



# Comparison of Thawing and Plating Methods for Cryopreserved Hepatocytes

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**BD Biosciences**

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# BD Gentest™ Hepatocyte Seminar Series

- Today's seminar is the first in a series of five hepatocyte seminars that BD will present throughout 2010 and 2011.
- The upcoming titles and dates are as follows:
  - Effect of BD Matrigel™ matrix overlay on hepatocyte health and CYP induction: **November 10, 2010**
  - CYP induction assays in plated hepatocytes - CYP activity and mRNA analysis: **January 12, 2011**
  - Plated hepatocytes for longer term metabolic stability assays: **March 9, 2011**
  - Drug transport efflux and uptake assays using plated and suspension hepatocytes: **May 5, 2011**



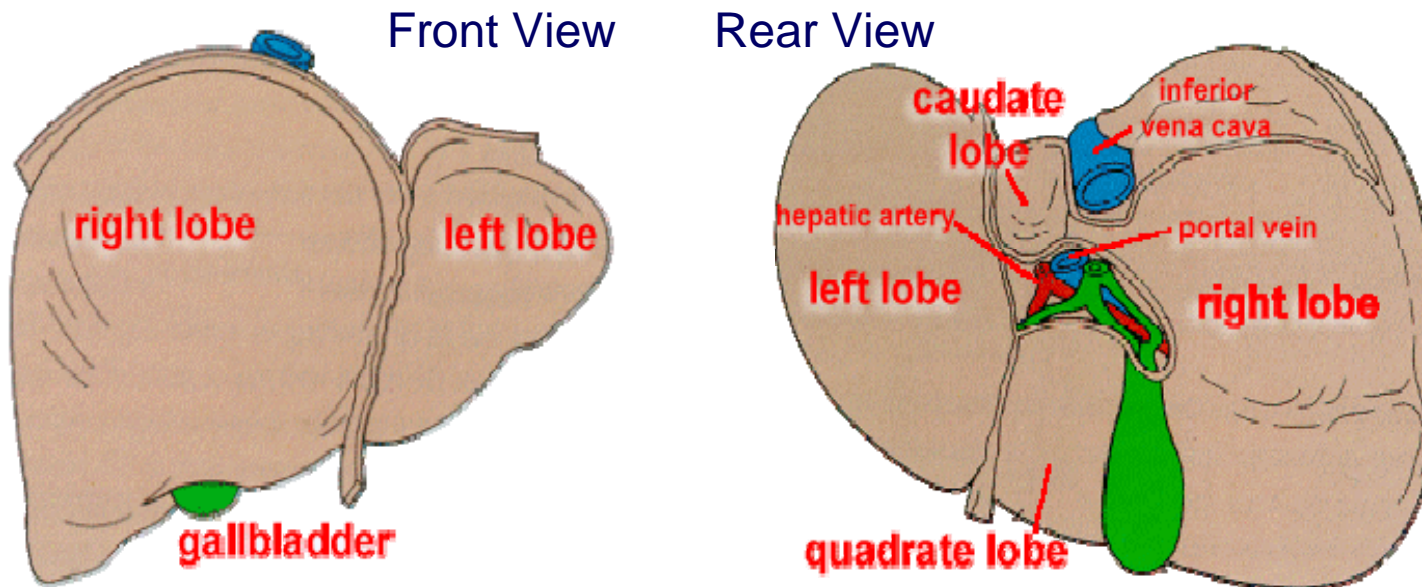
# Today's Topics

- Overview of hepatocyte structure and physiology – Liver Basics
- Hepatocyte applications and products
- Thawing and plating methods for platable cryopreserved hepatocytes
- Sandwich cultures
- BD Gentest™ high viability recovery medium for thawing and plating cells



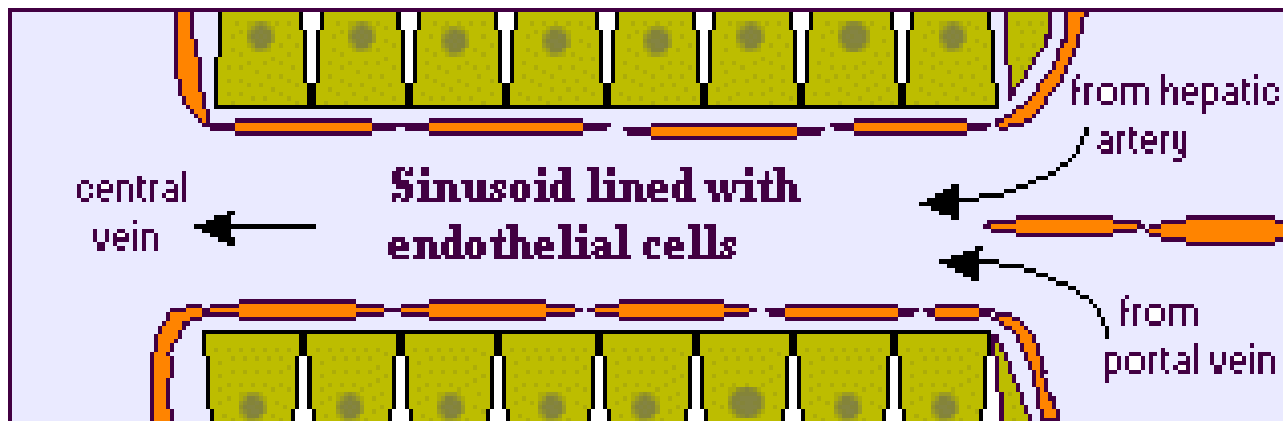
# The Liver—Structure

- The liver weighs ~1200 to 1500 gm. It is 1/50 of the total adult weight. Diseases such as liver cirrhosis can increase the weight of liver >6x.
- Structure:
  - 2 major lobes, left and right, separated by the round and falciform ligaments.
  - 2 smaller lobes- the caudate lobe and the quadrate lobe, separated by gallbladder fossa and the inferior vena cava fossa.
  - The Gall bladder is connected to the liver and a common bile duct delivers bile from the liver and into the duodenum.



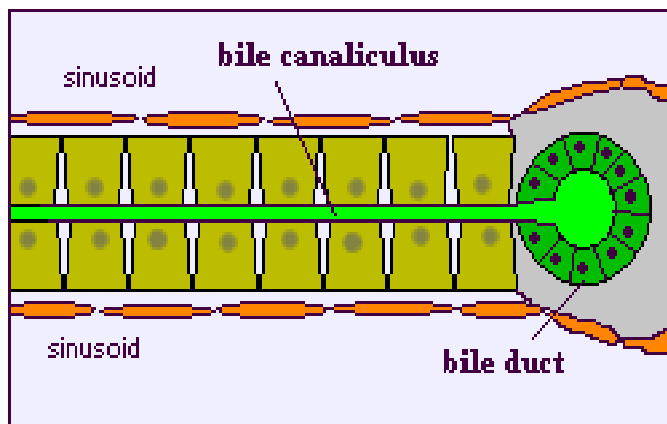
# The Liver—Its Blood Supply

- Has a Dual blood supply:
  - Portal vein: Provides 75% of blood flow (1500 mL/min) to the liver. Receiving blood from small intestine, stomach, pancreas and spleen such that liver is the first site to receive nutrients absorbed from intestine.
  - Hepatic artery: 25% of blood flow. This is the oxygenated source.
  - Portal vein and hepatic artery blood empty and mix together in the sinusoids.
- Sinusoids are irregular vascular channels lined with a boundary of endothelial cells.
- Hepatocytes line the sinusoids around the endothelial cells.



