This video describes the process of the cryopreserved suspension hepatocyte assay. assay commonly used to determine in vitro clearance.

What is hepatocyte clearance.
Metabolism can have an important effect on the toxicity and therapeutic range of a drug. Drug metabolism occurs in many tissues such as the kidneys, lungs, skin, gut wall, and blood. The liver is the principal site of metabolism with liver hepatocytes and microsomes being used as an effective way to determine in vitro drug clearance.

Hepatocyte clearance. Unlike microsomes, hepatocytes are full liver cells which contain the whole package of phase 1 and phase 2 drug metabolising enzymes. As a result, both phase 1 and phase 2 metabolism can be tested and determine clearance that reflects cellular metabolism. The use of in vitro metabolism studies to determine hepatocyte clearance, alongside other ADME parameters such as plasma protein binding, are a good resource for the prediction in vivo half-life and hepatic clearance of a drug. Various species of hepatocytes can be used to determine and understand interspecies differences, which is fundamental for carrying out in vivo dose predictions.

Materials and equipment.
For this experiment you will need, cryopreserved hepatocytes, CHRM which is cryopreserved hepatocyte recovery medium, Williams medium E with no phenol red, primary hepatocyte maintenance supplement CM 4000, trypan blue solution, dimethylsulfoxide or methanol, acetonitrile plus a suitable internal standard. Any suitable internal standard can be used, for example donepezil. This must be used at a concentration that does not saturate the MS. Internal standards are used to correct variability during LCMS analysis. These must be similar to the analyte but also distinguishable when analysed. You will also need 82:18 acetonitrile water and positive controls such as verapamil, 7-ethoxycoumarin and phtalazine. Controls are required to ensure the assay is working correctly and that the hepatocytes are alive and metabolizing as expected. The controls chosen should test each of the different metabolic pathways.

For this experiment, you will also need hemocytometers, 48-well plates for suspension cells, a water bath, a thermometer, pipettes which are either single or multichannel, 96-well deep well 2 mL blocks, 96-well 300 µL v-bottomed plates, 0.5 mL micro tubes, 50 mL centrifuge tubes, a timer, DMSO resistant plate seals, a centrifuge, microscope, an LCMS instrument which is liquid chromatography with tandem mass spectrometry, and an incubator with 5% CO_2 and a plate shaker. Alternatives can be used where necessary. For example, alternatives can be used in place of the 96-well deep well 2 mL blocks and 96-well 300 µL v-bottomed plates. You can use MS vials or make the chips as alternatives. If you are using alternatives, please ensure that the final carrier with your samples for MS analysis is suitable for use on the instrument. For example, use MS vials or 2mL deep well blocks with a pierceable seal.

Assay preparation.
Firstly, remove the WME and supplement pack CM 4000 from the fridge, add the whole supplement pack to the 500 mL bottle of WME. The adding of the CM 4000 supplement is only carried out when a new bottle of WME is opened. Once added the supplemented media can be stored at 2-8 degrees Celsius for up to one month and used as needed. Bubble the WME with carbogen, which is 5% CO_2 for at least 30 minutes prior to use. While the WME is bubbling take out three 48-well plates, label one cells, one WME and one no cell control. You can also heat up your water bath to 37 degrees Celsius and create your termination blocks by adding 100 µL of acetonitrile plus internal standard to a whole 96-well deep well 2 mL well block. Create the number of termination blocks depending on your number of compounds there are eight compounds per termination block. For example, 16 compounds equals two termination blocks.

Next, warm up your WME and CHRM in the incubator to 37 degrees Celsius for around 30 minutes prior to initiating your experiment. Ensure you check the temperature

with a thermometer before starting.

Compound preparation and cell thawing.
Firstly, gather your compounds and controls and prepare them to a 100 µM in DMSO. It is important that controls such as 7HC, which are metabolites, need to be prepared tenfold higher. These should be prepared to 1 mM. In the 48-well plate labeled WME prepare 1mL of a 1 µM working solution for compounds and controls in supplemented WME. However here as compounds such as 7HC need to be at a concentration ten-fold higher they will be prepared as 1mL of a 10 µM solution. Cover the plate with lid and incubate at 37 degrees Celsius, 5% CO_2 until use. Next retrieve one vial of cryopreserved hepatocytes from the liquid nitrogen store and transport on dry ice. Thaw according to the manufacturer's instructions, for example in a 37 degree Celsius water bath. Once thawed, tip the full hepatocyte vial into the preheated CHRM. Mix gently and centrifuge according to the manufacturer's instructions. Different species are centrifuged at different speeds. Check the manufacturer's instructions to ensure the hepatocytes are centrifuged at the correct speed.

