User Guide

TRANSIL

High Sensitivity Binding Kit

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TPB-0400-0335
TPB-0400-2535

Version 3, Revision 05

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Contents

1 Quick Protocol

1. Assay Specification

- Run and analyze the membrane affinity kit before the main experiment (refer to TRANSIL MA kit's protocol)
- Alternatively: Have the compound's membrane affinity from previous experiments at hand

2. Kit Thawing and preparation

- Thaw for 3h at room temperature (alternatively overnight).
- Spin vials quickly for 5 seconds at 750 g to collect all liquid at the bottom.
- Make sure the assay has a working temperature between 20°C and 25°C when starting the experiment.
- Open the lids only immediately before compound addition (step 5).

3. Drug Candidate Preparation

- Prepare 50x stock solutions of each compound in pure solvent (i.e. DMSO).
- The final compound concentration in the assay depends on the compounds solubility, analytical method and instrumentation: If permitted by compound solubility, use 5 µM final assay concentration. This requires a 250 µM stock solution.
- Since each compound is added in an aliquot of 10 µl to 5 x 7 vials and the calibration vials, we recommend preparing a volume of at least 600 µl stock solution for each compound.

4. Preparation of plasma and calibration sample pre-dilutions

- Prepare five sequential pre-dilutions (dilution step 1:3) for calibration in pure solvent (i.e. DMSO) at 50x concentration of the respective calibration concentration (c.f. section 14.2).
- Enter the compounds membrane in TRANSIL High Sensitivity binding assay spreadsheet to determine the optimal plasma dilution scheme (c.f. section 13). Prepare plasma pre-dilutions according to the instructions given in the spreadsheet.

5. Plasma and Drug Candidate Addition

- Mix the test compound and calibration curve stock solutions as well as plasma pre-dilutions carefully.
- Add 100 µl pre-diluted plasma to each of the 7 vials within each plasma dilution series.
- Add 100µl of pre-dilution with highest plasma concentration to the 5 calibration vials.
- Transfer 10 µl of the 50x test compound stock solution to each vial. Change tips after each compound transfer step to avoid carryover of beads.
- Transfer 10 µl of the respective 50x calibration pre-dilution to each calibration vial.
- Close each vial with its respective screw cap.

6. Incubation and Supernatant Sampling

- Incubate the tray with the glass vials on a plate shaker at 1000 rpm for 30 minutes and **make sure all beads resuspend**.

  Alternatively: mix by aspirating and suspending a volume of 350 µl for 20 times.
- Spin the vials in a swing-out centrifuge for 10 minutes at 750 g.
- Quantification by scintillation counting:

  Transfer up to 200 µl supernatant to scintillation vials and proceed with step 8.

  Quantification by LC-MS/MS:

  Transfer 100 µl supernatant to a new silanized glass vial for protein precipitation (vials provided).

7. Sample preparation

Use your method of choice or the provided protocol for plasma precipitation below. To avoid matrix effects we recommend adding excess human plasma to all samples before proceeding with any sample preparation method.

- Add 200 µl acetonitrile containing internal standard to each well. Vortex each vial for 5 seconds.
- Incubate 10 minutes at room temperature without agitation.
- Centrifuge 30 minutes at 3000 g to precipitate plasma.
- Transfer 100 µl of supernatant in a standard 96 well plate for analytical quantification.

8. Analysis

- Quantify supernatants by the method of choice.
- For evaluation of the results, please use the supplied MS Excel spreadsheet and refer to the operating instructions for data analysis.

2 Background

The plasma protein binding of drugs continues to be important for property pharmacokinetic evaluations and in routine clinical monitoring of drugs. This is because for many drugs, the therapeutic and toxic response correlates better with the concentration of diffusible, unbound drug than with the total drug concentration.

Several methods exist for assessing the unbound fraction of drug in plasma. Standard methods include dialysis, ultrafiltration, centrifugation and rapid dialysis with immobilized plasma proteins (TRANSIL HSA and AGP Binding kit). These methods differ in their limitations and are typically used as complimentary tools to assess the unbound fraction for a wide array of chemical structures. However, if the unbound fraction becomes very low and comprises only 1% of the total drug in plasma or even less, then all the mentioned methods become inaccurate mainly because of analytical problems. Plasma dilution can be used to increase the unbound fraction, however, care needs to be taken not to saturate the binding sites of the binding proteins in plasma that have only a very low abundance, such as α_1-acid glycoprotein, lipoproteins, or sex hormone binding protein.

Another problem that typically arises with highly lipophilic drugs is a deteriorating recovery of drugs in the experiment due to non-specific binding. The erythrocyte partitioning assay was developed to circumvent both the analytical problem and the problem on non-specific binding (Tucker et al. 1970, Trung et al. 1984, Urien et al 1990). This assay assesses plasma protein binding through competitive binding to cell membranes from erythrocytes.

Originally the erythrocyte partitioning assay was performed with erythrocytes from the same species as the plasma proteins. It turned out the assay's accuracy is not compromised when using human erythrocytes (Schuhmacher et al., 2000) or TRANSIL beads with immobilized membranes (Schuhmacher et al., 2004). Hence, the assay can be simplified and the cumbersome steps of washing and handling of erythrocytes can be eliminated altogether.

3 Applications of the TRANSIL High Sensitivity Binding assay

The TRANSIL High Sensitivity Binding Assay is an assay for estimating plasma protein binding of drugs and drug candidates. It accurately predicts the free fraction of drugs in plasma, even if the fraction is well below 1%. Also, it is designed to accurately determine the free fraction of highly lipophilic drugs or any other drugs that tend to bind in dialysis strongly to the assay system's setup.

The assay comes ready to use in pre-filled silanized glass vials. All liquid handling steps can be automated to increase the assay's throughput.

4 Basic assay principle

The TRANSIL High Sensitivity Binding assay determines the fraction of drug bound to plasma indirectly by determining the partitioning of drug between a plasma phase and a lipid membrane phase. The principle of the assay is equivalent to the erythrocyte partitioning assay (Schuhmacher *et al.*, 2004). The assay assesses plasma protein binding through competitive binding to synthetic cell membranes and has two key principles to obtain accurate binding estimates: (i) the membranes compete with the plasma proteins for finding the drug candidate, hence the more drug shifts away from plasma to membrane, the weaker the protein binding in comparison to the membrane affinity; (ii) the assay assess only the drug concentration in the membrane and the plasma/buffer fraction. A consequence of the latter is that there is no pure buffer phase in which the compound could precipitate and that the assay will not measure the unbound concentration of the drug directly. Assay principle (i) allows the accurate determination of the unbound fraction by measurement of the membrane affinity in different plasma dilutions.

The affinity of drugs to plasma is determined by measuring the change of bound to immobilized phosphatidylcholine membranes in response to changing the plasma concentration in the assay system. The membrane affinity is a distribution coefficient of drugs between a membrane phase and buffer (c.f. p. 16.6.1, Equation 3). When plasma is added to such a system, the amount of drug bound to plasma reduces the amount of free drug which can equilibrate with the membrane, hence less drug will be bound in the membrane phase. While the membrane affinity as a physico-chemical property of compounds remains constant, the apparent membrane affinity which is observed when measuring the total drug concentration in the buffer and plasma phase decreases with increasing plasma concentration in the assay system. How the plasma induced change of

apparent membrane affinity relates to the affinity of drugs to plasma and to the true membrane affinity is defined by Equation 1:

$$APA = \frac{n_t \cdot (\alpha \cdot P + K_D)}{\alpha \cdot P \cdot V_b + K_D \cdot (V_b + MA \cdot V_l)} \qquad \text{Equation 1}$$

Where *APA* is the experimentally determined drug concentration in the buffer and plasma phase, *P* the concentration of full plasma, *α* the chosen plasma dilution, *MA* the drug's membrane affinity fitted from the experimental data, K_D the affinity constant of drug to plasma, n_t the total amount of drug in the experiment and V_b and V_l the buffer volume and lipid volume of membrane, respectively. The concentration of full plasma was arbitrarily set to 600 µM and K_D and *MA* were obtained by fitting the experimental data to this model. The fitting process involves the minimization of the deviation between the experimentally determined drug concentration in the buffer and plasma phase (*APA*) and the predicted *APA* values (denoted as *APA**) using the given model. Starting with arbitrarily chosen values for *MA* and K_D, both were optimized via minimizing the sum of squared residuals by an iterative process. To obtain an optimal result squared residuals (SR) were weighted by means of the corresponding experimental free fractions before minimizing the sum of the residuals (SSR). The bins for the weighting approach are provided by Table 1. Frequently, it is observed that mass spectrometers exhibit a non-linear response even in concentration ranges up to 100x above the detection limit. Likewise, impurities of radiolabelled compounds can lead to similar effects when the impurity exhibits different properties from the parent compound. We use the weights to discount such deviations in the model fit. The calibration curve can be used as a guide to adjust the weights. If the relative peak area deviations in the low concentration range follow an obvious trend and exceed 40% we recommend to set lower weights for these concentrations (c.f. section 18.4/ Figure 14). The calibration curve and plot of relative peak area deviations can be found on the raw data tab. Note that this non-linearity issue is frequently hardly visible in the standard 5-point calibration curve. Therefore, if you notice hints of a non-linearity we recommend setting up a more detailed calibration curve with less spacing. Feel free to contact our technical support for guidance, in particular, because we advise to use the same buffer system for the calibration curve as for the assay.

Table 1: Weight cutoffs defined for the experimentally determined free fractions.

f_u' experiment	weight
0.00	0.00
0.05	0.05
0.10	0.10
0.15	0.50
0.20	1.00
0.80	1.00
0.85	1.00
0.90	1.00
0.95	1.00

The free fraction is calculated according to Equation 2 involving the K_D obtained from the model fit:

$$f_u = \frac{1}{1 + \frac{P}{K_D}}$$ Equation 2

Please note that the K_D determined form the model fit is a pseudo-K_D as it addressed the affinity of the drug to all different plasma proteins as a whole. Hence, the only truly meaningful result of the assay is the fraction unbound as calculated from Equation 2. The K_D will always depend on the choice of the molarity of the plasma. This choice is inherently difficult, since it relates to the sum of all plasma proteins while the binding will only occur to a small fraction of plasma proteins. Assuming that a typical HSA concentration in human plasma is 580 µM we arbitrarily chose a plasma concentration of 600 µM. Note that this choice only affects the calculated K_D, however, it has no effect on the final result of the free fraction as the free fraction calculation also depends on the assumed plasma concentration.

5 Limitations

The assay is extraordinarily well suited for assessing plasma binding of tightly bound, highly sticky, poorly soluble, lipophilic drugs. Accuracy increases the stronger the drugs bind to plasma and membranes. However, compounds with rather low or extremely high membrane

affinity (MA < 300 or MA > 30000) can be difficult to measure in this standard assay and require adapted assay conditions. Please contact our Sovicell staff for test compounds with the above specified properties.

6 Kit components

The table below lists the contents of the kit and Figure 1 shows the supplied prefilled vials.

