This video describes the process of the plasma protein binding assay, otherwise known as PPB. So first of all, what is plasma protein binding? The extensive binding of a compound to plasma proteins, blood cells, skin cells, brain tissue, and many other tissues, can affect the ability of a compound to be distributed through the body to the target active site. This in turn can affect the duration and extent of the therapeutic action of a compound. Plasma protein binding, also known as PPB, is the extent to which a compound binds to plasma proteins in the blood. The less bound a drug is to plasma proteins, the mode efficiently it can move across membranes through the body, elicit a pharmacological effect and be cleared. The determination of PPB is important for predicting the pharmacokinetic and pharmacodynamic profile of a compound that can also eat and determining the optimal oral dose for in vivo profiling as a higher dose may be needed if the compound is highly bound. The gold standard for determining the extent of binding by experiments such as PPB is the equilibrium dialysis method. The equilibrium dialysis methods consists of the use of a 96-well dialysis block, that is composed of Teflon bars labeled "A" to "I" and a metal holder with clamp. The 96-wells are sectioned in half with a semi-permeable membrane. In the top chamber of each well is sample which could either be plasma, blood or tissue homogenate and drug. In the bottom part of each well is dialysate, which could be buffer or artificial cerebrospinal fluid depending on which experiment is being run. Drug is incubated with sample and left to reach equilibrium for approximately 5 hours. Drug molecules which bind to the binding sites of the proteins in the sample will form a complex and being able to move across the semipermeable membrane. Drug molecules which are unbound are therefor free to move across the semi-permeable membrane to the dialysate chamber. Materials and equipment. For this experiment, you will need a dialysis block. Dialysis membranes, these membranes are available in various different pore sizes, 12 to 14 kilodaltons in molecular weight cut-off is standard for this assay. These membranes have a small enough pore size to stop the plasma protein moving to the dialysate but also have a large enough pore size to let the component through. Membranes can come either hydrated or dehydrated. Dehydrated membranes need to be hydrated before use. You will also need dialysis block adhesive seals, a beaker, pipettes, 96-well deep well 2mL blocks, 96-well 300 uL V-bottom plates, 0.5 mill Eppendorf tubes, 7 mL glass vials and lids, plasma, an orbital shaker and incubator, a centrifuge, and an LCMS instrument, which is liquid chromatography with tandem mass spectrometry. Alternatives to these can be used where necessary, for example, mass spectrometry vials or Eppendorf tubes of sufficient volume. Ensure that the final carrier for 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. You will also need deionized water, isotonic buffer at pH 7.4. This is required to keep the pH of the assay constant through the 5 hr incubation. Dimethylsulfoxide or methanol, acetonitrile plus suitable internal standard. Any suitable internal standard can be used. For example, donepezil, this must be used at concentration that does not saturate the MS. Internal standards are used to correct viability during LCMS analysis. These must be similar to the analyte, but also distinguishable when analysed. You will also need ethanol. Your compounds and controls, for example, Nicardipine and Warfarin. Controls are required to ensure the assay is working correctly and no experimental error has occurred. One tightly bound, for example, Nicardipine and one less tightly bound, for example, Warfarin control should be used. As mentioned previously, dehydrated membrane needs to be hydrated prior to use. This is recommended to be done the day before your experiment. For this you will need your membranes and a beaker. Fill the beaker with 400 mL of deionized water, then take out as many membranes as needed. Dehydrated membranes come as two membranes joined together. Therefore, for example, for a whole 96-well dialysis block, you will need 8 membranes in total. Therefore take out 4 of the joint together membranes, put these in your water and leave these on the bench for an hour. After the hour, add 100 mL of ethanol, cover the beaker and leave overnight in the fridge. Now, it is the next day and we are going to prepare the PPB assay. First, remove your plasma from the freezer and leave it to defrost at room temperature. This can take quite a while so it is