Cell preparation and counting.
Once centrifuged, tip the supernatant from the CHRM in one slow tipping motion, leaving the hepatocyte pellet. Gently resuspend the pellet in 1 mL of supplemented WME. To a microtube add 80 µL of supplemented WME, 10 µL of trypan blue, and 10 µL of resuspended cells, and mix. Add to both sides of a hemocytometer. Refer to the manufacturer's instructions for volume. Count the number of live and dead cells in both sides of the hemocytometer using a microscope.

To count your cells using the hemocytometer, please use the method as follows. Focus until you can see squares that look like this on either side of the hemocytometer. There should be four quadrants of 16 squares on each side. Using a hand counter, count the number of live cells. These are cells which have not been stained by the trypan blue. Also count the number of dead cells. These are cells which are stained by trypan blue. Trypan blue is a stain used to quantify live cells by staining dead cells. Trypan blue can only penetrate cells with damaged cells membranes and is therefore only able to penetrate dead cells. Please ensure that the same guidelines are used for counting each box of 16 squares. I.e. cells counted are fully within the squares. Count the cells in the 16 squares in each of the four quadrants on both sides of the hemocytometer. And use this to calculate cell viability.

Calculation of cell viability.
Note the number of dead and alive cells counted in each quadrant and calculate the total number of cells in each. Calculate the percent viability for each quadrant. Viability equals 100 divided by the total number of cells times the alive number of cells. Calculate the averages for the number of cells and percent viability.

Now you can calculate the volume required to dilute cells to 0.5 million cells per mL.

Dilution of cells to 0.5 million cells per mL.
To calculate the volume required to dilute cells to 0.5 million cells per mL please refer to the cell count previously determined. Firstly, multiply the average number of alive cells by their dilution factor to give cells per mL. The dilution factor is the factor which we diluted the resuspend cells before we counted them. For example, we added 10 µL of resuspended cells to 90 µL of trypan blue WME mix, therefore giving a dilution factor of 10. For example, in this case the number of live cells is 59, times by the dilution factor, which is 10. This will therefore give 590 cells per mL. Multiply by the hemocytometer factor and convert to million cells per mL. The hemocytometer factor will be stated in the manufacturer's instructions. For example, 590 times by the hemocytometer factor, which in this case is 10,000. This therefore gives 5.9 million cells per mL. Next, calculate the volume of WME needed to dilute cells to 0.5 million cells per mL. To do this, please follow the following equation. 5.9 million cells per mL divided by 0.5 million cells per mL, minus the volume of WME the cell mix is resuspend in, which

is 1 mL. This therefore gives 10.8 mL. Therefore, dilute the resuspended cells in 10.8 mL's of supplemented WME to get 0.5 million cells per mL.

Cell dilution and plating.
Dilute resuspended cells to 0.5 million cells per mL using the calculation on the previous slide. For example, dilute resuspended cells in 10.8 miles of supplemented WME. Next in the 48-well plate labeled cells add 200 µL of 0.5 million cells per mL to one well for each compound and control. A quick tip is to aliquot the cells in the same layout as the WME plate previously created. This will make sampling easier.

In addition aliquot an extra well for a cell viability check at the end of the experiment.

Next, incubate the plate for up to 10 minutes at 37 degrees Celsius, 5% CO_2, and gentle agitation approximately 90 RPM prior to use.

No cell control, initiation and sampling.
Firstly, label up each termination block with each of the timepoints, which are 0, 3,6,9,15,30,45,60,90 and 120 minutes. Column one is T0, column 2 is T3 and so forth. The last two columns on each termination block are used for the no cell control. No cell control samples are taken at 0 and 120 minutes. Please label these accordingly. Next, from the 48-well WME plate, remove 200 µL of the 1 µM compounds and controls in column A into column A of the 48-well no cell control plate already containing WME. Agitate this plate slightly. Immediately sample 20 µL into the appropriate column in the appropriate termination block, this is no cell control T0. For example column A equals termination block 1. Repeat these steps for column B and sample into the appropriate termination block. Column B equals termination block 2. Once all columns have been sampled place the no cell control and WME plates back in the incubator.