No.	Qty.	Item
1	7	Prefilled silanized glass vials for determination of membrane affinity in plasma dilution 1 (A1 to A7, color code: ●).
2	5	Prefilled silanized glass vials for calibration curve in plasma dilution 1 (cal-1 to cal-5, color code: ●).
3	7	Prefilled silanized glass vials for determination of membrane affinity in plasma dilution 2 (B1 to B7, color code: ■).
4	7	Prefilled silanized glass vials for determination of membrane affinity in plasma dilution 3 (C1 to C7, color code: ◆).
5	7	Prefilled silanized glass vials for determination of membrane affinity in plasma dilution 4 (D1 to D7, color code: ⌘).
6	7	Prefilled silanized glass vials for determination of membrane affinity in plasma dilution 5 (E1 to E7, color code: □).
7	40	Labelled silanized glass vials as receiver after plasma precipitation having the same labels and color codes as the donor vials.
8	5	Blank vials for setting up the calibration pre-dilution
9	1	Instruction manual
10	1	CD with spreadsheet calculation

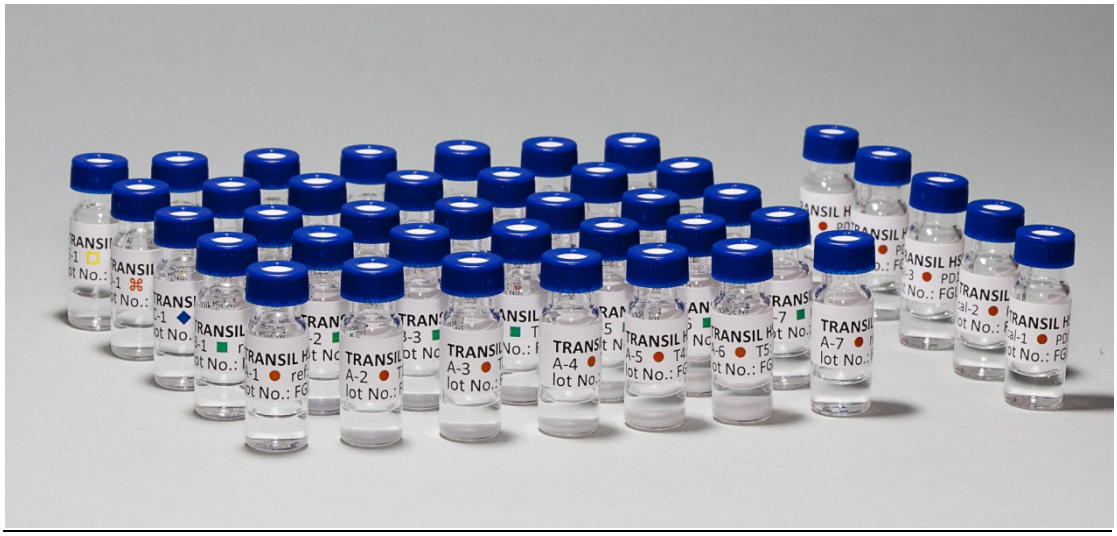


Figure 1: Prefilled vials supplied with the TRANSIL High Sensitivity Binding assay kit. For each plasma dilution there the kit supplies 5 vials with varying TRANSIL content and 2 reference vials. For the lowest and highest plasma dilution the kit supplies 5 vials for preparing a 5-point calibration curve.

7 Abbreviations

AAP	human α_1 acid glycoprotein, synonymous to AGP
AGP	human α_1 acid glycoprotein, synonymous to AAP
APA	Concentration of the drug in buffer and plasma phase (HSB assay)
clogP	Calculated logP
conc	Concentration
fu	Free fraction of drug in plasma
HSA	Human Serum Albumin
HSB	TRANSIL High Sensitivity Binding assay
K_D-value	The affinity constant K_D is directly related to the ratio of the forward rate constant over the reverse rate constant of the binding interaction A + B = AB.

MA	Membrane affinity: distribution coefficient of a compound between membrane and buffer
PBS	Phosphate buffered saline
SR	Squared residuals
SSR	Sum of squared residuals
TQI	TRANSIL Quality Index
wSSR	Sum of weighted squared residuals

8 Reagents

No.	Reagent	Specification
1	Full plasma	A total of 1.4 ml plasma is required per compound
2	Dulbecco's PBS (1x)	For dilution of plasma
3	50x sample stock solution	
4	Sample dilution for calibration	Prepare appropriate sample dilutions for a 5 point calibration curve

9 Equipment

No.	Instrument	Specification
1	Vortexer	
2	Test tube rotator or roller	
3	Pipette	
4	Centrifuge	Including rotor for ND11 vials

10 Assay kit preparation

Upon receipt the assay kit should be stored at -20°C.

Before use, thaw the assay kit either at 4°C for a period of 12 hours (overnight) or, at 20°C for a period of 3 hours. Make sure the vials have reached room temperature (between 20° and 25°C) prior to the assay.

11 Drug candidate preparation

A 50x stock solution has to be prepared for each drug candidate. The DMSO concentration should not exceed 10% in the final assay. Please consider the following:

Concentration

The TRANSIL High Sensitivity Binding assay kit can be used in conjunction with various analytical methods and instruments. These include HPLC with a UV or diode array detector, LC/MS/MS, as well as other methods such as scintillation counting. Please note that the lower limit of the compound concentration in the assay is only limited by the detection limit and dynamic range of the analytical system used. However, we advise to choose a compound concentration high enough to assure that the quantification is fully within the linear range of the instrument. Alternatively, it is advised to prepare detailed calibration curves for all plasma dilutions to account for non-linearities. Please contact the customer service for further advice on the best approach to the particular compound and situation.

The upper limit of the compound concentration in the assay is limited by the compounds solubility as well as the saturation of individual beads or the entire bead

suspension with the test compound. Therefore, we recommend using final assay concentrations of 5 µM or less for quantification via LC-MS/MS and concentrations of 25 µM or less for HPLC.

Volume

It is necessary to have at least 350 µl of the stock solution for each compound drug candidate. Since 10 µl of compound is added to 5 x 7 vials. We recommend preparing a volume of at least 600 µl per compound to accommodate for preparation of the calibration curves as well.

12 Replicates

The TRANSIL High Sensitivity Binding assay kit is designed such that the membrane affinity of one compound in one plasma dilution is determined in 7 vials – two references and 5 vials with increasing lipid membrane surface area. Therefore, the assay provides 5-fold determination of the assay parameters. Thus, it is not necessary to run more than one row per compound to obtain replicates for statistical validity.

However, since analytical accuracy has a direct influence on the accuracy of the estimated free fractions, it can be beneficial to perform duplicate or triplicate injections of the samples from the assay when using LC-MS/MS for quantification.

13 Plasma Dilutions

The assay utilizes a series of 5 lipid membrane surface areas and 5 plasma dilutions to determine the affinity of drugs to plasma via measuring how much drug binds to the membrane under the resulting 25 different experimental conditions. The gradient of lipid membrane surface areas is supplied with the kit, however, the gradient of plasma dilutions has to be set up individually. Compound properties, the lipid gradient and the plasma dilution series influence the accuracy of plasma binding results. Therefore, care must be taken to choose an appropriate plasma dilution series. We recommend using a dilution series based on four equal dilution steps (Figure 2).

Plasma Dilution Steps

logMA (Transil^{XL} Membrane Affinity Kit)	4,50
highest plasma conc (<= 1:5): 1:	5
subsequent dilution steps: 1:	4
plasma volume per well:	100 µL
plasma volume per preparation:	1000 µL

	final dilution	Assay	dilution	Pre-dilution ratio	Pre-dilution	buffer [µL]	pure plasma [µL]
No 1	1: 5		0,200000	1: 1	1,00000	0,0	2000,0
No 2	1: 20		0,050000	1: 4	0,25000	750,0	250,0
No 3	1: 80		0,012500	1: 16	0,06250	937,5	62,5
No 4	1: 320		0,003125	1: 64	0,01563	984,4	15,6
No 5	1: 1280		0,000781	1: 256	0,00391	996,1	3,9
						sum:	2332,0

Figure 2: Screen shot of the table “Plasma dilution steps” from the “main” tab of the spreadsheet for analyzing data from the TRANSIL High Sensitivity Binding Assay. The table shows calculated

volumes (blue shaded area) for setting up the plasma pre-dilution series based on the choice of dilution series specified in fields D21 and D22. The final dilution is obtained when 100 µl of each plasma pre-dilution are added to 400 µl assay volume making a total assay volume of 500 µl.

To assess the optimal plasma dilution knowledge of the test item's membrane affinity is required (input field D20 of the "main" tab). For the determination of the membrane affinity of the test compounds we offer the TRANSIL Membrane Affinity Kit. Use the guidance scheme in Figure 3 for the optimal choice of plasma dilutions and possibly choice of assay kit design.

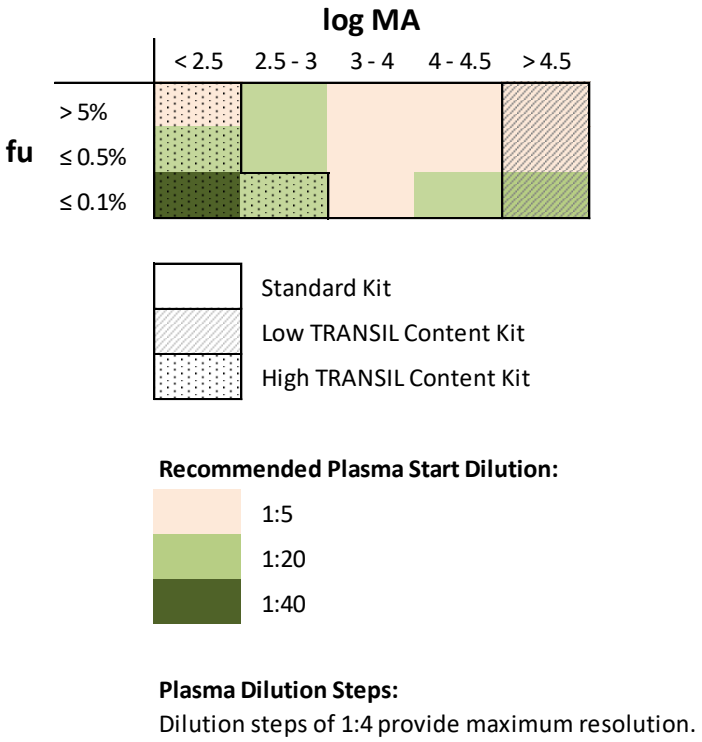


Figure 3: Design matrix for plasma dilutions and optimal choice of kit versions. Standard TRANSIL High Sensitivity Binding kits cover most common laboratory situations. However, special kits are available for compounds with either very high membrane affinity (hatched areas) or unusually low membrane affinity (dotted areas). Depending upon the compound's membrane affinity and it's expected unbound fraction we recommend plasma starting dilutions ranging from 1:5 (addition of pure plasma, beige), to 1:20 (addition of 1:4 pre-diluted plasma, light green) and in the extreme case of low membrane affinity and very strong plasma binding up to 1:40 (dark green).

If no preliminary compound property information is available, we recommend starting with a 1:5 dilution for the highest plasma concentration (corresponds to pure plasma without any pre-dilution) and four equal dilution steps with a dilution factor of 4. In that case, the final choice of the plasma dilution series has to be entered manually in fields D21 and D22 of the "main" tab. Based on this dilution series definition the spreadsheet automatically

calculates a pipetting scheme for setting up the plasma pre-dilution series in PBS buffer (Figure 2).

If the assay is performed without prior expectation or inadequate expectation of the final results for the membrane affinity, it may be possible to further optimize the free fraction estimate by iterating the assay procedure with more adequate assay parameters in the second round.