advised do this as soon as possible. While the plasma is defrosting prepare your compounds and controls to 1 mg/mL in DMSO or methanol. Once the plasma has defrosted, centrifuge it at 3750 RPM for 10 minutes at 22 degrees Celsius to pellet the fibrogen. Next we're going to dilute our compounds and controls to 10 micrograms per mL. To do this, we will need to take out as many 7 mL vials as needed. For example, if we have 6 compounds and controls in total, we will need 6 vials labeled between "1" to "6". To dilute our compounds, we need to add 990 uL of supernatant plasma to each of the vials. Next, we will add 10 uL of our 1 mg/mL compounds and controls to the relevant viles. For example, compound 1 will go into the compound "1" vial and so forth. These files will then be left on the bench for 10 minutes to equilibriate. Whilst the compounds and controls are equilibrating on the bench, the membranes and dialysis block can be prepared. First. To prepare our membranes, we must remove them from the fridge. Then we need to rinse the membranes with deionized water to ensure all ethanol is moved. Finally separate the membranes gently and ensure that we have all membranes. Remember, this is an example. Always use the number of membranes required for experiment to avoid any waste. It is important that the membranes are left in deionized water until use to avoid them from drying out. The dialysis block can be assembled according to the manufacturer's instructions. The instructions from HT dialysis are as follows. To assemble the Teflon bars with dialysis membranes first, we must lay the first Teflon bar labeled "A" flat on the bench. Insert the two stainless steel connecting rod so that they are perpendicular to the Teflon bar. Place the membrane on the Teflon bar ensure that the membrane is approximately 2 mm below the top edge of the bar and the lower membrane edge overlaps the bottom of all wells. Repeat layering dialysis membranes and Teflon bars until the unit is fully assembled. Assemble Teflon bars in alphabetical order. Note although the number of membranes inserted can be varied to suit the experimental requirements, all the Teflon bars must be assembled before loading the teflon block into the base. Now to load the teflon block into the base. First, make sure the clamp is in the open position before loading the Teflon block into the base, insert the teflon block into the base then place the stainless steel pressure plate between the teflon block and the cam as shown. Always tighten the assembled unit with even pressure by using both hands to rotate the cam levers. Now your unit is fully assembled, it is important to immediately add buffer to the dialysis site of the wells to prevent dehydration of the membranes before the test samples are added. As previously stated, it is important to dispense dialysate immediately into the appropriate wells to ensure membrane do not dry out. To do this, we must add a 150 uL of buffer to the bottom half of each well. Dispense this in a N = 3 and N = 6 fashion for as many compounds as needed. That should be done in both halves of the dialysis block in the same layout. Here's an example of an N of 3 for 6 compounds. Be careful not to touch the membrane when pipetting as it can affect the membrane integrity. Now, we are going to carry out the assay pre-incubation. Here is our dialysis block with our buffer in the bottom chamber of the appropriate wells. First, we're going to deal with the left-hand side. Also known as the samples side. On the left-hand side, also known as the sample size of our dialysis block. We have a 150 uL of isotonic buffer in the bottom chamber of each well. Now into the top chamber of each well we are going to add a 150 uL of equilibrated plasma and compound, or a control, to the appropriate wells. Do this in an N of 3 for each compound. Here's an example of an N of 3 for each compound. For example, in A1, A2, and A3 is compound 1, and B1, B2 and B3 is compound 2, and so forth. Remember to keep a log of the order of your compounds to avoid any error. Once the left-hand sample side of the dialysis block has been dealt with. The stability controls must be made. To do this, we must add a 150 uL of each10 ug/mL compound or control into 2 separate Eppendorf tubes. These must then be labeled according to the compound number. One of the Eppendorf's must be incubated at 4 degrees Celsius for the duration of the experiment. The other must be incubated at 37 degrees Celsius. This is important to check the stability of your compounds during the assay. Now, we're going to deal with the right-hand side of the dialysis block, also known as the control side. In the right side of the dialysis block, we have a 150 uL of isotonic buffer in the