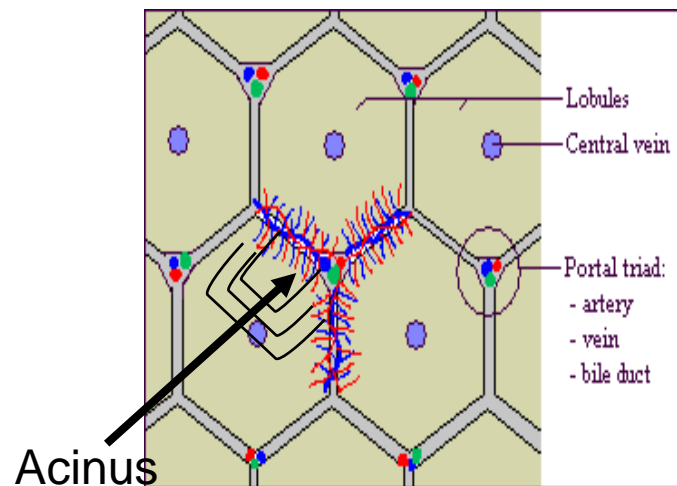
# The Liver—The Biliary System

- **Function: Elimination pathway**
  - It helps to remove lipophilic compounds out of the hepatocyte
- **Structure:**
  - The biliary system is a series of channels that conveys bile which is a secretory product of hepatocytes, from lumen to small intestine.
  - Bile canaliculus is not a “duct” per se. It is the intercellular space between adjacent hepatocytes that is defined by tight junctions.
  - Small ducts anastomose into larger and larger ducts to eventually form the common bile duct.
- The Gall Bladder serves as a place to store excess bile.

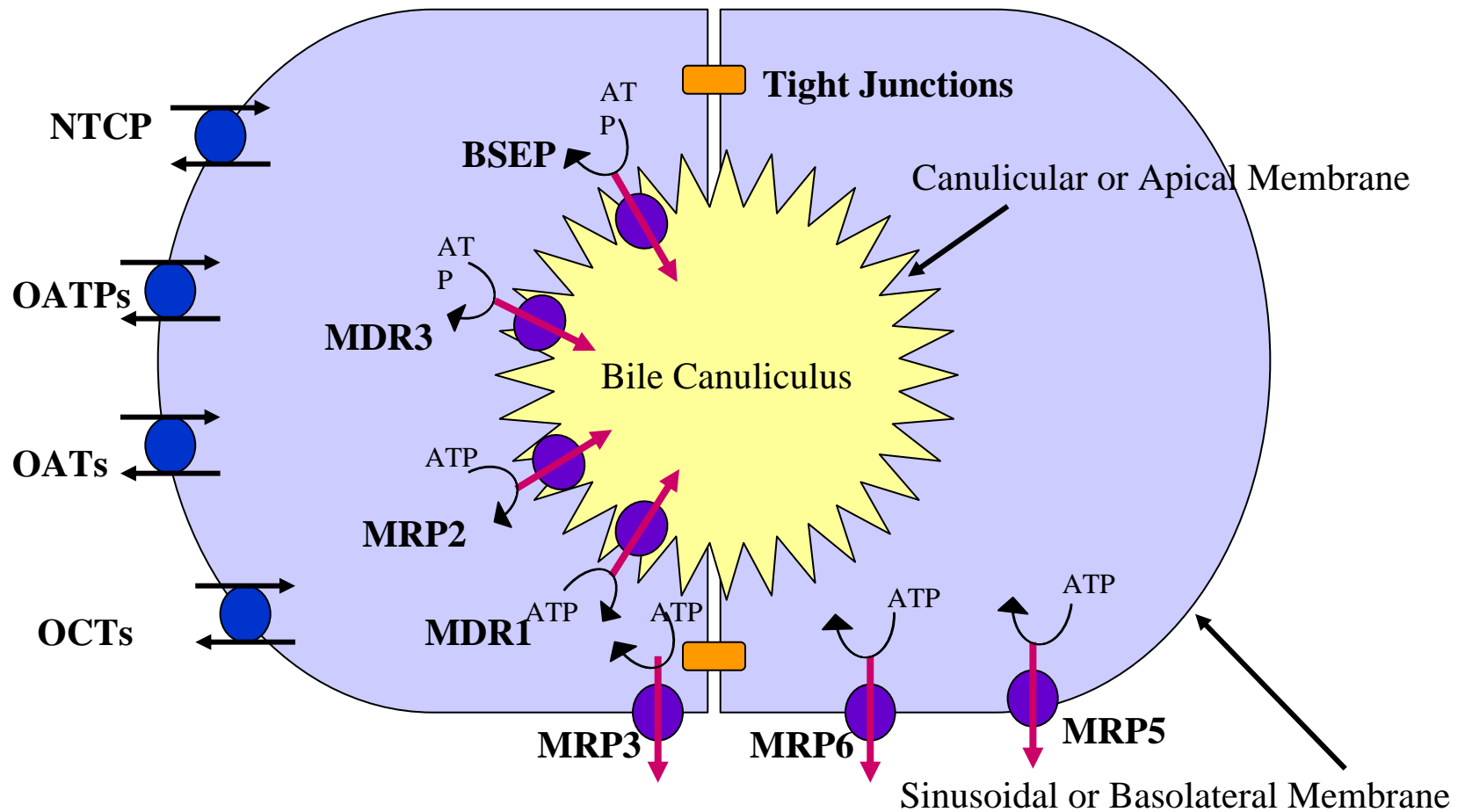


# Liver—Physiology

- Hepatic Lobule: comprised of portal triad and central vein. Differential function based on hepatocyte location within lobule.
- Hepatic acinus- orients the cells to vascular system.
  - Zone 1: (Periportal) nearest to arterioles, oxygenated, most metabolically active, first to “see”/absorb potential toxins. Oxidative metabolism—generation of ATP.
  - Zone 2
  - Zone 3: Pericentrally localized hepatocytes, more hypoxic: synthesis of glycogen, fatty acid and bile salts. Detoxification by reduction.



# Hepatobiliary Transport





# The Liver—Physiology

## Cell types:

- Hepatocytes – Comprises the bulk of liver (~80%). Responsible for Metabolism.
- Endothelial cells – Form loose layer of cells around sinusoids. Lack typical basement membrane of vascular endothelium. Have role in lipoprotein metabolism.
- Kupffer cells – Macrophages; modulate immune response through release of mediators and cytotoxic agents. Associated with endothelium.
- Perisinusoidal fat-storing cells (Lipocytes or Ito cells) – Located in sinusoids. Contribute to hepatic fibrosis with liver injury.
- Pit cells: Natural Killer lymphocytes associated with endothelium, highly mobile (least common cell type in liver).



# Liver Function—Metabolism and Energy Production

- **The liver is the primary organ for metabolism**
  - Metabolizes carbohydrates, fats and protein
  - Synthesizes lipoproteins, cholesterol and phospholipids
  - Produces blood coagulation factors
  - Stores energy: fatty acids/glycerol and glycogen
  - Maintains constant blood-glucose levels  
(glycogenesis  $\leftrightarrow$  glycogenolysis)
  - Bile Production
  - Converts  $\text{NH}_3$  (toxic byproduct of amino acid breakdown) to Urea
  - Storage of iron, vitamins and trace elements
  - *Drug Detoxification, Metabolism, Transport*
    - Most drugs interact with one or more SLC or ABC transporter proteins for entry or exiting hepatocytes
    - Drug-induced liver injury is the most frequent cause of acute liver failure in the United States