14 Assay procedure

The TRANSIL HSB binding assay consists of the following steps: (i) addition of 100 µl of pre-diluted plasma to each of the 7 vials within one of the 5 plasma dilution series and to each of 5 vials for calibration (ii) addition of 10 µL compound to each of the 7 sample vials per plasma dilution and to the 5 calibration curve vials, (iii) mixing by vortexing and 30 minutes incubation while rotating vials, (iv) separation of beads and buffer by centrifugation of the assay plate for 10 minutes at 750 g, and (v) transfer of 100 µl supernatant and plasma protein precipitation (vi) analytical quantification of the assay's supernatant after precipitation. The assay principle is illustrated by Figure 4.

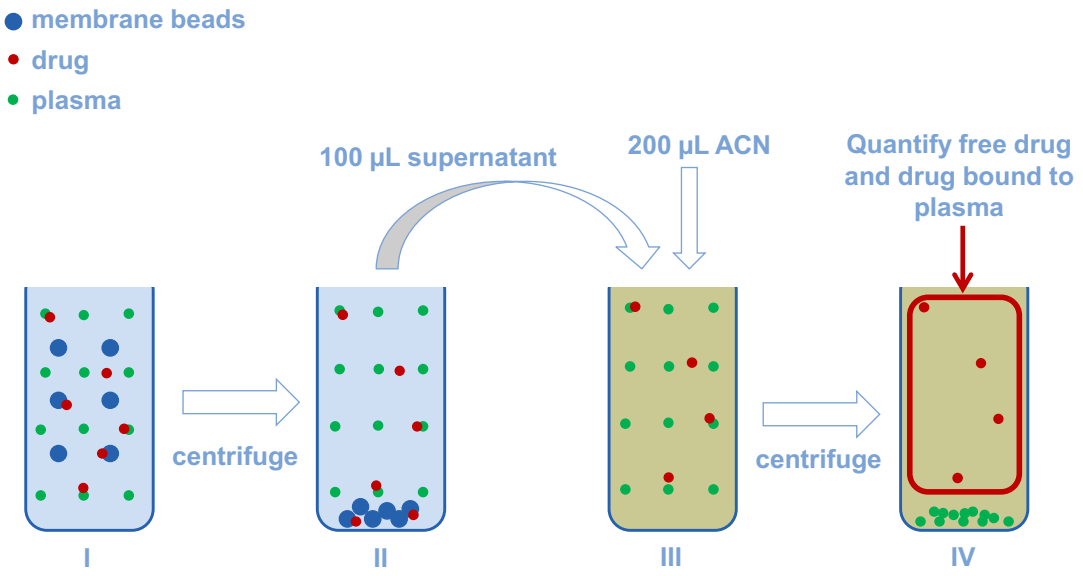


Figure 4: Schematic illustration of the TRANSIL High Sensitivity Binding assay to assess plasma protein binding of sticky or hardly soluble drugs. The assay principle consists of two competitive binding events of the drug candidate to immobilized phosphatidylcholine membranes and to the plasma proteins. The affinity of drugs to plasma is determined by measuring the change of drug bound to the membranes in response to changing the plasma concentration in the assay system. The higher the amount of plasma that is added to this system, the more drug binds to plasma. This reduces the amount of free drug which can equilibrate with the membrane. Hence, less drug will be bound in the membrane phase. After equilibration of the reaction setup (I), TRANSIL membrane beads are captured by centrifugation (II) and the supernatant, containing both free drug and drug bound to plasma proteins, is transferred to a new reaction vial (III). Acetonitrile is then used to precipitate the plasma proteins (III) and the drug concentration is quantified in the supernatant of the centrifugation sample (IV). The concentration of the drug in the supernatant (framed in red) is referred to as APA in the data analysis section.

14.1 Preparation of reagents

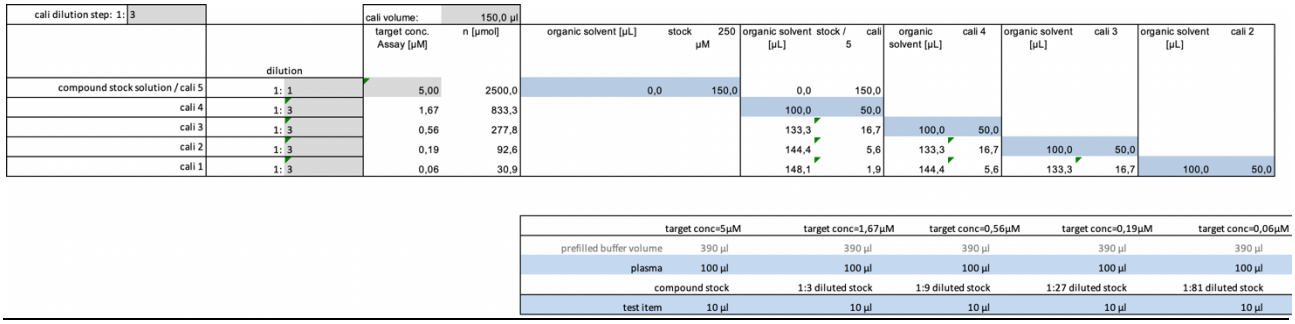
Table 2: Preparation of reagents for the TRANSIL High Sensitivity Binding assay.

No.	Reagent
1	A volume of 100 µl plasma will be added to each assay vial. Therefore, plasma should be pre-diluted to obtain the final desired plasma dilutions. Pre-dilutions need to be prepared as 5x stock solutions.
2	50x sample stock solution.
3	Sample dilution for calibration.

14.2 Calibration

The calibration curve in the TRANSIL High Sensitivity Binding assay is only used to assess apparent recovery. The assay supplies 5 vials for the calibration curve at the highest plasma concentration in the assay. The calibration curve with the highest plasma concentration is used to assess the maximal apparent recovery in the assay. We recommend preparing the calibration curves with 1:3 dilution steps between the different calibration samples. An automatic calculation of the pre-dilutions, being concentrated 50x higher than the final concentrations in the calibration samples vials, is integrated in the “main” tab of the spreadsheet delivered with the Kit (Figure 5).

The spreadsheet also provides an approximation of the absolute recovery based on calculating apparent recovery at the highest plasma concentration (assuming this is close to 100% absolute recovery) and by evaluating the decrease of peak-area to internal standard ratios in reference vials from the highest to the lowest plasma concentration.



cali dilution step: 1:	3		cali volume:	150,0 µl													
		dilution	target conc. Assay [µM]	n [µmol]	organic solvent [µL]	stock µM	250	organic solvent [µL]	stock / 5	cali	organic solvent [µL]	cali 4	organic solvent [µL]	cali 3	organic solvent [µL]	cali 2	
compound stock solution / cali 5	1:	1	5,00	2500,0	0,0	150,0		0,0	150,0								
cali 4	1:	3	1,67	833,3				100,0	50,0								
cali 3	1:	3	0,56	277,8				133,3	16,7		100,0	50,0					
cali 2	1:	3	0,19	92,6				144,4	5,6		133,3	16,7	100,0	50,0			
cali 1	1:	3	0,06	30,9				148,1	1,9		144,4	5,6	133,3	16,7	100,0	50,0	

	target conc=5µM	target conc=1,67µM	target conc=0,56µM	target conc=0,19µM	target conc=0,06µM
prefilled buffer volume	390 µl	390 µl	390 µl	390 µl	390 µl
plasma	100 µl	100 µl	100 µl	100 µl	100 µl
compound stock		1:3 diluted stock	1:9 diluted stock	1:27 diluted stock	1:81 diluted stock
test item	10 µl	10 µl	10 µl	10 µl	10 µl

Figure 5: Screen shot of the table “Dilution for calibration curve” from the “main” tab of the spreadsheet for analyzing data from the TRANSIL High Sensitivity Binding Assay. The table shows calculated volumes (highlighted in blue) for setting up the calibration dilution series of the test compound. All calculations are run automatically after the test compound concentration has been specified in the “main” tab of the spreadsheet. We recommend preparing a dilution series with 1:3 dilution steps starting with the 50x compound stock solution. The example provided here refers to an assay run at a final concentration of 5 µM with a corresponding 50x stock solution of 250 µM. Please note that the volume and dilution ratios in the fields highlighted in grey can be adjusted if other calibration concentrations are used. Vials for the calibration curve are provided with 390 µl buffer. Add 100 µl plasma (using the same dilution as in the highest plasma concentration) and add 10 µl of the test item in the appropriate dilution.

14.3 Plasma Dilutions

Please refer to section 13 on plasma dilutions for details. Prepare 5 different plasma dilutions such that adding 100 µl of plasma stock solution to the prefilled assay volumes of 400 µl result in the desired final dilution.

Add 100 µl of pre-diluted plasma to each of the 7 vials within one of the 5 plasma dilution series.

Add 100 µl of pre-diluted plasma to the 5 vials provided for calibration.

14.4 Compound addition

Add 10 µL sample to each vial (assay and calibration) and vortex for 5 seconds.

14.5 Incubation

Incubate for 30 minutes while rotating vials. Incubation can be performed at room temperature or at 37°C. Temperature has little – if any – effect on dissociation constants and membrane affinity. More importantly, it has no effect on species differences of plasma protein binding. Thus, the assay temperature is a matter of individual choice and preferred convention.

14.6 Separation of beads

Centrifuge vials at 750 g for 10 minutes to remove the TRANSIL beads and transfer 100 µl of the supernatants to new silanized glass vials with the same sample label as the donor vial. Steps 13.7 to 13.9 can be skipped for radiolabelled compounds quantified by scintillation

counting. In that case, supernatants will be analyzed directly after this centrifugation step separating beads and the plasma/buffer phase.

14.7 Internal standard and Plasma precipitation

Add 200 µL of acetonitrile (ratio 2:1; acetonitrile/sample) containing internal standard to each vial and vortex for 5 seconds. The choice of internal standard depends on the compound structure and the ionization state. We recommend a standard structurally similar to the test compound and having essentially the same ionic charge.

14.8 Incubation

Incubate for 10 minutes at room temperature without agitation.

14.9 Precipitation of plasma

Centrifuge for 30 minutes at 3000 g to precipitate the plasma.

14.10 Sampling of supernatant

Sample supernatant for quantification. Vials may be placed directly in an autosampler for LC/MS/MS or HPLV-UV analysis.

15 Sample quantification

Use your analytical technique of choice for quantifying the compound concentration in the supernatant obtained in the last assay step.

Depending upon the compound properties LC-MS/MS measurements can have up to 20% error margin. If high peak area variation is noticed in the internal standard or test compound (as indicated by the reproducibility of the reference peak areas as well as the quality of the calibration curve) we recommend double or triple injections to obtain more robust data.

16 Data analysis

The TRANSIL High Sensitivity Binding Kit comes with a data analysis spreadsheet that calculates free fractions of drug in a given plasma species from the experiment. Open the supplied spreadsheet for data analysis and follow the steps below to obtain the results for the TRANSIL High Sensitivity Binding Kit. Only the fields with light green shading require user input. Cells with light gray shading are optional.

The spreadsheet contains a very large data table for parameter optimization. We therefore strongly advise to turn off automatic recalculation of tables in Excel's options menu. When automatic recalculation is turned off it will be necessary to recalculate the data tables after data entry by pressing <SHIFT F9> on each tab containing a data table. The tabs are marked accordingly.