bottom chamber of each well. Now we will dispense 150 uL of control plasma into the top chamber of the relevant wells. Do this in the same layout as the right-hand sample side. Here's an example. Once sample has been loaded onto both sides of the dialysis block, it is now ready to be sealed. Seal the block using the manufacturer's adhesive sealing film. Ensure that the seal covers all wells effectively and doesn't overhang. This can lead to leakage and contamination of other rails when shaking. Now, incubate your dialysis block at 37 degrees Celsius, 100 RPM for 5 hours to reach equilibrium. It is important to carry out protein check prior to sampling to take the membrane integrity. This can be quantitative or visual. The following example is a visual check which uses the pierce BCA protein assay, which includes reagent A and reagent B. For a full 96 well plate, add 10 mL of reagent A and 600 uL of reagent B and vortex to mix and turn green. Alter these volumes where appropriate to accommodate the number of samples and avoid any waste. Now into fresh 96 well plate, add 100 uL of BCA mix and 5 uL of buffer from the sample side and the control side of the dialysis block. Ensure that the tips are changed between each buffer sample to avoid cross-contamination of wells. Now leave on the bench for 15 minutes. After the 15 minutes, if all wells are green then the protein check is good. However, if there are purple wells, then this can indicate protein contamination in the buffer. These wells should be discounted or the assay repeated if there are a large number of purple wells. So why does the well turn purple? The copper ion is reduced in the presence of protein to the cuprous cation. This when mixed with the BCA mixture undergoes chelation in turns a purple colour. Therefore, the more protein present, the darker the purple colour. This can also be quantified using the quantitative protein check. Once the protein check has been carried out, post incubation sampling can occur. First, we're going to deal with the left-hand sample side of the dialysis block. Into a fresh 96-well 2 mL deep well block, we're going to add 50 uL of dialysed plasma and drug into columns 1-3. We are then going to add 50 uL of dialysed sample buffer and columns 4-6 for all compounds. It is important that the buffer and plasma samples with with drug are dispensed into separate wells. For an example, for row A, columns 1-3, we have the dialysed plasma and drug samples for compound 1. Then in columns 4-6, we have the dialysis buffer samples also for compound 1. Now we're going to look at the right hand control site of the dialysis block. Into our 96-well 2 mL block, where we have our dialyse plasma and drug samples and our dialysed buffer samples, We are now going to add 50 uL of control dialysed buffer into the the wells with the dialyse plasma and drug, which are the columns 1-3. We are then going to add 50 uL of control dialysed plasma into the wells with the dialysed buffer samples, which are columns 4-6. This is carried out to normalise the samples to reduce any ion suppression that may be seen on the mass spec. Once the 5 hour incubation time and post incubation sampling has occurred, the stability control samples can be removed from the fridge and from the incubator. These will then be added into our 96-well deep well 2 mL block, which also includes our post incubation samples of buffer and plasma. First, we need to add 50 uL of each 37 degree control for each compound into individual wells. Take 50 uL of each 4 degree control for each compound into individual wells. Dispense the stability control samples beside the buffer and plasma samples for the same compound. For example, in row A columns 1-3, we have our plasma samples with our dialyse control buffer for compound 1. In columns 4-6, we have our buffer samples with our dialysed control plasma for compound 1. Then and column 7, we have our 37 degrees stability control sample for compound 1. And in column 8, we have our 4 degree stability control sample also for compound 1. Next, we need to add control buffer to the stability control samples to normalise the experiment as previously described. To do this, we need to take 50 uL of control dialysed buffer and dispense it into the wells with 37 degree and 4 degree stability control samples. Now, we have our 2 mL 96-well deep well block containing all samples. To this block add 200 uL of acetonitrile with internal standard. Next centrifuge the block at 3750 RPM for 10 minutes at 22 degrees Celsius to pellet any remaining fibrinogen. Whilst this block is in the centrifuge, get a clean 96 well 2 mL deep well block, and add 50 uL of deionized water into the same positions as the sample

block. Once the centrifuge has completed, take a 150 uL of supernatant and remove it into the block with the deionized water. Now the block can be sealed with an appropriate seal and is ready for LCMS analysis. Once the experiment is complete, the dialysis bit must be cleaned according to the manufacturer's instructions. The instructions from HT dialysis are as follows. To clean the Teflon bars, open the clamp by rotating the calm levers 45 degrees. To ensure even pressure, always use two hands to operate the clamp. Remove the entire teflon block assembly from the clamp, then remove the stainless pressure plate. Remove one or two Teflon bars from one end of the assembly, then pull out both stainless steel connecting rods. Teflon bars well then dissemble. Clean separated Teflon bars with