# **Hepatocyte Products and Applications for Drug Metabolism and Transport Studies**

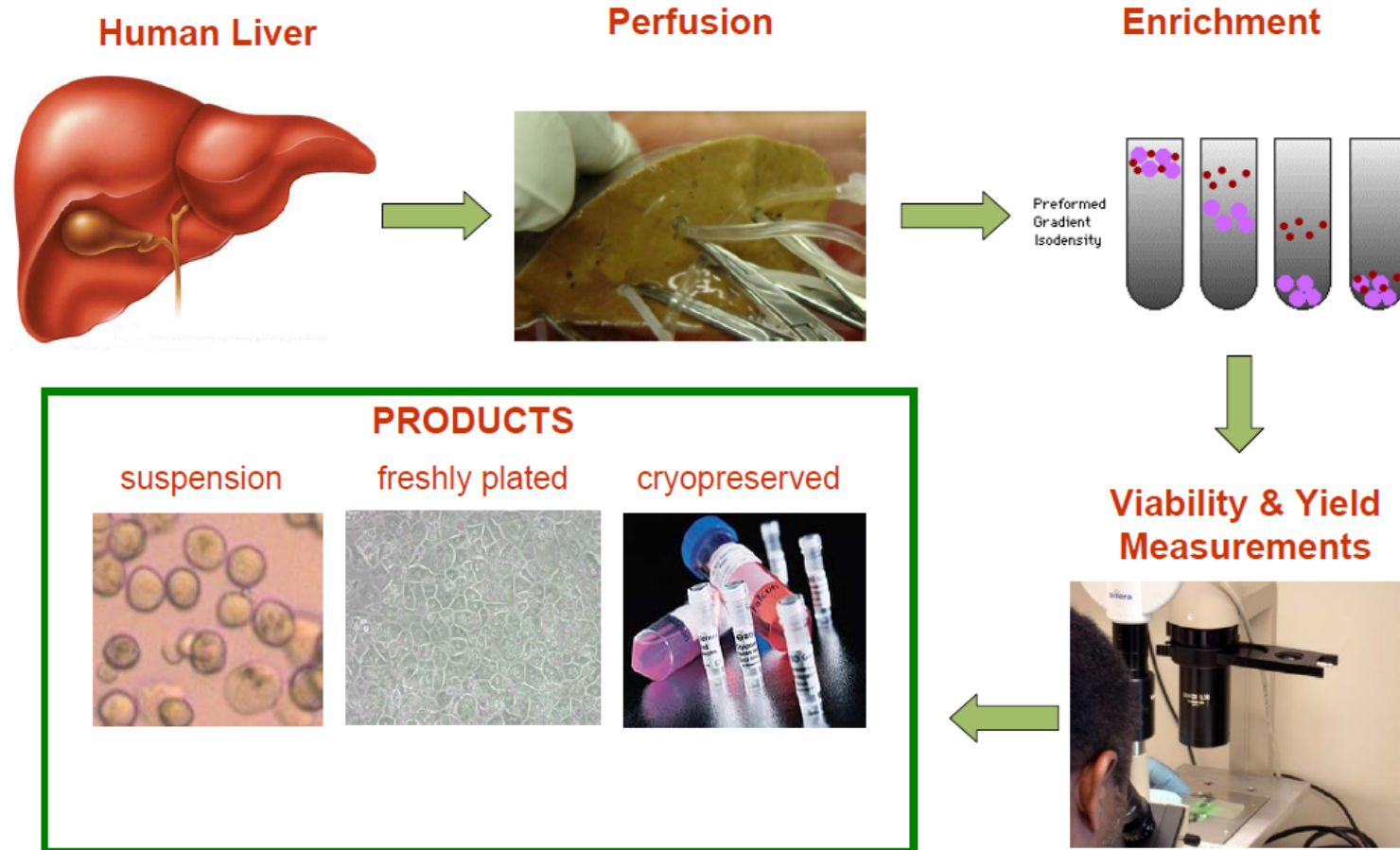


# Human Liver Tissue for Hepatocyte Isolation

- All human tissue materials originate from US Organ Procurement Organizations. Informed consent for research use has been provided.
- Livers rejected for organ transplant.
- Obtain medical history of the donor and serologies. Only donors which meet certain criteria are accepted. Internal processes are in place to ensure that details which can identify the patient are eliminated.
- Prior to use in cryopreserved hepatocytes, tissue is tested by PCR for HIV1/2, HBV, HCV, HTLV1/2 and CMV.



# Hepatocyte Isolation



# Advantages and Disadvantages

- **Advantages**

- Considered the Gold Standard model for metabolism studies
- Contain all the hepatic enzymes, including transporters and co-factors (NADPH, UDPGA, GSH, PAPS etc.) needed for drug metabolism/hepatic transport studies
- Studies typically carried out with Hepatocytes include:
  - Enzyme induction
  - Metabolite ID
  - Drug transport studies (uptake and efflux)
  - Toxicity studies
  - DDI studies (recent application)

- **Disadvantages**

- Loss of viability for cells in suspension.
- Lose P450 enzyme expression within 2-3 days of plating
- Not all lots of cryopreserved hepatocytes will replate
- Variability between levels of P450 activity between human donors