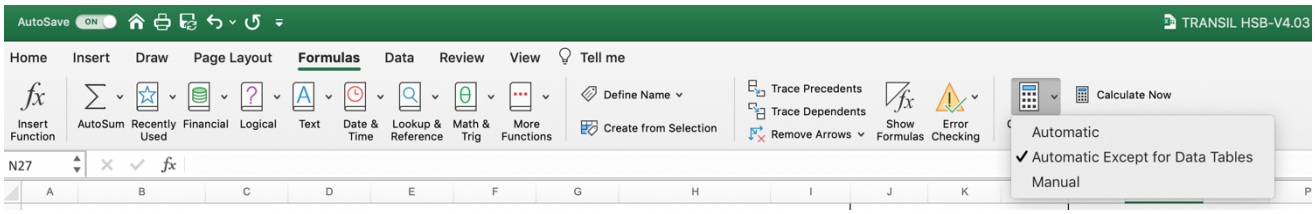


Figure 6: Screen shot of Excel's formula menu. It is best to choose the workbook calculation modus "automatic except for data tables" for the spreadsheet to avoid slowing down of the computer during data entry. To calculate the tables in the "fu_calc" and "95%CI_calc" tabs press the button "calculate Sheet" next to the calculation options button, or press <shift> F9. Remember to change the setting back to "automatic" after finishing your work with the spreadsheet to avoid unexpected results of other tasks performed with MS Excel.

16.1 Presets

The default requirement for good references is that both measurements will not deviate more than 15%. This assumption can be changed by setting the margin in cell L3 in the "main" tab of the spreadsheet to a different value. If the references differ more than this threshold of 15% the spreadsheet uses the highest reference value. However, if the highest reference value is lower than the concentration determined in the TRANSIL well with the lowest membrane surface area, then the spreadsheet discards the reference measurements and selects the first TRANSIL measurement as reference and eliminates this TRANSIL measurement from the calculation of the membrane affinity. When this approach is used, the reported membrane affinity will be higher or equal the true membrane affinity.

16.2 Assay parameters

After opening the spreadsheet navigate to the "main" tab and enter the lot specific parameters from the certificate of analysis that came with the assay kit. Also, enter the lot number and the assay date (Figure 7). All assay kits provided after April 2020 come with a certificate of analysis that specifies the lipid volume in each of the 5 Transil wells. Enter the volumes of Transil well 1 (T1 to well 5 (T5) in cells C7 to G7. You can still use previous kits with this spreadsheet by entering lipid content and γ value in cells L9 and L10 respectively.

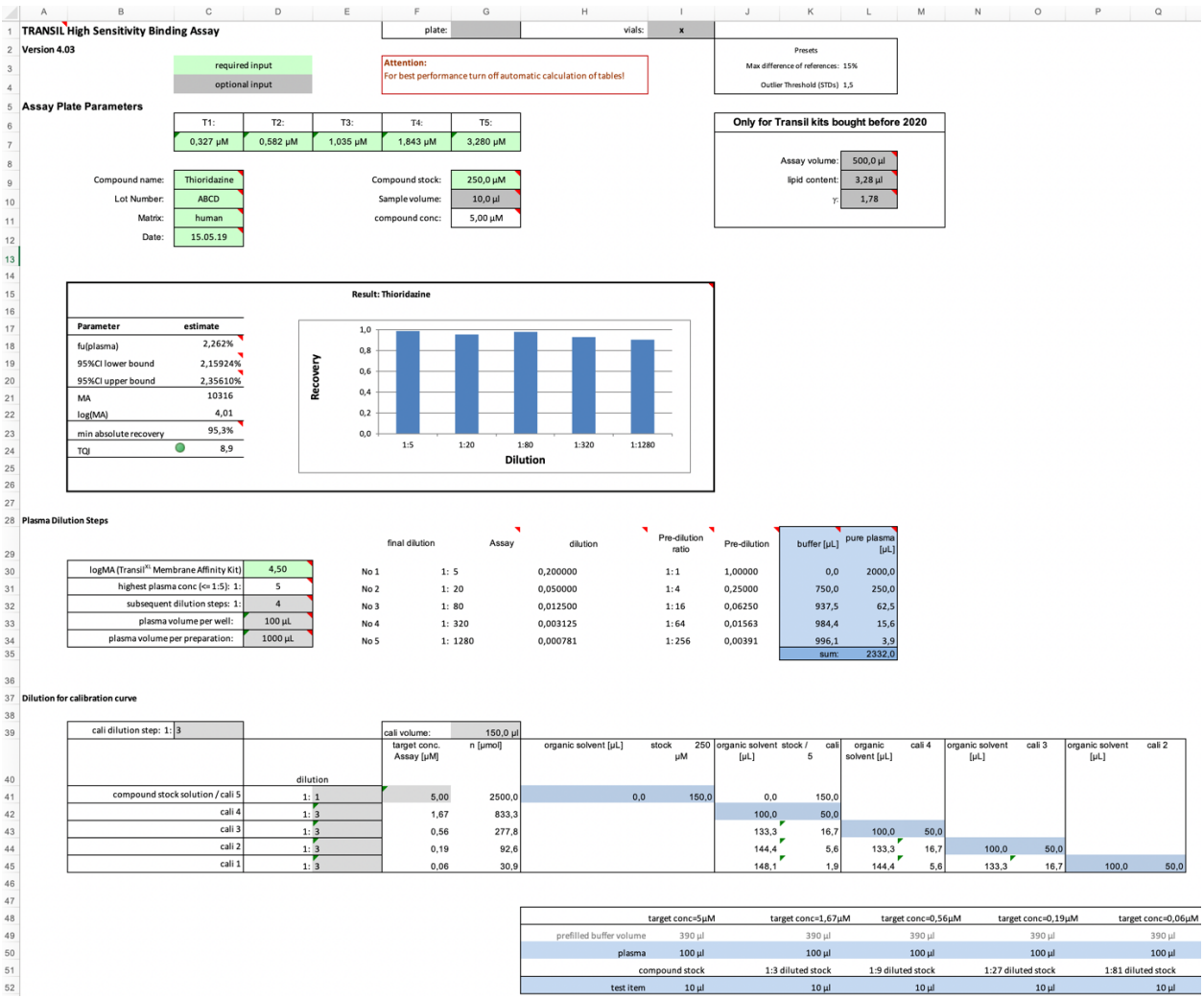


Figure 7: Screen shot of the spreadsheet's main tab where data from the certificate of analysis needs to be entered and the plasma dilution has to be specified. The tab then provides pipetting schemes for the plasma dilution and the calibration curve. The main tab also provides the final results. Note however, that it is necessary to initiate the calculations on the "fu_calc" and "95%Ci_calc" tab.

16.3 Compound information and plasma dilutions

Please enter the name of the compound in cell C9 and enter the pre-determined membrane affinity in field D30 of the "main" tab.

16.4 Raw data from sample quantification

Please enter the raw data from the sample quantification in the "raw data" tab of spreadsheet. The spreadsheet provides sections for each plasma dilution step and fields for entering the peak areas form quantifying the drug as well as from quantifying the internal standard. If no internal standard is used, please enter "1" in all fields requiring internal standard input data. In case of missing values enter "NaN" in the respective field.

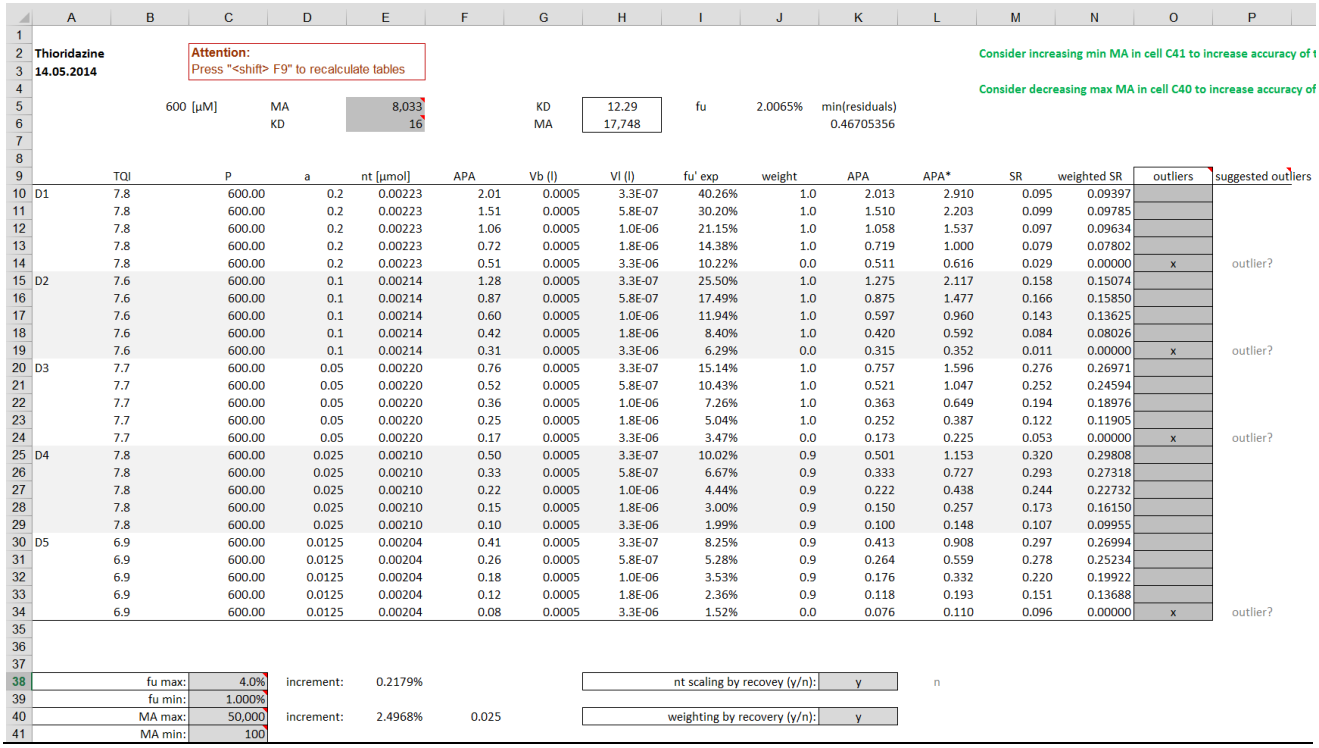
Figure 8: Screen shot of the spreadsheet's "raw data" tab where quantification data need to be entered. The tab provides basic analysis of data quality. A calibration curve is shown that highlights the calibrations' deviation from linearity (the instrument response is fully linear across the concentration range measured when the red dashed line aligns fully with the black regression line). The percentage deviation from linearity is shown in the bar chart below the calibration curve. The coefficient of variation of the internal standards is shown for all plasma dilutions and the calibration (lower left bar chart). And a plot of the scaled peak areas versus the peak areas highlights potential unwanted trends in the data such as instrument drift, that may adversely affect the results. The measurements should be inspected in more detail when the data points for the analyte and/or the internal standard are not clustered together, but have individual points in discrete locations.

16.5 Free fraction calculation

The free fraction of a drug in plasma from one species is calculated using the "fu_calc" tab of the spreadsheet (Figure 9). The spreadsheet performs a global parameter search in the range of free fractions between 0.1% and 30% as well as membrane affinities between 100 and 50,000. This range can be adjusted as needed using the optional input fields C38 to C41. The top 3 rows of the "fu_calc" tab will make recommendation on when and how to adjust the range when necessary.

