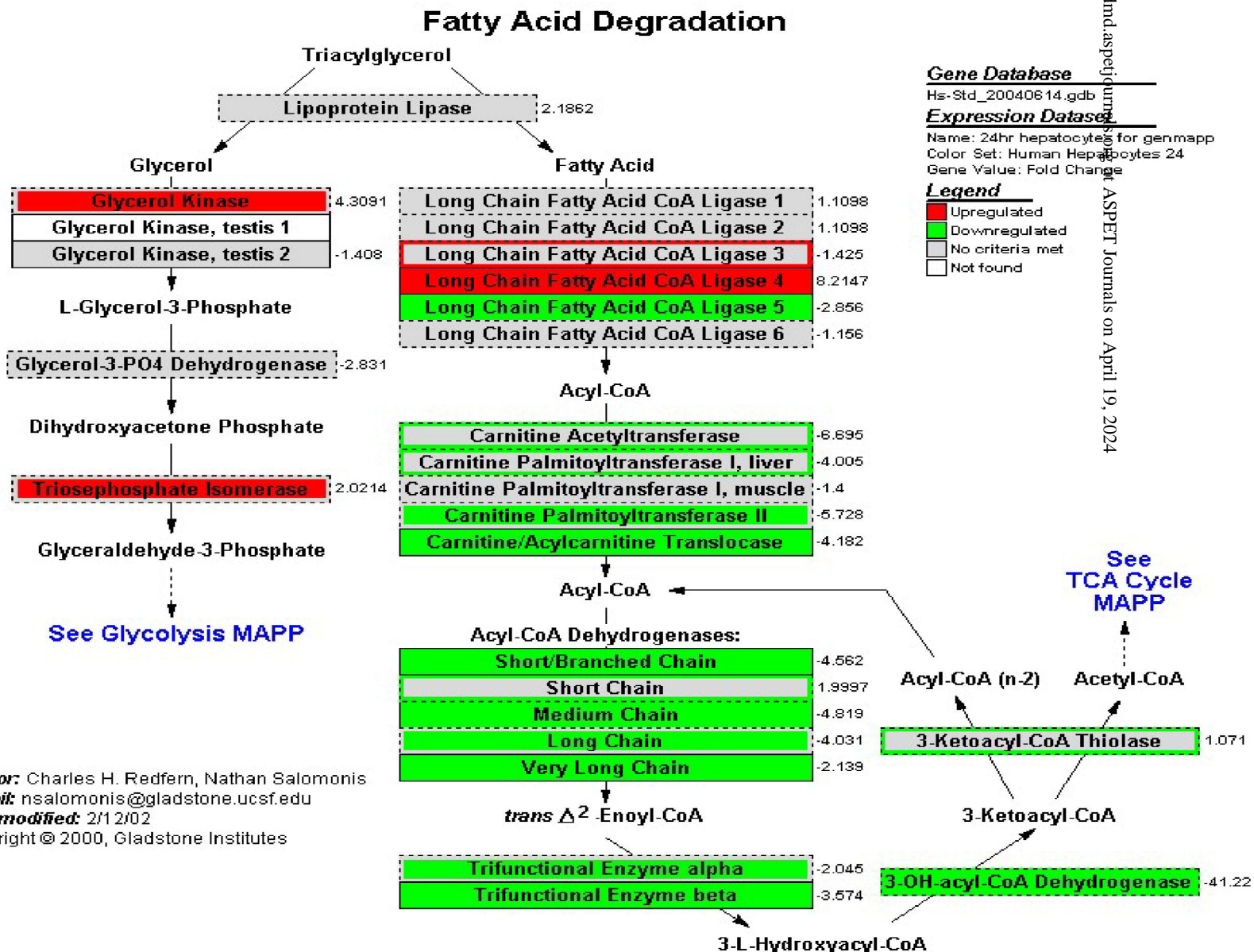
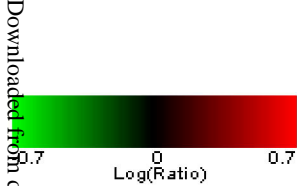