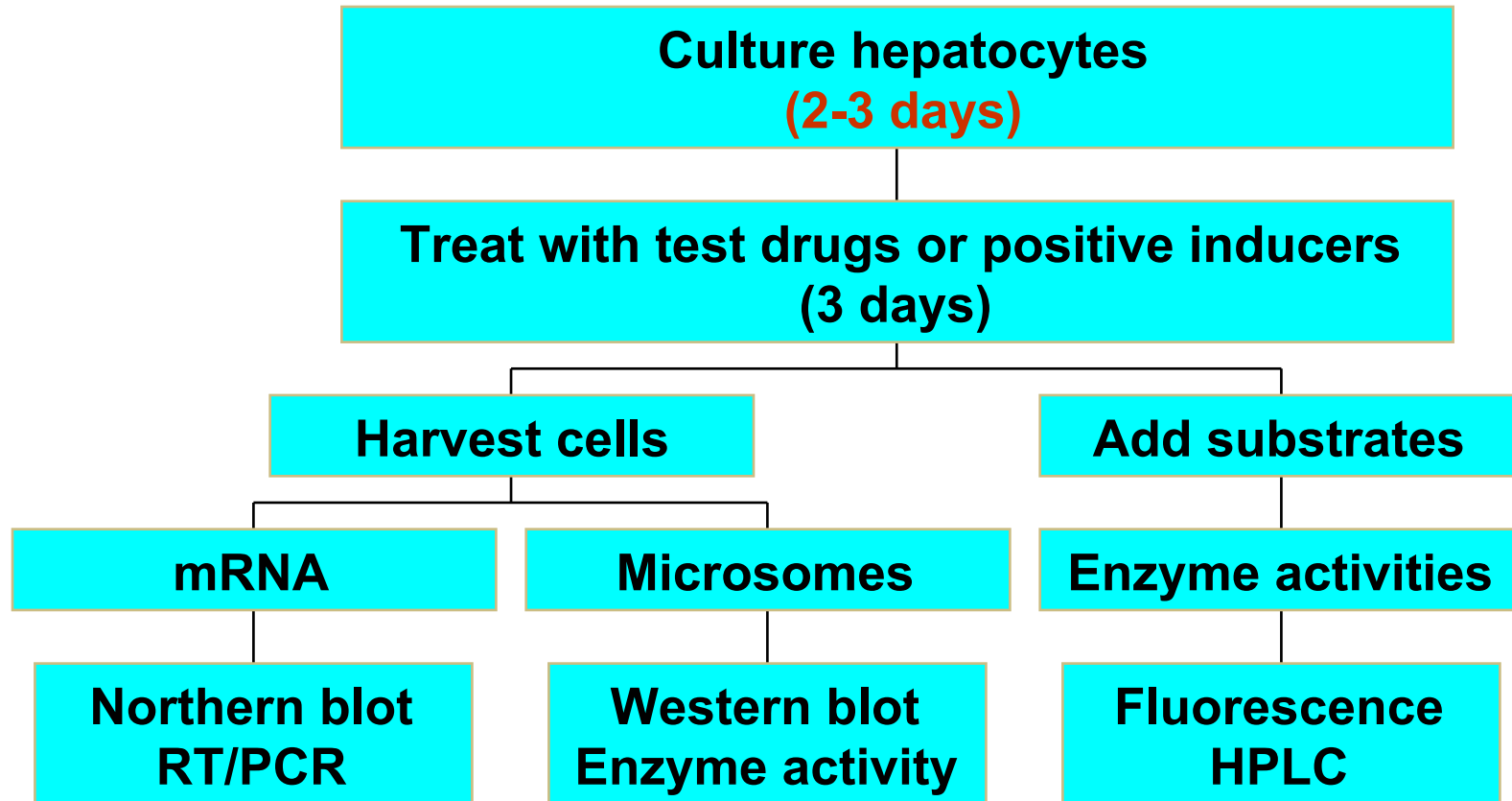


# Human Hepatocyte Products and Applications

- **Hepatocytes are prepared from fresh human livers (organ donors)**
- **Hepatocytes are sold as:**
  - Fresh cells on tissue culture plates
    - Primarily used for Induction studies
    - Drug efflux studies (with sandwich culture)
  - Fresh cells in suspension
    - Metabolic stability, transport studies
    - Metabolite ID
  - Cryopreserved hepatocytes (more convenient vs fresh cells)
    - Metabolic stability (drug half-life); characterized for all the major CYPs and UGTs
    - Drug uptake studies; characterized for important SLC uptake transporters
    - Cryopreserved hepatocytes that plate
      - CYP induction studies (CYP3A4, 1A2 and 2B6)
      - Drug efflux studies using sandwich culture (P-gp, MRP2, BSEP)
      - Metabolic stability studies (slowly metabolized drugs requiring long incubation period in order to observe metabolites)

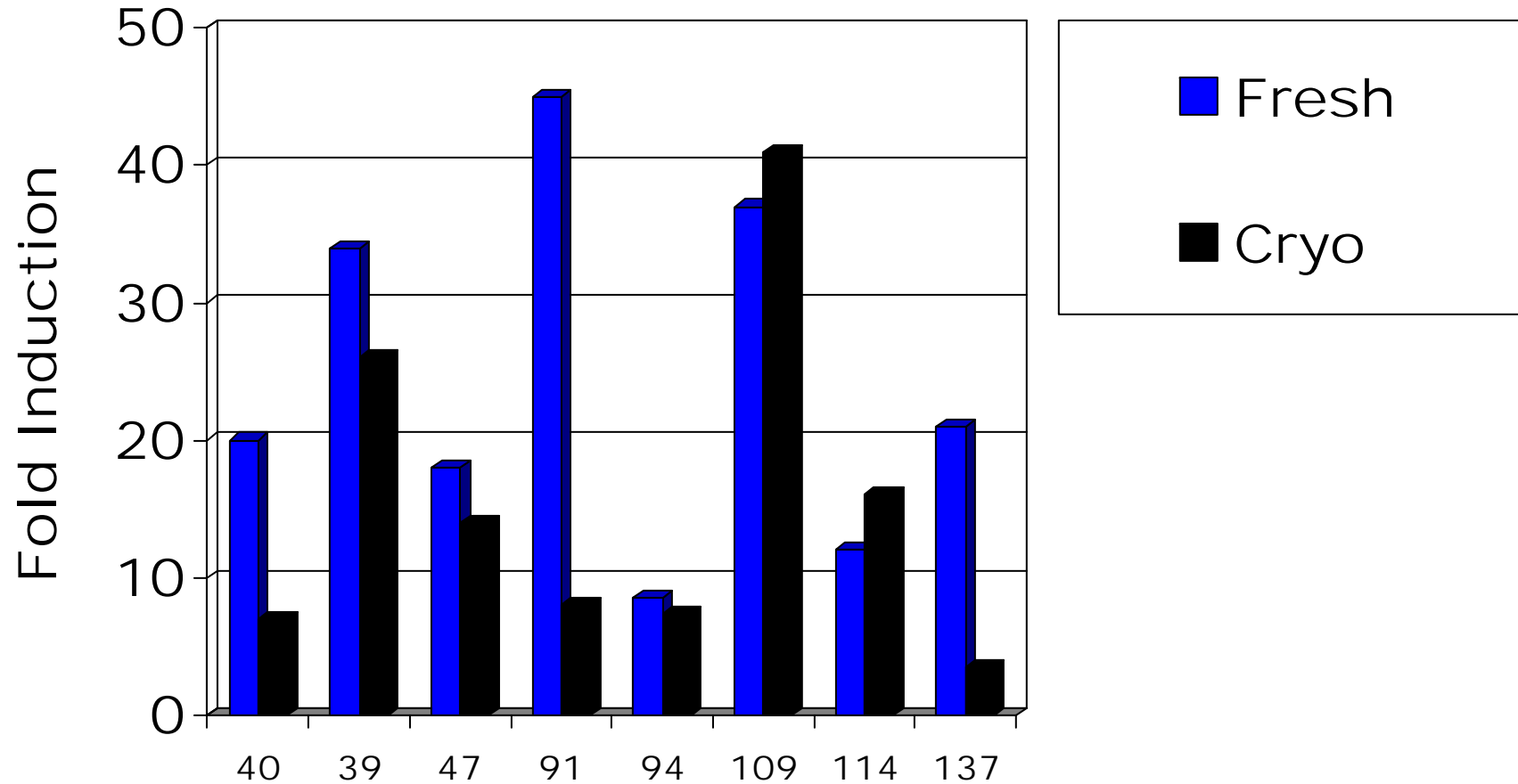


# CYP Induction Procedures (Hepatocyte Model)



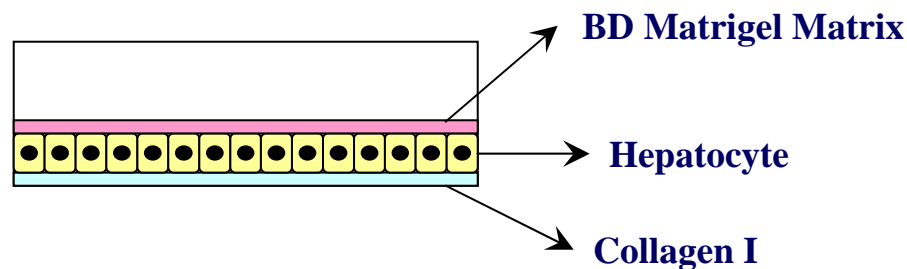


# Comparison of Fresh vs. Cryopreserved Hepatocytes on 3A4 Induction



# BD Matrigel Matrix Overlay Format for Efflux Transporter Studies

- Hepatocyte collagen/BD Matrigel matrix sandwich culture
- Plated hepatocytes in sandwich culture form Biliary Canaliculi and express major efflux ABC transporter proteins (P-gp, MRP2, BSEP)
- BD Matrigel matrix overlay procedure
- Prepare BD Matrigel matrix solution on ice: 0.25mg/mL in overlay medium (WME)
  - BD Matrigel matrix/WME mixture should be added to cells on day 1, after 2-4 hour incubation in Hepatocyte Culture medium
  - Add BD Matrigel matrix /WME mixture to cells @ 500ul/well (0.25mg/ml)
  - Incubate the plates 5% CO<sub>2</sub> at 37oC over night
  - Replace BD Matrigel matrix/WME with Hepatocyte Culture Medium and proceed to application (e.g. CYP induction or drug efflux assays)