non-ionic laboratory detergent. Not always rinse away all detergent residue with distilled water before re-use. Once the blocks have been run and the raw results have been received, data analysis can then occur. The following describes the calculation of the fraction unbound, also known as the percent Fu. To calculate the percent Fu, we will need our raw results. Our response, which is our analyte and internal standard response ratio. This is used to reduce any analytical variability of results. We will also need the following calculations for percent bound and precent Fu. To calculate a percent fraction unbound. First, we need our raw analytical data and a table for each compound, similar to the one shown here. This table details the compound name, each replicate number, buffer response, plasma response, percent bound, percent unbound, and the average for both of these. First in the table, we put compound plasma response for each corresponding replicate. Next, do the same for buffer response. From this, we can now work out our percent bound by using the following equation. Percent bouned equals PL minus BU over PL times a 100, where PL equals plasma response and BL equals buffer response. So for example, percent bound for replicate 1 would be 2.32 minus 0.363 over 2.32 times a 100. This would therefore give us 84.35 percent bound. Do the same for all other replicates and average at the bottom. From this, we can then work out percent unbound. For example, for replicate 1, unbound is a 100 minus percent bound, which we've just calculated to be 84.35. This would therefore give us 15.65% unbound. You can do the same for all other replicas and average at the bottom. For fraction unbound. So for example, fraction unbound is a 100 minus the percent bound. However, in this case, as we have several replicates, it would be a 100 minus the average percent bound. So for this compound, fraction unbound would be a 100 minus the average percent bound, which we've just calculated to be 80.12. This would therefore give us 19.88, or when rounded, 20 % fraction unbound. You can also calculate standard deviation to check that there is not too much variability between the replicates. The greater that percent Fu, the more compound unbound to plasma proteins and therefore available to be distributed to the target site and incur a therapeutic action. Now, for the calculation of percent recovery. For this, you will need your raw data with your areas and the following equations. One, detailing precent recovery at 37 degrees and the other detailing precent recovert at 4 degrees. Percent recovery is calculated at 37 and 4 degrees Celsius to check compound stability in the assay. We can calculate percent recovery for each compound at 37 and 4 degrees. For this, you will need your raw data with your areas and a table detailing replicate number, buffer area, plasma area, total and average. First into the table we're going to put a buffer areas for each replicate. Next, do the same for the plasma areas. Total these and average these at the bottom. Next, we can create a new table detailing the temperature control, area and percent recovery. First, into this table we're going to put the area for the 37 degree temperature control. Next, do the same for the 4 degree temperature control. Now we're ready to calculate recovery using the following equations. First, for the 37 degree temperature control, percent recovery at 37 degrees is the sum of peak areas, both buffer and plasma, divided by the area of the 37 degree compound temperature control, times 100. So for example, percent recovery at 37 degrees is the average of the total of the buffer areas and the plasma areas divided by the area of the 37 degree temperature control times a 100. This would therefore give us 108% recovery at 37 degrees. Next, do the same for the 4 degree temperature control using the 4 degree equation. So percent recovery at 4 degrees is the sum of peak areas, both buffer

and plasma, divided by the area of the 4 degree temperature control times 100. So here for 4 degrees. the percent recovery is the average of the totals of the buffer and plasma areas divided by the area of the 4 degree temperature control times 100. This would therefore gave us a 100 % recovery at 4 degrees. Percent recovery between 70-120 % is deemed acceptable. If percent recovery is outwith this, your compounds may have binding or stability issues. Finally, some advantages and disadvantages of the Teflon block method for PPB. First, advantages. It is less expensive than other methods such as rapid equilibrium dialysis and ultracentrifugation. Minimal amount of compound is needed. It is less susceptible to experimental artifacts.

For example, nonspecific binding. Various membrane pore sizes are available to accommodate different experiments. Now disadvantages. It has a 5 hour incubation time needed to reach equilibrium. It is not high throughput and a relatively large volume of plasma is needed.