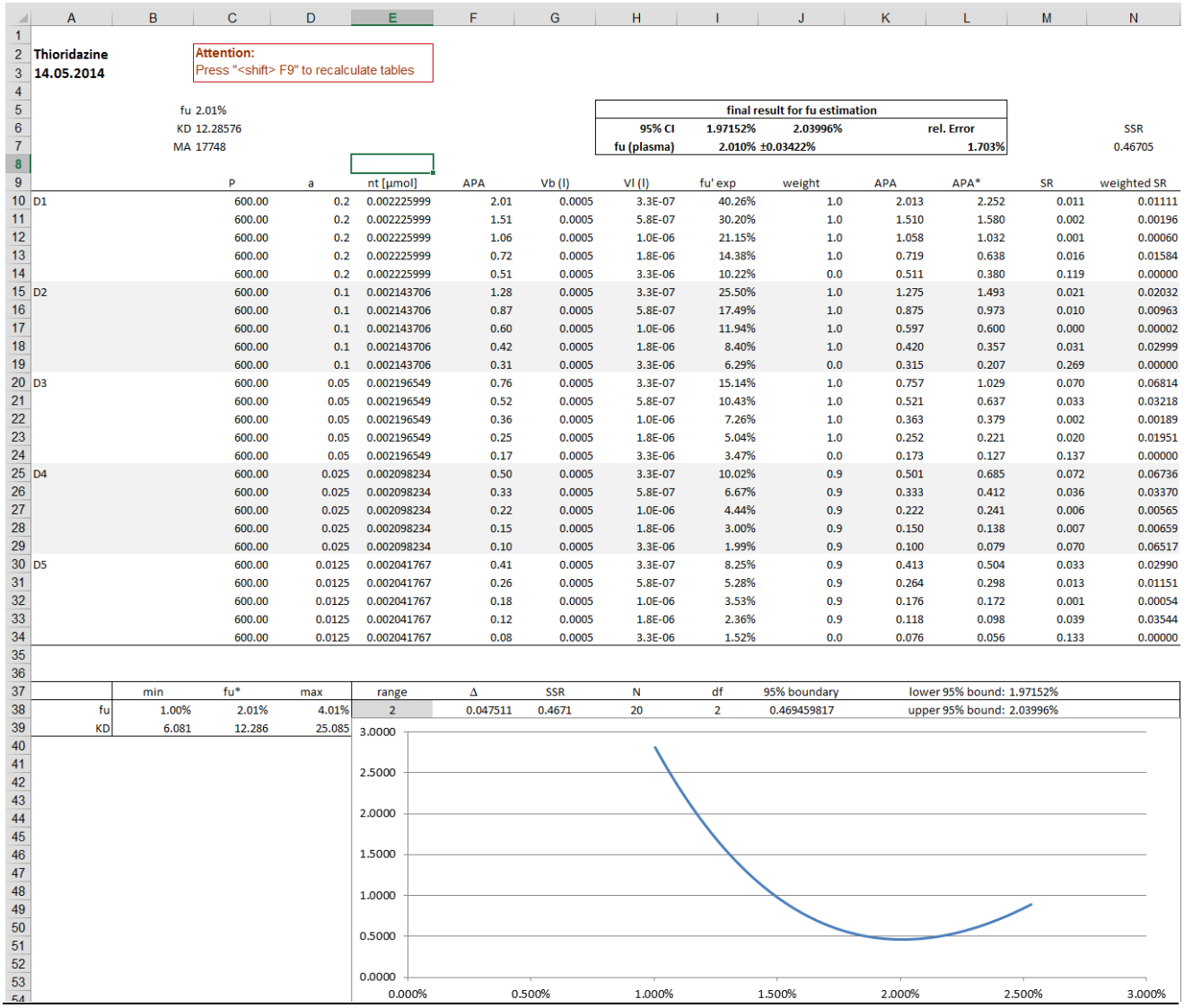
Initiate the fu calculation by pressing <SHIFT F9> on the "fu_calc" tab. Then move on to the "95%CI_calc" tab and press <SHIFT F9> for calculation of the confidence interval of the f_u estimate and the measurement error. Check it any warning messages appear in cells G1 to G3, or if any recommendations regarding the parameter range appear in cells M1 to M4. If such warning messages appear, change the parameters accordingly and recalculate the

sheet. Then move on to the “95%CI_calc” tab and press <SHIFT F9> for calculation of the confidence interval of the f_u estimate and the measurement error (Figure 10).



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
2	**Thioridazine**		**Attention:**										Consider increasing min MA in cell C41 to increase accuracy of t			
3	**14.05.2014**		Press "<shift> F9" to recalculate tables													
4													Consider decreasing max MA in cell C40 to increase accuracy of			
5			600 [µM]	MA	8,033		KD	12.29	fu	2.0065%	min(residuals)					
6				KD	16		MA	17,748			0.46705356					
9		TQI	P	a	nt [µmol]	APA	Vb (l)	Vl (l)	fu' exp	weight	APA	APA*	SR	weighted SR	outliers	suggested outliers
10	D1	7.8	600.00	0.2	0.00223	2.01	0.0005	3.3E-07	40.26%	1.0	2.013	2.910	0.095	0.09397		
11		7.8	600.00	0.2	0.00223	1.51	0.0005	5.8E-07	30.20%	1.0	1.510	2.203	0.099	0.09785		
12		7.8	600.00	0.2	0.00223	1.06	0.0005	1.0E-06	21.15%	1.0	1.058	1.537	0.097	0.09634		
13		7.8	600.00	0.2	0.00223	0.72	0.0005	1.8E-06	14.38%	1.0	0.719	1.000	0.079	0.07802		
14		7.8	600.00	0.2	0.00223	0.51	0.0005	3.3E-06	10.22%	0.0	0.511	0.616	0.029	0.00000	x	outlier?
15	D2	7.6	600.00	0.1	0.00214	1.28	0.0005	3.3E-07	25.50%	1.0	1.275	2.117	0.158	0.15074		
16		7.6	600.00	0.1	0.00214	0.87	0.0005	5.8E-07	17.49%	1.0	0.875	1.477	0.166	0.15850		
17		7.6	600.00	0.1	0.00214	0.60	0.0005	1.0E-06	11.94%	1.0	0.597	0.960	0.143	0.13625		
18		7.6	600.00	0.1	0.00214	0.42	0.0005	1.8E-06	8.40%	1.0	0.420	0.592	0.084	0.08026		
19		7.6	600.00	0.1	0.00214	0.31	0.0005	3.3E-06	6.29%	0.0	0.315	0.352	0.011	0.00000	x	outlier?
20	D3	7.7	600.00	0.05	0.00220	0.76	0.0005	3.3E-07	15.14%	1.0	0.757	1.596	0.276	0.26971		
21		7.7	600.00	0.05	0.00220	0.52	0.0005	5.8E-07	10.43%	1.0	0.521	1.047	0.252	0.24594		
22		7.7	600.00	0.05	0.00220	0.36	0.0005	1.0E-06	7.26%	1.0	0.363	0.649	0.194	0.18976		
23		7.7	600.00	0.05	0.00220	0.25	0.0005	1.8E-06	5.04%	1.0	0.252	0.387	0.122	0.11905		
24		7.7	600.00	0.05	0.00220	0.17	0.0005	3.3E-06	3.47%	0.0	0.173	0.225	0.053	0.00000	x	outlier?
25	D4	7.8	600.00	0.025	0.00210	0.50	0.0005	3.3E-07	10.02%	0.9	0.501	1.153	0.320	0.29808		
26		7.8	600.00	0.025	0.00210	0.33	0.0005	5.8E-07	6.67%	0.9	0.333	0.727	0.293	0.27318		
27		7.8	600.00	0.025	0.00210	0.22	0.0005	1.0E-06	4.44%	0.9	0.222	0.438	0.244	0.22732		
28		7.8	600.00	0.025	0.00210	0.15	0.0005	1.8E-06	3.00%	0.9	0.150	0.257	0.173	0.16150		
29		7.8	600.00	0.025	0.00210	0.10	0.0005	3.3E-06	1.99%	0.9	0.100	0.148	0.107	0.09955		
30	D5	6.9	600.00	0.0125	0.00204	0.41	0.0005	3.3E-07	8.25%	0.9	0.413	0.908	0.297	0.26994		
31		6.9	600.00	0.0125	0.00204	0.26	0.0005	5.8E-07	5.28%	0.9	0.264	0.559	0.278	0.25234		
32		6.9	600.00	0.0125	0.00204	0.18	0.0005	1.0E-06	3.53%	0.9	0.176	0.332	0.220	0.19922		
33		6.9	600.00	0.0125	0.00204	0.12	0.0005	1.8E-06	2.36%	0.9	0.118	0.193	0.151	0.13688		
34		6.9	600.00	0.0125	0.00204	0.08	0.0005	3.3E-06	1.52%	0.0	0.076	0.110	0.096	0.00000	x	outlier?
38		fu max:	4.0%	increment:	0.2179%				nt scaling by recovey (y/n):		y	n				
39		fu min:	1.000%													
40		MA max:	50,000	increment:	2.4968%	0.025			weighting by recovery (y/n):		y					
41		MA min:	100													

Figure 9: Screen shot of the spreadsheet’s “fu_calc” tab. where the numerical solutions are calculated in a large data table below the area shown. The initial conditions for the calculations are set in cells E5 and E6. There is no need to change them as the search is global. The results for the parameter search are shown in cells H5 (KD) and H6 (membrane affinity). The fu that results for the estimated KD is reported in cell J5. The parameter space (range of possible MA and KD values) that is searched for the optimum fit is defined in cells C38 to C41. It may be necessary to change the default values in these fields to obtain a valid result. When the spreadsheet can’t find a result in this parameter range error messages will pop up in fields G1 to G3. The fields M1 to M4 may show recommendations to narrowing the search space, which can result in more accurate final parameter estimates.