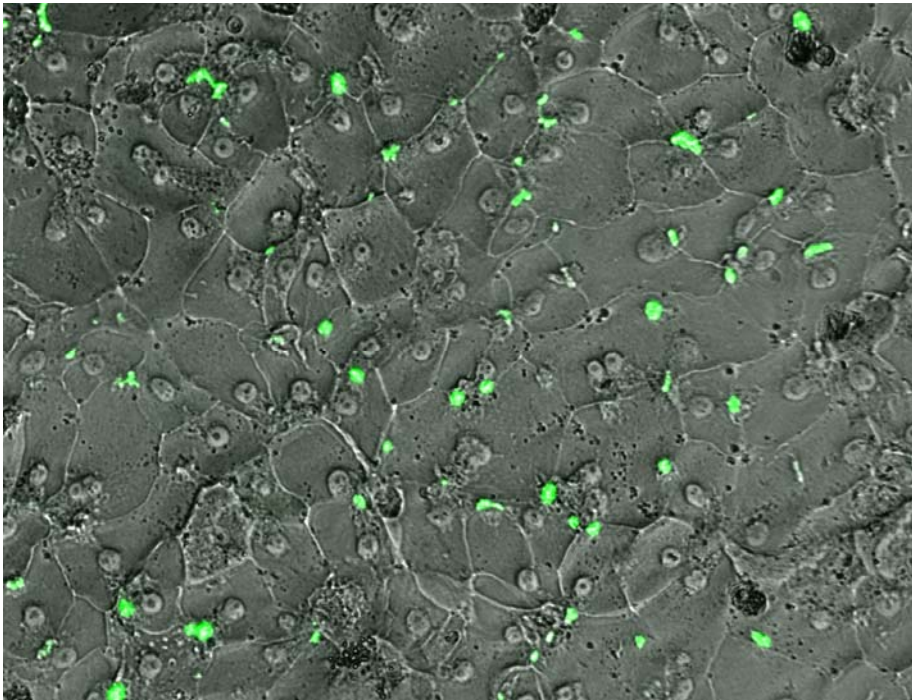


# Applications for Hepatocyte Sandwich Cultures

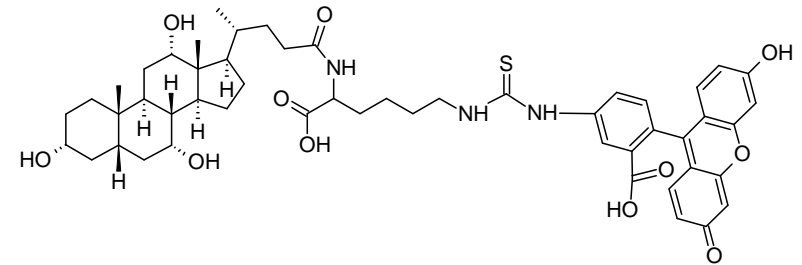
- BD Matrigel matrix sandwich cultures can improve cell morphology and health
- CYP basal activity (in absence of inducer) is generally higher with sandwich culture format
- Sandwich culture is required for the formation of biliary canaliculi
  - Supports biliary efflux assays (P-gp, MRP2 and BSEP are expressed on apical membrane in sandwich environment)



# Accumulation of BSEP Fluorescent Substrate (CLF) in Bile Canaliculi



BD Gentest human cryopreserved hepatocytes (lot 141)



Cholyl Lysyl Fluorescein



# Thawing and Plating Methods for Cryopreserved Hepatocytes

## Density Gradients



# Considerations for Choosing a Thawing/Plating Method

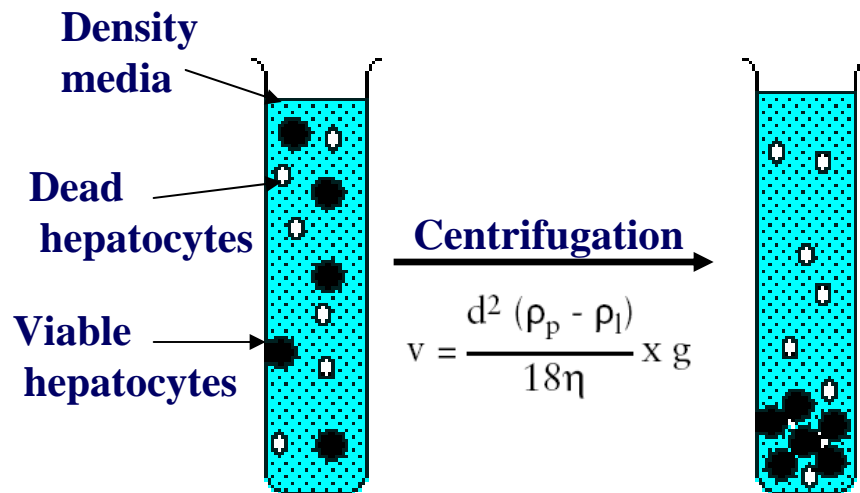
- Method should be fast (as few steps as is possible to minimize loss in cell viability and save researchers time)
- Non-toxic to cells, no changes to hepatocyte physiological function
- Maximize cell viability and recovery
  - Traditional methods generally use a density gradient to remove dead cells (e.g. Percoll™)

# Cryopreserved Hepatocyte Plating Methods

- Direct plating
  - Fast
  - DMSO is included when plating
  - Low viability, large amount of dead cells effect cell attachment
- Cell Culture Media
  - DMSO is removed
  - One-step centrifugation step
  - Low to Moderate viability; high recovery
  - Can have significant amount of dead cells which can effect cell attachment
- Percoll Purification Media
  - High viability and low/moderate recovery
  - Generally employs two centrifugation steps
  - Percoll can be cytotoxic to certain cell types, e.g. sperm cells
    - High endotoxin levels and unstable PVP coating
    - Contaminating PVP can impact plating efficiency
- **The new BD Gentest™ High Viability Recovery Kit**
  - Proprietary non-cytotoxic recovery media
  - Low endotoxin levels
  - One centrifugation step
  - Superior viability and recovery (vs Percoll™)
  - Maximizes cell plating efficiency



# Density Gradient Centrifugation



## Sedimentation rate:

- Proportional to the difference between the density of the particle and that of the surrounding media
- Proportional to particle size.

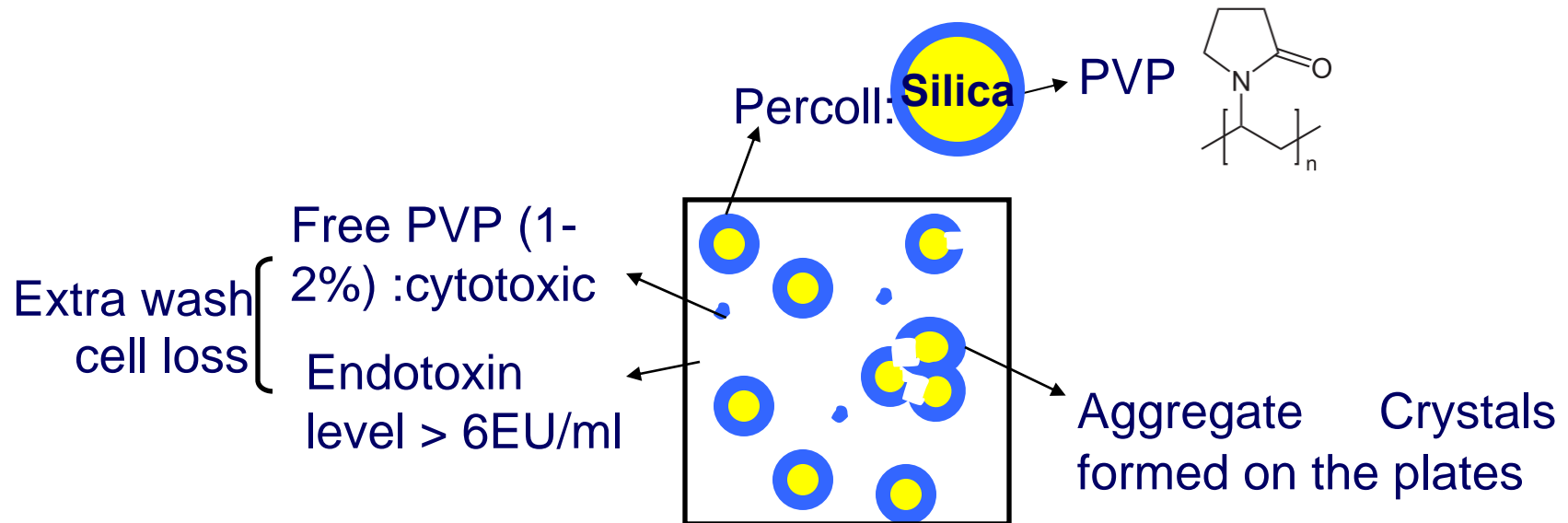
## Hepatocyte:

- Live cells are dense, while dead cells are light
- A gradual decrease in density when cells die
- Different sizes

In general, as the density of the media increases, the viability increases, but yield will decrease



# Percoll™: Silica coated by Polyvinylpyrrolidone (PVP)



Other cell damaging effects: possible membrane alterations, induce inflammatory responses.