Figure 5A



Author: Charles H. Redfern, Nathan Salomonis
E-mail: nsalomonis@gladstone.ucsf.edu
Last modified: 2/12/02
 Copyright © 2000, Gladstone Institutes

Figure 5B

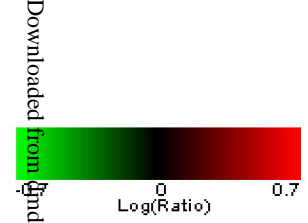


Filtered for $p \leq 0.05$
*T=0 Hr. Hepatocytes used as baseline

Downloaded from dmnd.aspetjournals.org at ASPET Journals on April 19, 2024



Figure 5C



Filtered for $p \leq 0.05$
T=0 Hr. Hepatocytes
used as baseline



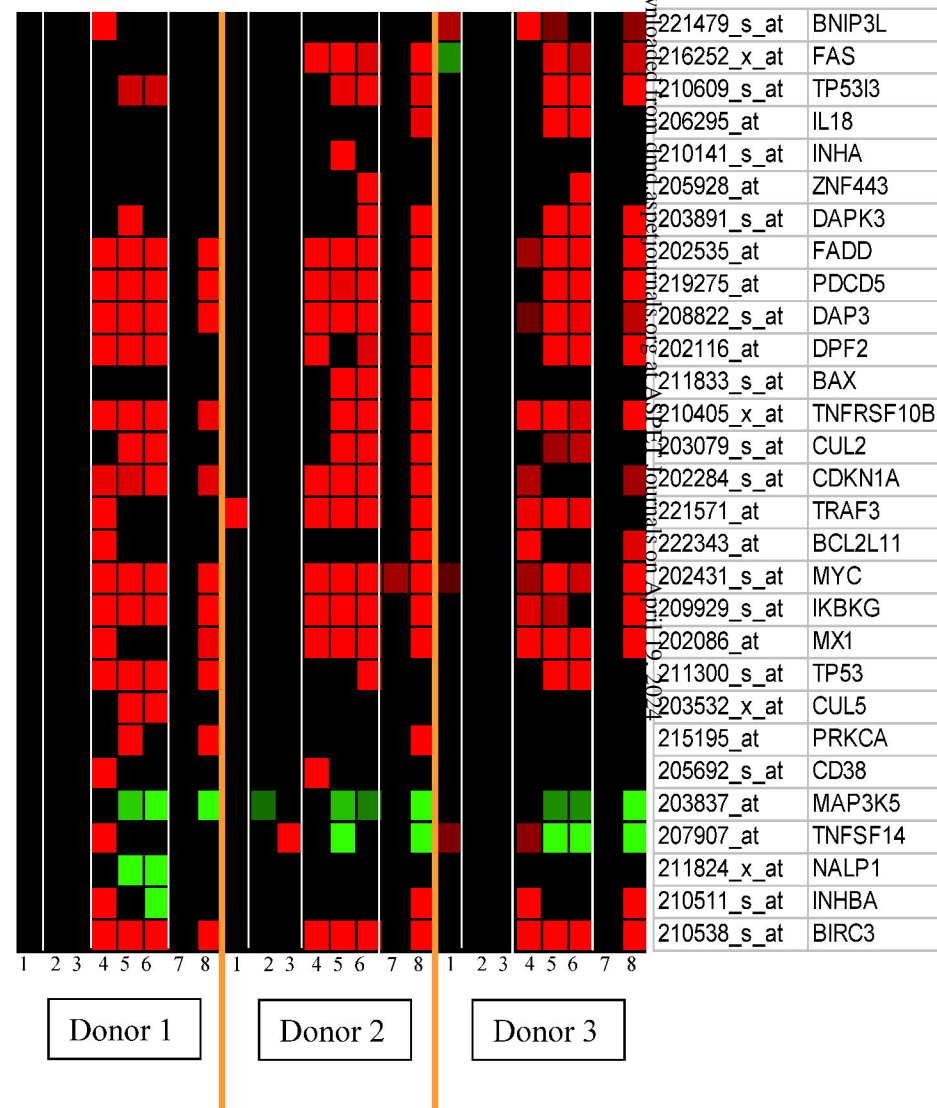
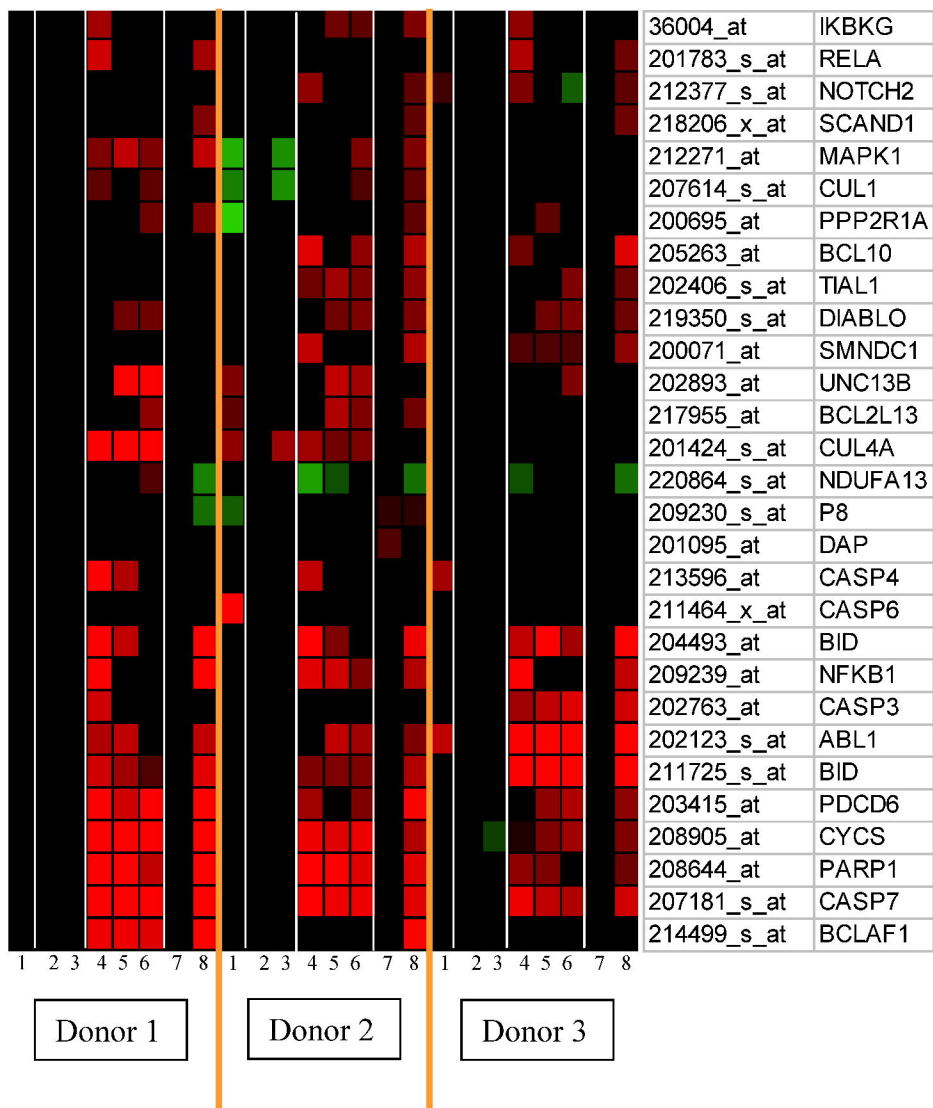


Figure 5D



Filtered for $p \leq 0.05$
*T=0 Hr. Hepatocytes
used as baseline

- 1) Intact Human Liver
- 2) 3 Hour UW
- 3) 24 Hour UW
- 4) 24 Hour Monolayer
- 5) 48 Hour Monolayer
- 6) 72 Hour Monolayer
- 7) Cryopreserved- immediately after thaw
- 8) Cryopreserved- 24 Hr Monolayer