Thioridazine
14.05.2014

Attention:
Press "<shift> F9" to recalculate tables

fu 2.01%
KD 12.28576
MA 17748

final result for fu estimation				
95% CI	1.97152%	2.03996%	rel. Error	SSR
fu (plasma)	2.010%	±0.03422%	1.703%	0.46705

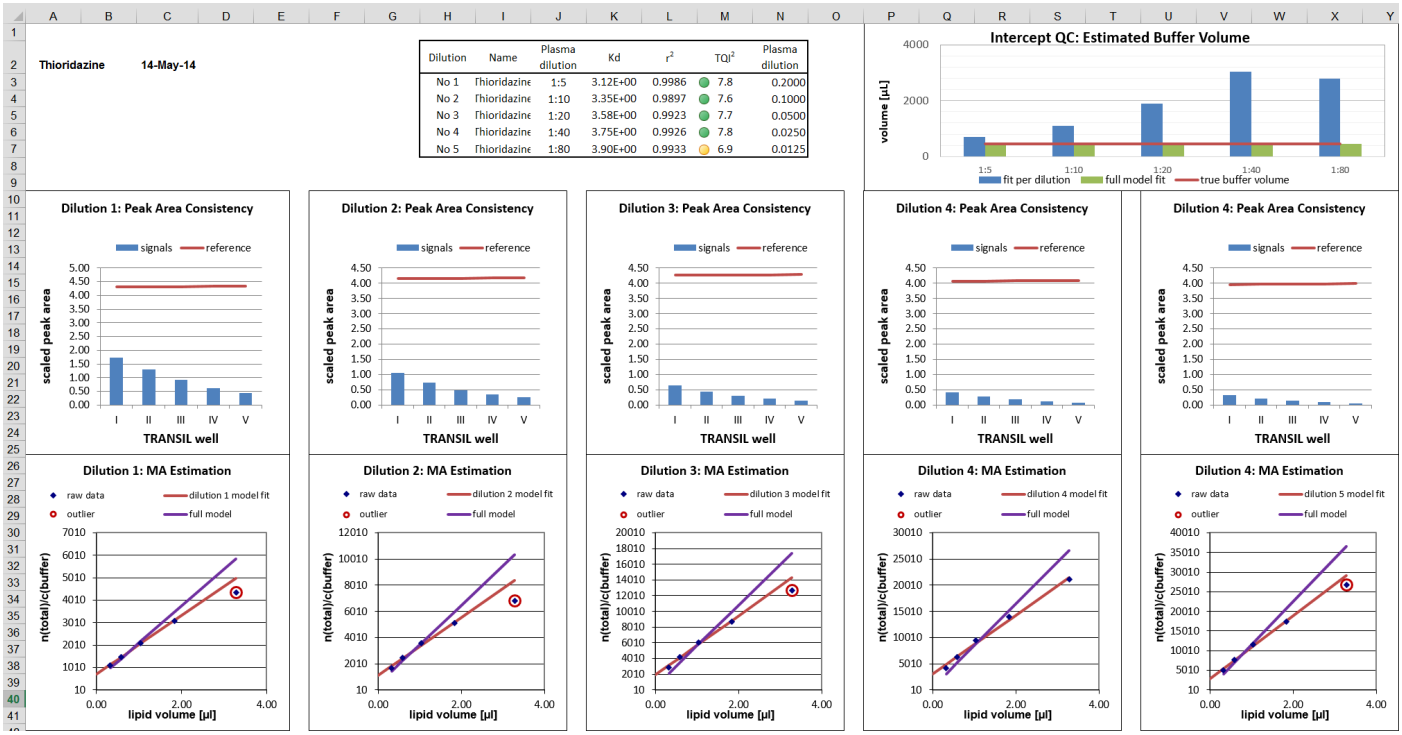
	P	a	nt [µmol]	APA	Vb (l)	Vl (l)	fu' exp	weight	APA	APA*	SR	weighted SR
D1	600.00	0.2	0.002225999	2.01	0.0005	3.3E-07	40.26%	1.0	2.013	2.252	0.011	0.01111
	600.00	0.2	0.002225999	1.51	0.0005	5.8E-07	30.20%	1.0	1.510	1.580	0.002	0.00196
	600.00	0.2	0.002225999	1.06	0.0005	1.0E-06	21.15%	1.0	1.058	1.032	0.001	0.00060
	600.00	0.2	0.002225999	0.72	0.0005	1.8E-06	14.38%	1.0	0.719	0.638	0.016	0.01584
	600.00	0.2	0.002225999	0.51	0.0005	3.3E-06	10.22%	0.0	0.511	0.380	0.119	0.00000
D2	600.00	0.1	0.002143706	1.28	0.0005	3.3E-07	25.50%	1.0	1.275	1.493	0.021	0.02032
	600.00	0.1	0.002143706	0.87	0.0005	5.8E-07	17.49%	1.0	0.875	0.973	0.010	0.00963
	600.00	0.1	0.002143706	0.60	0.0005	1.0E-06	11.94%	1.0	0.597	0.600	0.000	0.00002
	600.00	0.1	0.002143706	0.42	0.0005	1.8E-06	8.40%	1.0	0.420	0.357	0.031	0.02999
	600.00	0.1	0.002143706	0.31	0.0005	3.3E-06	6.29%	0.0	0.315	0.207	0.269	0.00000
D3	600.00	0.05	0.002196549	0.76	0.0005	3.3E-07	15.14%	1.0	0.757	1.029	0.070	0.06814
	600.00	0.05	0.002196549	0.52	0.0005	5.8E-07	10.43%	1.0	0.521	0.637	0.033	0.03218
	600.00	0.05	0.002196549	0.36	0.0005	1.0E-06	7.26%	1.0	0.363	0.379	0.002	0.00189
	600.00	0.05	0.002196549	0.25	0.0005	1.8E-06	5.04%	1.0	0.252	0.221	0.020	0.01951
	600.00	0.05	0.002196549	0.17	0.0005	3.3E-06	3.47%	0.0	0.173	0.127	0.137	0.00000
D4	600.00	0.025	0.002098234	0.50	0.0005	3.3E-07	10.02%	0.9	0.501	0.685	0.072	0.06736
	600.00	0.025	0.002098234	0.33	0.0005	5.8E-07	6.67%	0.9	0.333	0.412	0.036	0.03370
	600.00	0.025	0.002098234	0.22	0.0005	1.0E-06	4.44%	0.9	0.222	0.241	0.006	0.00565
	600.00	0.025	0.002098234	0.15	0.0005	1.8E-06	3.00%	0.9	0.150	0.138	0.007	0.00659
	600.00	0.025	0.002098234	0.10	0.0005	3.3E-06	1.99%	0.9	0.100	0.079	0.070	0.06517
D5	600.00	0.0125	0.002041767	0.41	0.0005	3.3E-07	8.25%	0.9	0.413	0.504	0.033	0.02990
	600.00	0.0125	0.002041767	0.26	0.0005	5.8E-07	5.28%	0.9	0.264	0.298	0.013	0.01151
	600.00	0.0125	0.002041767	0.18	0.0005	1.0E-06	3.53%	0.9	0.176	0.172	0.001	0.00054
	600.00	0.0125	0.002041767	0.12	0.0005	1.8E-06	2.36%	0.9	0.118	0.098	0.039	0.03544
	600.00	0.0125	0.002041767	0.08	0.0005	3.3E-06	1.52%	0.0	0.076	0.056	0.133	0.00000

	min	fu*	max	range	Δ	SSR	N	df	95% boundary	lower 95% bound: 1.97152%
fu	1.00%	2.01%	4.01%	2	0.047511	0.4671	20	2	0.469459817	upper 95% bound: 2.03996%
KD	6.081	12.286	25.085							

Figure 10: Screen shot of the spreadsheet's "95%CI calc" tab. This tab calculates the confidence interval around the plasma protein binding estimate. Occasionally, the pre-determined range within which the spreadsheet searches the confidence interval is not sufficient. In that case an error message pops up in cell H3 and the default value for the range in cell E38 needs to be increased.

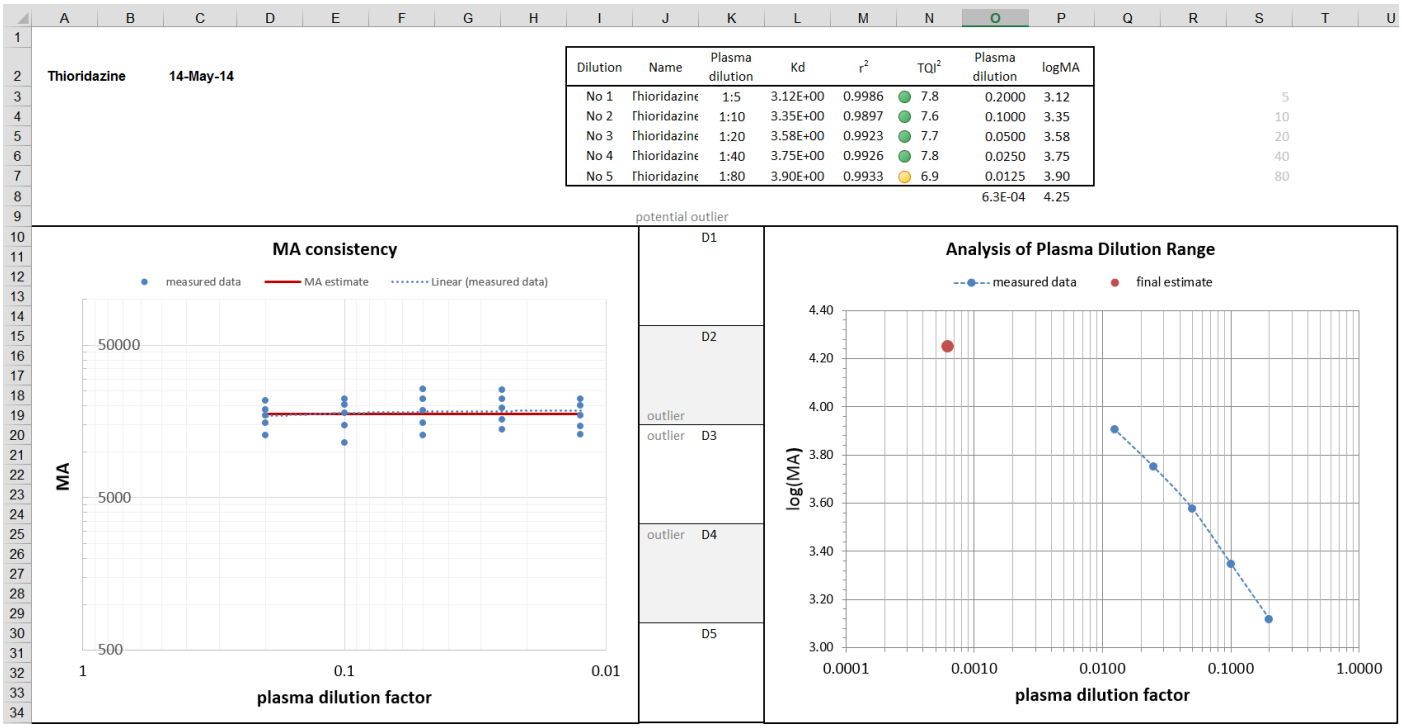
16.6 Final Results

After initiating the calculations on the "fu_calc" and "95%CI_calc" tabs the spreadsheet reports all final results along with the TRANSIL Quality Index (TQI) on the "main" tab. The spreadsheet also contains a "report" tab where the raw data, transformed data, calculated results, spreadsheet settings and error or warning messages are compiled. To assess the validity of the results the tabs "MA_d1", "MA_d2", "MA_d3", MA_d4" and "MA_d5" can be inspected to analyze the data quality of the measurements from each plasma dilution. Those tabs are summarized in the "QC plots 1" tab (Figure 11). The "QC plots 2" provides additional insight into the data quality (Figure 12).



Dilution	Name	Plasma dilution	Kd	r^2	TQI^2	Plasma dilution
No 1	Thioridazine	1:5	3.12E+00	0.9986	7.8	0.2000
No 2	Thioridazine	1:10	3.35E+00	0.9897	7.6	0.1000
No 3	Thioridazine	1:20	3.58E+00	0.9923	7.7	0.0500
No 4	Thioridazine	1:40	3.75E+00	0.9926	7.8	0.0250
No 5	Thioridazine	1:80	3.90E+00	0.9933	6.9	0.0125

Figure 11: Screen shot of the spreadsheet's "QC plots 1" tab. This tab summarizes the graphs from the separate analysis of each individual plasma dilution. The bar chart in the top right corner compare the intercept estimates in each plasma dilution with the expected value (=buffer volume of the assay).