**BD Gentest™ High Viability Recovery Medium  
for Plating Cryopreserved Hepatocytes**



# BD Gentest High Viability Recovery Kit: *Non-cytotoxic recovery medium*



- A ready-to-use kit
- 2 tubes of Recovery Media (45 mL)
- 2 tubes of Plating Media (45 mL)
- Data sheet and instructions on how to use

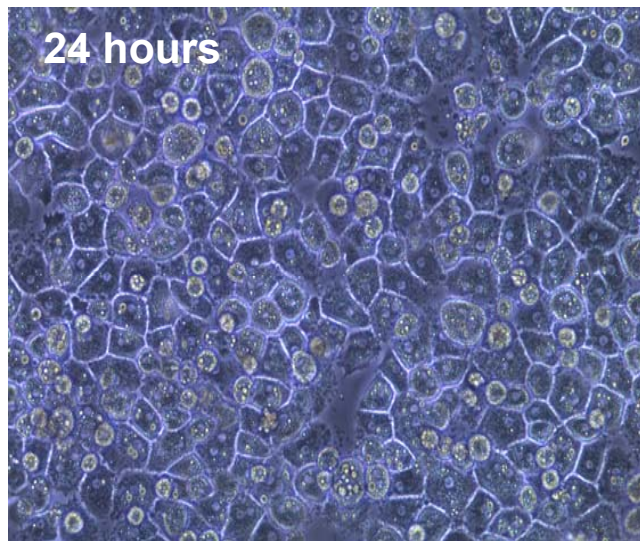
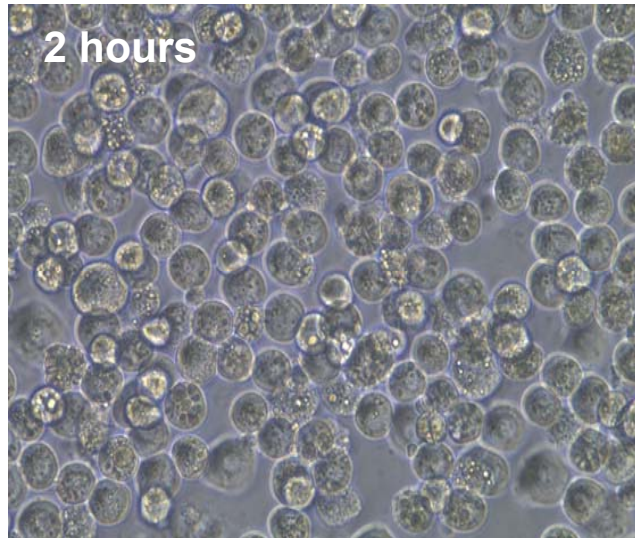


# How to Use the Kit for Plating Cells

1. Add 5 mL FBS to the Plating Media tube, warm the Recovery Media and Plating Media to 37°C.
2. Thaw human cryohepatocytes in 37°C water bath, transfer the contents to the Recovery Media tube, rinse the vial with Recovery Media and combine the wash.
3. Centrifuge at 100 g for 10 minutes (middle acc/low brake).
4. Aspirate the supernatant, resuspend the cell pellet in 2 mL of Plating Media/10% FBS.
5. Measure volume and cell viability / recovery.
6. Plate cells on 24-well collagen I coated plate @ 400,000/well.
7. Incubate cells with 5% CO<sub>2</sub> at 37°C for 2-4 hours for initial attachment.
8. Feed cells with BD™ hepatocyte culture medium @ 400 µL/well and incubate overnight with 5% CO<sub>2</sub> at 37°C.
9. CYP Induction assays should be initiated on day 2, after confluent monolayer has formed.



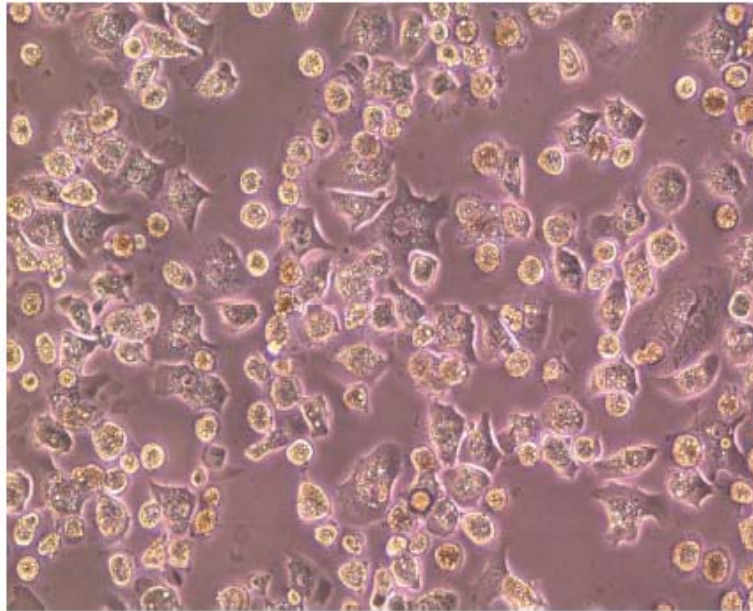
# Cell Reattachment Post Thaw



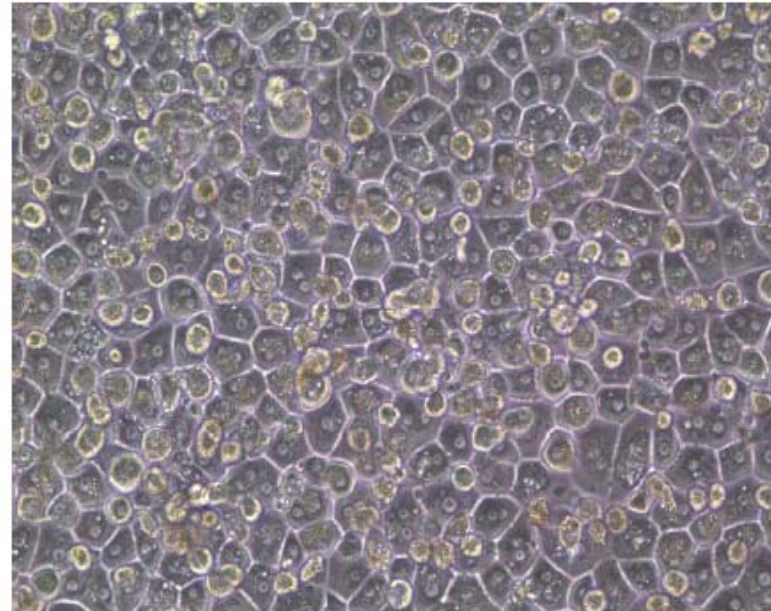
- Cells will not be completely adherent after 2 hours, but they should be attached and be in the process of spreading.
- After 24 hours, cells should be in cuboidal morphology.
- Cryopreserved cells need to be used right away for induction assays.