Experiment initiation and sampling.
From the 48-well WME plate remove 200 µL of the 1 µM compounds and controls in column A into column A of the 48-well cell plate containing 0.5 million cells/mL. Agitate this plate slightly. The final working solution concentration is 0.5 µM and the final cell concentration is 0.25 million cells/mL. Immediately sample 20µL into the appropriate column in the appropriate termination block. This is T0, for example column A goes into termination block 1. Ensure that after sampling you start the timer. Repeat these steps for column B and sample into the appropriate termination block. Column B goes into termination block 2. From the cell plate: Sample 20µL of the 0.5 µM working solution at 3,6,9,15,30,45,60,90 & 120 mins for each of the columns into the relevant termination block.

It is important that you ensure that you stagger your sampling so that each column is accurately sampled at the correct time.

No cell control T120 sampling.
At the end of the experiment sample 20µL of the no cell control working solution into the 120min column in the appropriate termination block, for example if you are sampling from NCC plate column A, dispense into termination block 1. Repeat these steps for column B and sample into the appropriate termination block. Column B goes into termination block 2.

T120 viability check.
Carry out a viability check at end of the experiment to ensure cells are viable. To a micro tube add 90µL of cell mix from the extra well in the cells plate and 10µL

of trypan blue, mix this gently. Add mix to both sides of a hemocytometer and count cells in the same way as previously carried out. You can now calculate the T120 viability for the end of the experiment. Calculate using the same formula as used for the viability check at the beginning

82:18 addition.
Add 100µL of 82:18, acetonitrile: water, to each of the termination blocks. Centrifuge the blocks at 3750 rpm for 10mins.

Finally, seal your samples with an appropriate seal and they are now ready to be analysed by LC-MS.

Data analysis: Creating an exponential decay curve.
When your samples have been run you will receive raw mass spec data. From your raw data, use the response at each timepoint to create an exponential decay curve of response against time in minutes. This can be done using a graphical package for example Xcel fit or Grafit. Response is the analyte and internal standard response ratio. Response is used to reduce analytical variability of results. Once plotted your graph should look as follows.

Data analysis: Calculation CLi(mL/min/gliver).
Clearance in mL/min/g liver equals k divided by V times the hepatocyte scaling factor, where k is rate per minute and v is million cells per mL. K is the decay rate derived from the exponential decay equation. The exponential decay equation is Y = a((b)x) where a is the initial amount, Y is the final amount, x is number of time intervals passed and b is the decay factor (b equals 1 minus r) where r equals rate which is also written as k. V is the final concentration of cells used in the experiment in million cells/mL. In our case this is 0.25 million cells/mL. Hepatocyte scaling factor is a constant which changes depending on which species is used. Mouse, rat and human is 120 million cells/g liver and dog is 240 million cells/gliver.

Data analysis: Example calculation CLi(mL/min/gliver).
Below we have our equation and we have our key. Our example equation uses mouse hepatocytes. k which is calculated from our exponential decay curve using the data previously shown was given as 0.044 per min, V is 0.25 million cells/mL and the hepatocyte scaling factor, as our species is mouse, is 120 million cells/g liver. Clearance in mL/min/g liver is calculated as 0.044 divided by 0.25 times 120. Therefore, clearance equals 21 mL/min/g liver.

Data analysis: Example calculation of half-life.
Half life also knows as t half equals ln2 divided by k. Where k is the rate per minute and ln2 is the natural log of 2, calculated as 0.693. Half-life is the time taken for half of a drug to be broken down/metabolised by the hepatocytes. For example, k equals 0.044 per minute which we have calculated previously from our exponential decay curve and ln2 is the natural log of 2 which is 0.693, this is a constant. Therefore, t half in minutes equals 0.693 divided by 0.044. Therefore, giving a half-life of 16 minutes.

What to do with hepatocyte clearance results?
You can utilise the data as an early stage screening tool to triage compound progression. You can correct for other ADME parameters such as plasma protein binding (PPB) and scale in vitro clearance data to predict in vivo hepatic clearance. You can use data to compare and investigate inter species differences in clearance, or the hepatocyte method can be adapted to carry out other techniques such as metabolite identification.

Advantages and disadvantages of suspension hepatocytes.
Advantages. Hepatocytes are full liver cells which contain the whole package of phase 1 and phase 2 drug metabolising enzymes. Both phase 1 and phase 2 metabolism

can be tested, and clearance determined that reflects cellular metabolism. Hepatocytes provide an effective in vitro model for predicting in vivo metabolism. Minimal amount of compound is needed and additional cofactors are not needed for enzyme activity. Disadvantages. Hepatocytes are expensive compared to other methods of determining metabolism for example microsomes. The assay is not high throughput and suspension hepatocyte assays can only be taken out to around 2 hours before cell viability and metabolic competency is reduced. Therefore, resulting in suspension hepatocytes not being optimal for determining an accurate clearance value for low turnover compounds.