Dilution	Name	Plasma dilution	Kd	r^2	TQI^2	Plasma dilution	logMA
No 1	Thioridazine	1:5	3.12E+00	0.9986	7.8	0.2000	3.12
No 2	Thioridazine	1:10	3.35E+00	0.9897	7.6	0.1000	3.35
No 3	Thioridazine	1:20	3.58E+00	0.9923	7.7	0.0500	3.58
No 4	Thioridazine	1:40	3.75E+00	0.9926	7.8	0.0250	3.75
No 5	Thioridazine	1:80	3.90E+00	0.9933	6.9	0.0125	3.90
						6.3E-04	4.25

Figure 12: Screen shot of the spreadsheet's "QC plots 2" tab. This tab shows the consistency of the membrane affinity estimates across all 25 assay wells (left graph). Ideally the final estimate (red line) aligns fully with the regression line (dotted blue line). Also, make sure the data variability is heterogeneous across all plasma dilutions. The right plot shows how the membrane affinity estimate converges from high to low concentrated plasma towards the full model estimate. The data may be compromised if this is not a clear conversion towards the final estimate.

16.6.1 Detailed measurement results –membrane affinity

Detailed measurement results can be found for each drug can be found on the spreadsheet's tabs "MA_dX" with the index from 1 to 5 for each respective dilution step. Figure 13 illustrates the information reported on each individual drug tested.

The membrane affinity is a partitioning coefficient of drug between membrane and buffer. It is defined as the concentration of drug in membrane over the concentration of drug in buffer:

$$MA = \frac{c_l}{c_b}. \qquad \text{Equation 3}$$

The membrane affinity is calculated from the assay data using the mass balance equation:

$$n_t = c_b \cdot V_b + c_l \cdot V_l \qquad \text{Equation 4}$$

which is rearranged such that the membrane affinity can be determined from the slope of plotting the ratio of total amount of drug (n_t) over remaining concentration in supernatant (c_b) against the lipid membrane volume present in each well:

$$\frac{n_t}{c_b} = \frac{c_l}{c_b} \cdot V_l + V_b = MA \cdot V_l + V_b \qquad \text{Equation 5}$$

The results for the membrane affinity are reported in the individual tabs "MA_dX" along with the TRANSIL Quality Index (TQI).

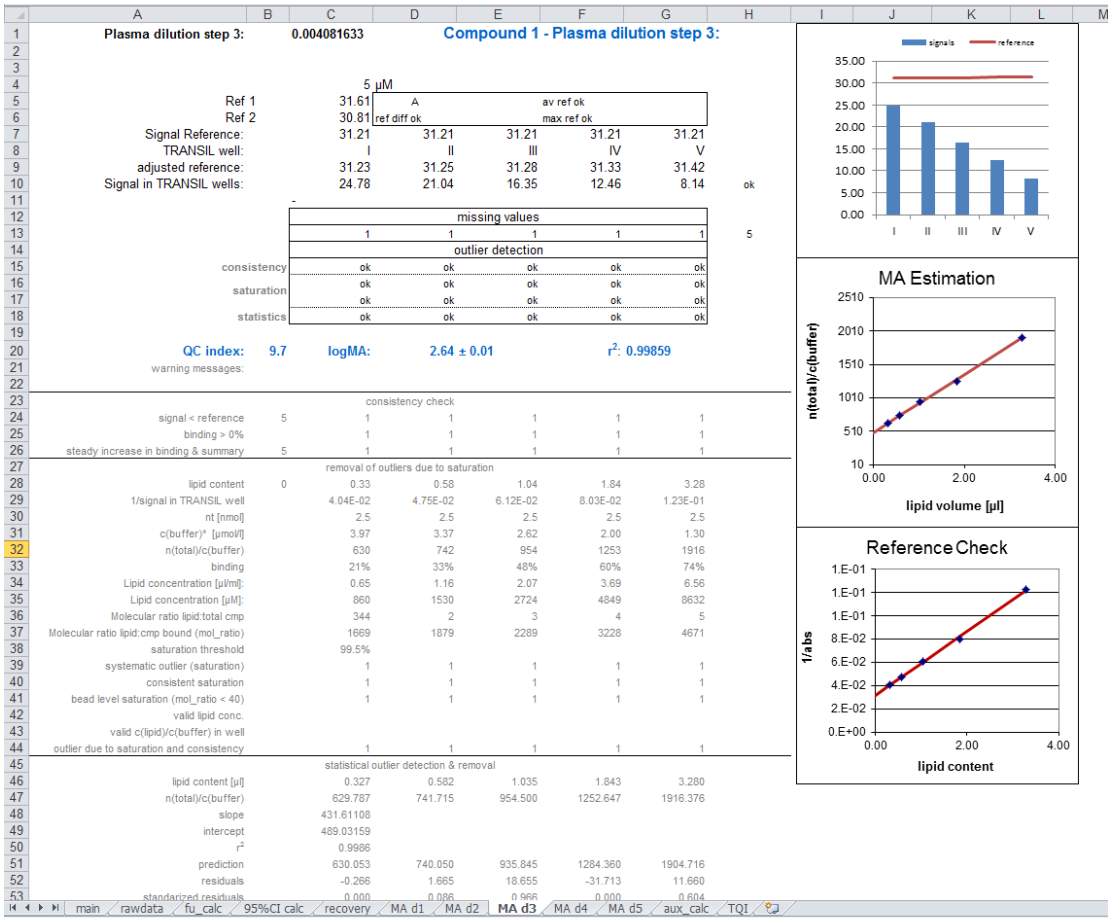


Figure 13: Screen shot of the "MA_dx" tab of the spreadsheet for analyzing data from the TRANSIL High Sensitivity Binding Assay. The "MA_dX" tab shows calculated concentrations in each well and all calculations performed to derive the affinity to membranes as well as three plots indicating the experiment performance. Calculations and outlier detections performed on these tabs have no influence on the final data analysis. These tabs serve mainly the purpose of visual data quality inspection.

16.6.1.1 TRANSIL Quality Index for Membrane Affinity

The TRANSIL Quality Index (TQI) is based on five independent measures derived from the data analysis. For each individual measure a partial quality score on a scale between 0 and 10 is attributed to the estimate. 0 represents lowest quality, while 10 represents highest quality. The final quality index is a weighted average of the partial quality scores.

16.6.1.1.1 Model fit

The membrane affinity is calculated by fitting the experimental data to the rearranged mass balance equation:

$$\frac{n_t}{c_b} = MA \cdot V_l + V_b \qquad \text{Equation 6}$$

where denotes the membrane affinity. Fitting optimal data to Equation 6 will yield a slope that exactly represents the true membrane affinity, MA, and the buffer volume used in the experiment. In fact, a biased estimation of the slope will typically result in a biased estimation of the intercept as well. Since the intercept equals the buffer volume used in the experiment, the estimated intercept is used as a quality control parameter. If the estimated buffer volume is within an interval ±10% around the true value a partial quality score of 10 is attributed. If the estimated buffer volume is within an interval ±50% around the true value a partial quality score of 5 is attributed.

The partial quality score for the model fit has a weight of 3 in the total quality index.

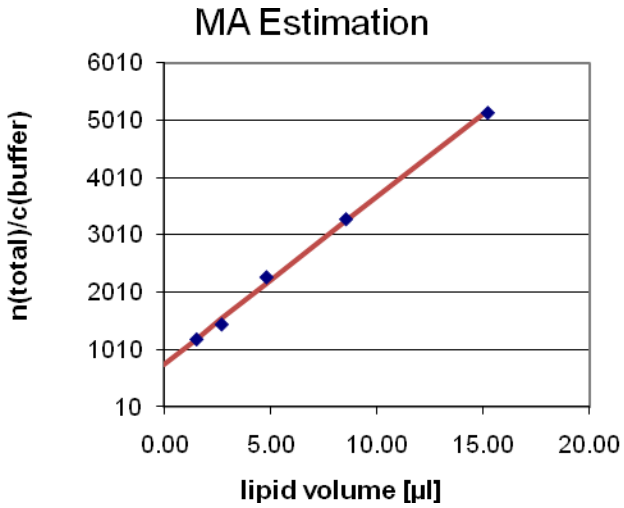


Figure 14: Illustration of fitting experimental data to Equation 6 to determine the membrane affinity. A buffer volume of 500 µL has been used in the experiment, the intercept was estimated to 747 µL, hence a quality score of 5 was attributed to the model fit.

16.6.1.1.2 Match of measured versus predicted reference signal

When determining the membrane affinity via 5 different lipid volumes using TRANSIL beads along with 2 reference estimates without TRANSIL beads, the expected peak area resulting from quantification of the references can be calculated from the peak areas from the TRANSIL wells by linear regression, since lipid binding can be assumed to be a non-cooperative process (Figure 15). This score has a weight of 3 in the TQI.

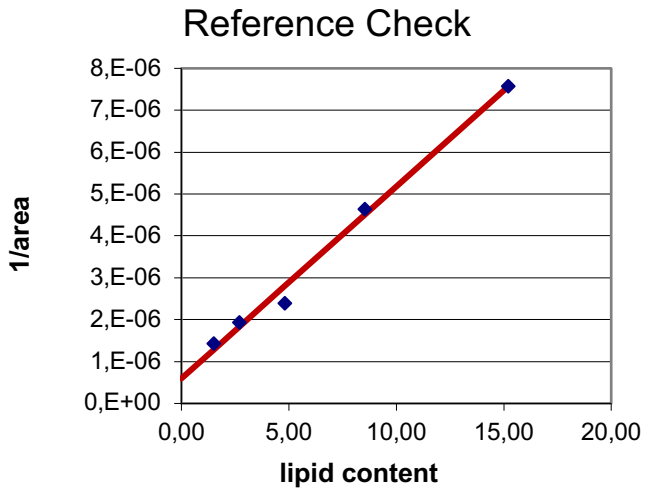


Figure 15: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the lipid content. The inverse of the intercept represents the expected peak area of the references.

A deviation of the measured from the expected reference peak area can be due to a non-linear calibration curve or unspecific binding, which will be more pronounced in the references without the lipid phase of the TRANSIL beads than in the TRANSIL wells.

Table 3 lists the partial quality scores for deviations of the reference peak areas from expected reference peak areas.

Table 3: Partial quality scores for deviations of the reference peak areas from expected reference peak areas.

Deviation	Score
10.0%	10
20.0%	9
50.0%	8
100.0%	7
200.0%	5
500.0%	3
>500.0%	0

16.6.1.1.3 Correlation coefficient

The correlation coefficient from fitting the experimental data to Equation 6 also contributes as a partial quality score (Table 4). This score has a weight of 3 in the TQI.

Table 4: Partial quality scores for the least square model fit of the experimental data to Equation 6.

r2	Score
0.9999	10
0.999	9
0.99	8
0.9	7
0.8	6
0.7	5
0.6	4
0.5	3
<0.5	0

16.6.1.1.4 Number of outliers or missing data

The number of data points used to calculate the membrane affinity is also used as partial quality score (Table 5). This score has a weight of 2 in the TQI.

Table 5: Partial quality scores for the number of data points used in the model fit of the experimental data to Equation 6.