# Cell Monolayer Examples

Not all lots of cryopreserved hepatocytes will plate



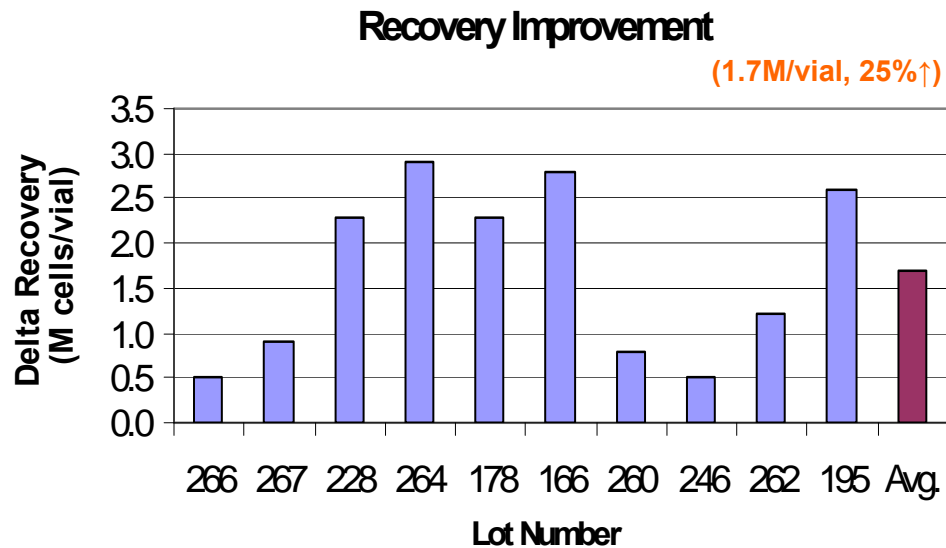
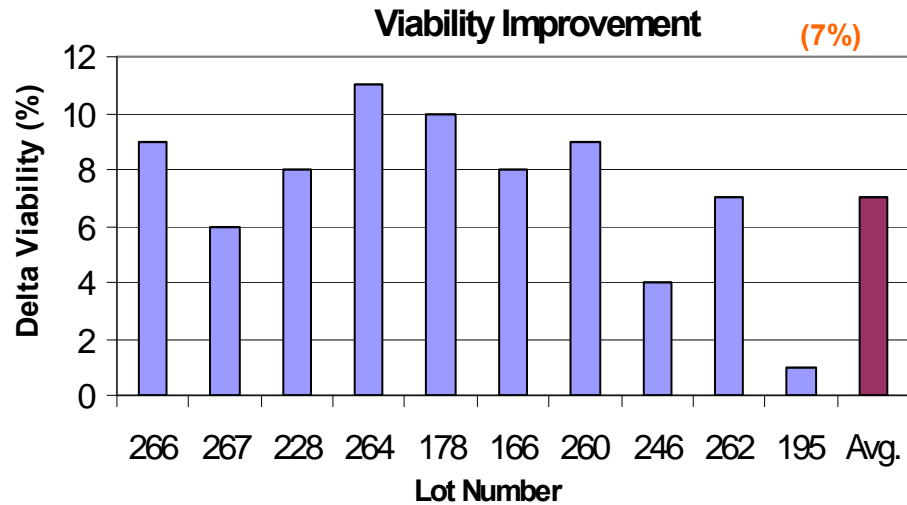
Lot did not pass plating criteria



Lot 114 passed plating criteria and QC.



# BD Gentest High Viability CryoHepatocyte Recovery Kit (vs BD Percoll-based purification kit)



For 10 representative BD inducible lots:

- Viability increases on average 7% ( $p < 0.05$ ,  $n = 10$ )
- Recovery increases on average 1.7 Million/vial, a 25% increase



# BD Gentest Inducible-Qualified Human CryoHepatocytes Inventory

Catalog No.	BD Lot No.	Donor Number	High Viability Recovery Kit	
			Viability	Recovery
454550	142	HH224	95%	6.5
	193	HF281	93%	6.2
	118	HH199	87%	4.9
	170	HH253	86%	3.5
	258	HMC414	86%	3.9
	262	HFC418	95%	6.4
	264	HFC422	91%	7.2
	277	HFC438	89%	7.0
454551	106	HH187	92%	5.3
	141	HH223	88%	14.9
	166	HH248	93%	7.9
	94	HH177	83%	5.1
	107	HH188	81%	6.2
	195	HF284	90%	9.4
	178	HH261	90%	8.9
	178*	HH261	90%	7.6
	226	HF373	80%	17.3
	228	HF382	90%	7.5
	246	HMCP402	86%	12.9
	260	HMAAP41 6	90%	10.1
	266	HMC424	88%	6.9
	267	HFC425	83%	6.5
	271	HMC429	88%	7.9
	276	HF AA437	90%	8.0
	281	HFC443	90%	7.7

- > 80% post-thaw viability for all lots. Average viability : 89%
- The increases of viability and recovery with the new high viability kit are both statistically significant (vs Percoll media)



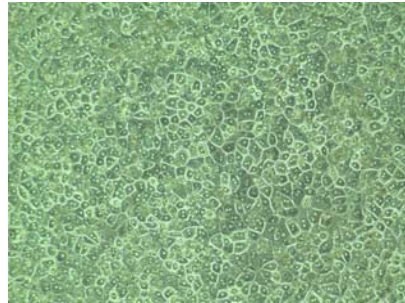


# Plating Efficiency

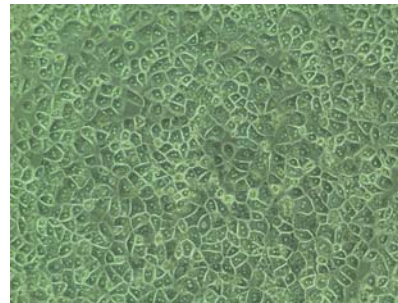
High Viability Recovery Kit

Percoll Purification

**Lot 281**  
24 hr, 100X

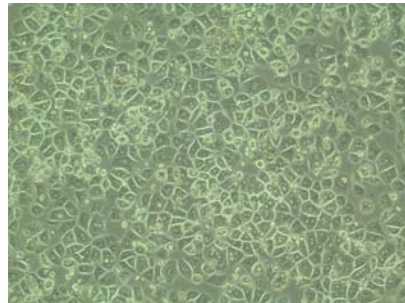


Confluency: 95%

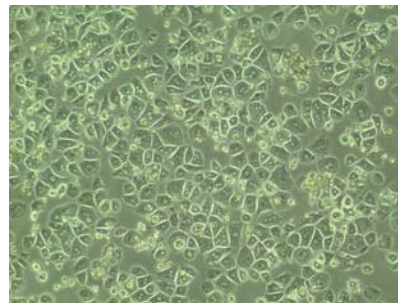


95%

**Lot 261**  
24 hr, 100X

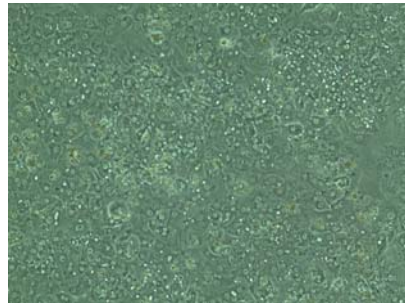


Confluency : 85%

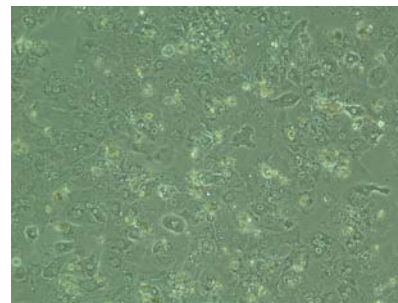


80%

**Lot 261**  
96 hr, 100X



Confluency : 85%



33

75%

- BD Gentest high viability cryohepatocyte recovery kit maximizes plating efficiency for some lots



# Midazolam Metabolism Assay

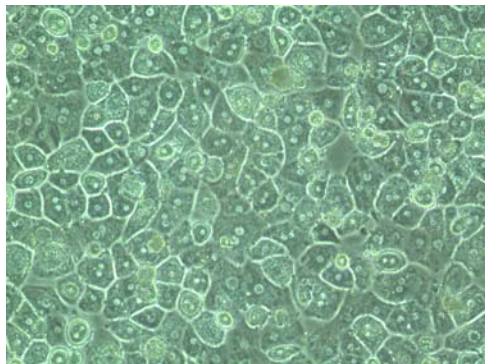
## Lot 281

Recovery Media	% Viability	Yield, E6/vial	24h, % Conflency	MDZ,CL, ml/hr/e6 cell	% MDZ remaining @8h
Cell Culture Media	73	8.4	90	0.222	8
Percoll	80	5.6	85	0.201	9.3
The New Media	83	6.8	90	0.204	5.1

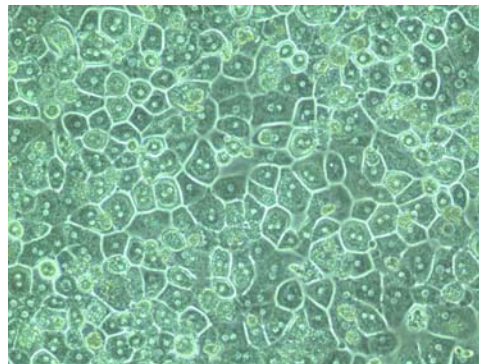
- All three methods gave similar results for Midazolam Clearance

## 24-hr Cell Morphology and Conflency (24 h, 20X)

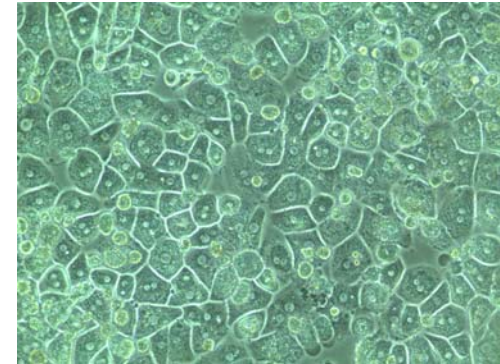
Cell Culture Media



The New High Viability Media

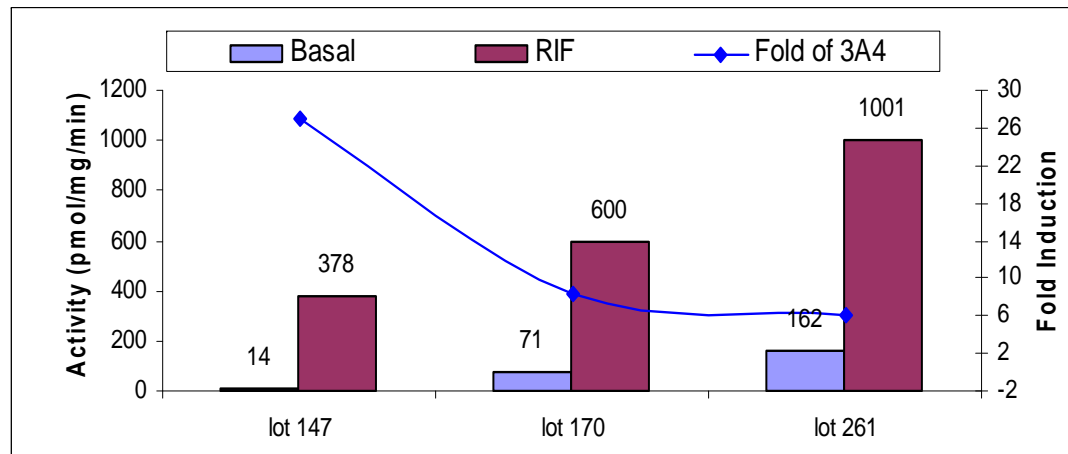


Percoll Purification Media

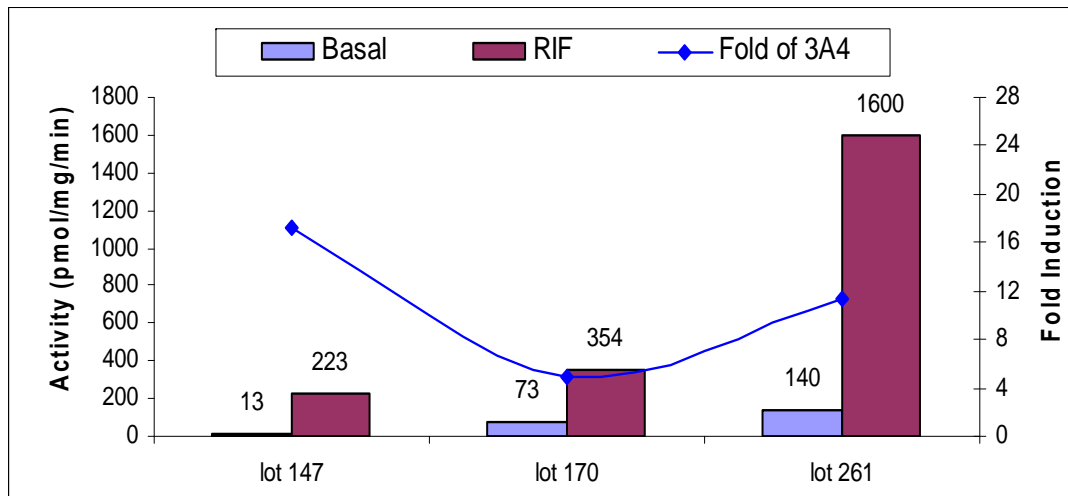


# 3A4 Induction Assays

**High Viability Recovery Media**



**Percoll Recovery Media**



- 3A4 induction assay results are consistent (tested on 3 lots)



# Number of Vials which can be Processed per 45 mL Recovery Medium

Up to 5 vials can be processed per tube of recovery media

	Lot 251		Lot 247	
vials / tube	Viability (%)	Recovery (M/vial)	Viability (%)	Recovery (M/vial)
1	84.2	6.7	80.8	11.8
2	84.5	7.4	79	12.3
3	83.9	6.8	81.7	12.5
4	81.3	6.6	76.7	12.3
5	81.3	6.8	80.3	13.3
6	78.4	6.5	75.5	12.4

Greater than 5 vials (6) of cryopreserved hepatocytes per tube of recovery media demonstrated a 5% drop in viability.



# Lot-to-lot Consistency

Viability is within **4%** with 3 different lots

	Viability (%)			Recovery (M/vial)		
	Kit Lot 1	Kit Lot 2	Kit Lot 3	Kit Lot 1	Kit Lot 2	Kit Lot 3
Lot 178	89.0	88.6	85.4	6.89	6.71	8.44
Lot 246	83.9	85.7	84.9	10.85	11.45	10.65
Lot 260	88.8	89.7	89.1	6.42	5.33	6.73



# Benchmark

Primary criteria is viability - indicative of cell health and ability of cells to attach.

	Lot 142		Lot 170		Lot 261	
	Viability (%)	Recovery (M/vial)	Viability (%)	Recovery (M/vial)	Viability (%)	Recovery (M/vial)
<b>High Viability Recovery Kit</b>	95	7.3	84	2.9	73	4.3
<b>BD Percoll Kit</b>	90	3.7	81	3.1	50	2.7
<b>Competitor 1</b>	91	7.5	74	4.4	58	5.5
<b>Competitor 2</b>	64	9.1	49	7.9	30	5.4



# Summary

- Several methods exist for thawing and plating cryopreserved hepatocytes
  - Individual methods generally not optimized for all critical features: speed/ease-of-use, non-cytotoxic, high viability, and high recovery, etc.
- Cell viability, recovery and plating efficiency tended to vary the most between the various methods, while fold-induction and metabolic activity varied the least.
- BD Gentest high viability recovery medium showed improved viability and cell recovery compared to conventional methods such as Percoll density gradients.



# Contact Us



## Questions?

### Contact information:

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