Data points	Score
5	10
4	9
3	6
2	1
1	0

16.6.1.1.5 Data consistency

With increasing lipid volume, i.e. increasing lipid membrane surface, the binding the test items to the membrane should increase proportionally. At least the binding should increase with increasing lipid volume. If the measured peak area suggests decreased binding compared to binding in the TRANSIL well with the next lower lipid volume, then this data point is considered to be inconsistent with the fundamental assumption about lipid binding. If this happens for more than one consecutive TRANSIL well, the data point will be excluded from the calculation. Irrespective of inclusion or exclusion, a partial quality score will be attributed to the data set based on consistency according to Table 6. This score has a weight of only 1 in the TQI as it may affect also the number or data points.

Table 6: Partial quality scores for the number of consitent data points used in the model fit of the experimental data to Equation 6.

No. of consistent data points	Score
5	10
4	5
3	2
2	0

16.6.2 Recovery

The apparent recovery is determined from the calibration curve at the highest plasma concentration. Alternatively, an approximation of the absolute recovery is calculated based on the assumption that the apparent recovery at the highest plasma concentration in the assay is close to 100% absolute recovery. The decrease in absolute recovery due to further plasma dilution is then measured by assessing the change in peak-area to internal standard ratios in the references in each further plasma dilution step. The resulting scaled recoveries are averaged using mean weights from the f_u's in the experiment (see weighting approach applied in section 4) to obtain the final weighted absolute recovery.

16.6.3 Total TRANSIL Quality Index

The TRANSIL Quality Index for the free fraction estimation of one individual plasma species depends upon the average of the five quality indices of the membrane affinity estimates based on the plasma dilution series, recovery as well as on the model fit of the fu estimation. All different parameters contribute to the total score with a weight of 1.

The contribution of the final weighted absolute recovery to the TQI calculation is summarized by Table 7.

Table 7: Partial quality scores for the final weighted absolute recovery.

Apparent recovery	Score
>=90%	10
>=80%	9
>=70%	8
>=60%	7
>=55%	6
>=50%	3
>=30%	1
<30%	0

17 Storage and shelf life

The assay kits are shipped in a frozen state and should be stored at -20 °C. TRANSIL materials are stable for several months when stored as recommended. Once thawed and at room temperature, the kit should be used within 24 h.

18 Trouble shooting

18.1 Recovery

18.1.1 Challenges and problem identification

Absolute recovery is estimated as described in section 15.6.2. Recovery in all 5 plasma dilutions is shown graphically in the “main” tab of the spreadsheet within the result window. Figure 11 displays typical situations that may occur in the assay. Increasing recovery with decreasing plasma concentration suggests instability of the test compound in plasma (e.g. metabolism by esterases in plasma). While plasma instability issues are rare due to the short assay time of only 30 minutes, solubility issues may be observed more frequently even though the assay is optimized for lipophilic compounds. Decreasing solubility is indicated by lower recoveries towards lower plasma concentrations. Increased unspecific binding as a result of lower plasma concentrations may lead to similar recovery pattern, however, the assay is designed to minimize these effects. The model fit and thus the final reported results may be affected by impaired recovery once the observed approximated absolute recovery is 70% and lower in more than one plasma.

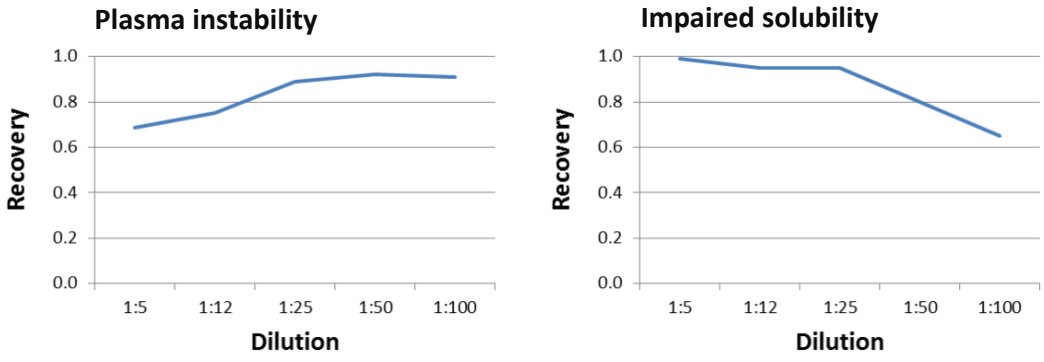


Figure 16: Illustration of the recovery curve shapes that can observed in the TRANSIL High Sensitivity Binding assay. While increasing recovery indicates certain instability of the test compound in plasma, decreasing recovery can visualize impaired solubility and other instabilities.

18.1.2 Problem-solving approaches

i. Sovicell support team can assist you in checking the plausibility of the data if recovery problems are observed. In any case, for optimization of the assay parameters it will be helpful to know the solubility of the test compounds in pure buffer solutions.

ii. A separate vial of the test compound at the same final concentration as used in the assay in pure plasma or pure solvent as matrix can be included in the assay to detect the extend of recovery reduction. This control helps in particular when low recovery is observed even in the highest plasma concentration. Please note that comparing the absolute peak areas should be done with caution because of matrix effects.

iii. If only the two lowest plasma dilutions are affected by suboptimal recovery, plasma dilution steps can be reduced from 1:4 to 1:3 to maintain higher plasma concentrations even in the lowest plasma dilution steps.

iv. DMSO content can be increased. The assay tolerates up to 10% DMSO. This requires the addition of a higher volume of test compound increasing the total assay volume. Please contact our support team how to adapt the spreadsheet accordingly.

v. Test compound concentration can be reduced, however, it has to be considered that running the assay with lower compound concentrations increases the likelihood of running out of the linear range of the instruments (c.f. section 10).

Before repeating the whole assay you may check the success of recommendations given in iii. to v. by setting up an individual small control experiment. It is recommended to use the same assay buffer and vials to ensure comparability. Please contact Sovicell to receive vials with assay buffer at appropriate volumes.

18.2 General data quality

18.2.1 Challenges and problem identification

The TRANSIL High Sensitivity Binding assay contains several internal quality controls (Figure 12). The variation coefficient of the internal standard is calculated for each plasma dilution series within the raw data tab. Variation coefficients of high quality data will generally be smaller than 10%. In addition, the duplicate measurements of references of plasma dilution 2 to 4 and triplicate measurements of the first plasma dilution (2 references and the

corresponding data point from the calibration) should ideally produce the same values. Significant variations can indicate analytical variations or stability problems.

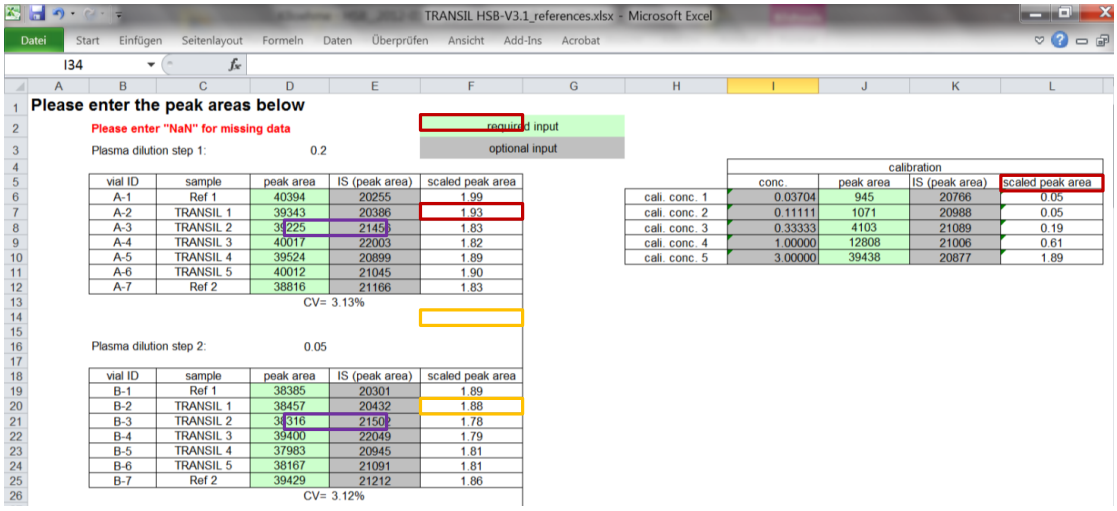


Figure 17: The replicate measurements of the references as well as the correlation coefficient of the internal standard serve as internal quality controls. The triplicate determination at the highest plasma concentration is shown in red (two references and the calibration point 5). The references at plasma dilution 2 to 4 are measured in duplicates (orange). The coefficient of variation is calculated for each individual plasma dilution and framed in purple.

18.2.2 Problem-solving approaches

Multiple injections can reduce the analytical variations. Adjusting test compound concentration may also help to improve measurement window and increase data robustness. Check for other problems like recovery which are generally associated with inconsistencies of the quality controls described in this section.

18.3 Quality control by graphic data analysis

18.3.1 Challenges and problem identification

The spreadsheet contains a graphic analysis based on the apparent membrane affinities calculated for each individual plasma dilution series. The inverse of apparent membrane affinities correlates linearly with the plasma concentration (Figure 13).

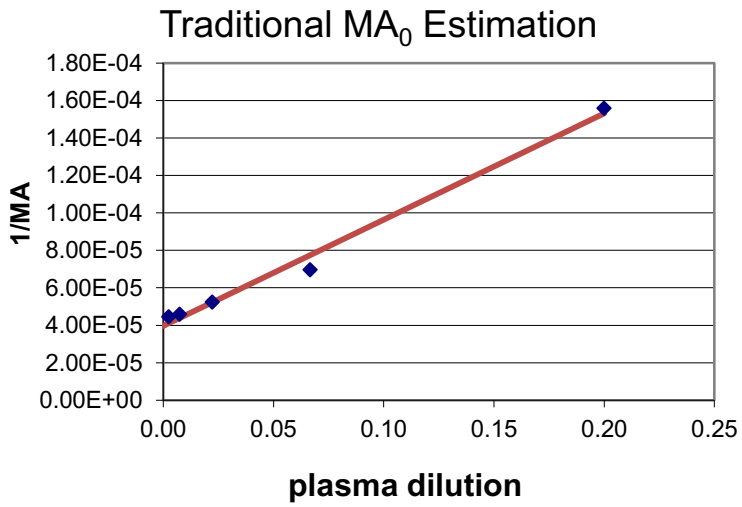


Figure 18: Graphical analysis of the TRANSIL High Sensitivity Binding assay. Plasma dilution correlates linearly with the inverse of apparent membrane affinities calculated at each plasma dilution. The intercept provides inverse of the true membrane affinity in pure buffer. The latter corresponds to the membrane affinity provided as finals assay result using the mathematical fitting approach and serves as additional quality control parameter.

18.3.2 Problem-solving approaches

Significant deviations from linearity can be caused by several independent issues. Outliers due to underestimated membrane affinities (overestimated 1/MA values) may occur at high plasma dilutions and are regularly associated with reduced recovery (see section 17.1). Poor regression fits may further be caused by analytical challenges (see section 17.4).

18.4 Non-linearity of the response

18.4.1 Challenges and problem identification

Non-linear responses as described in section 3 may occur in the assay. The “Calibration curve” plot (Figure 14) in the raw data tab of the spreadsheet as well as the “MA estimation” plots within the individual data analysis tabs MAd1 to Mad5 (Figure 15) can visualize such issues.

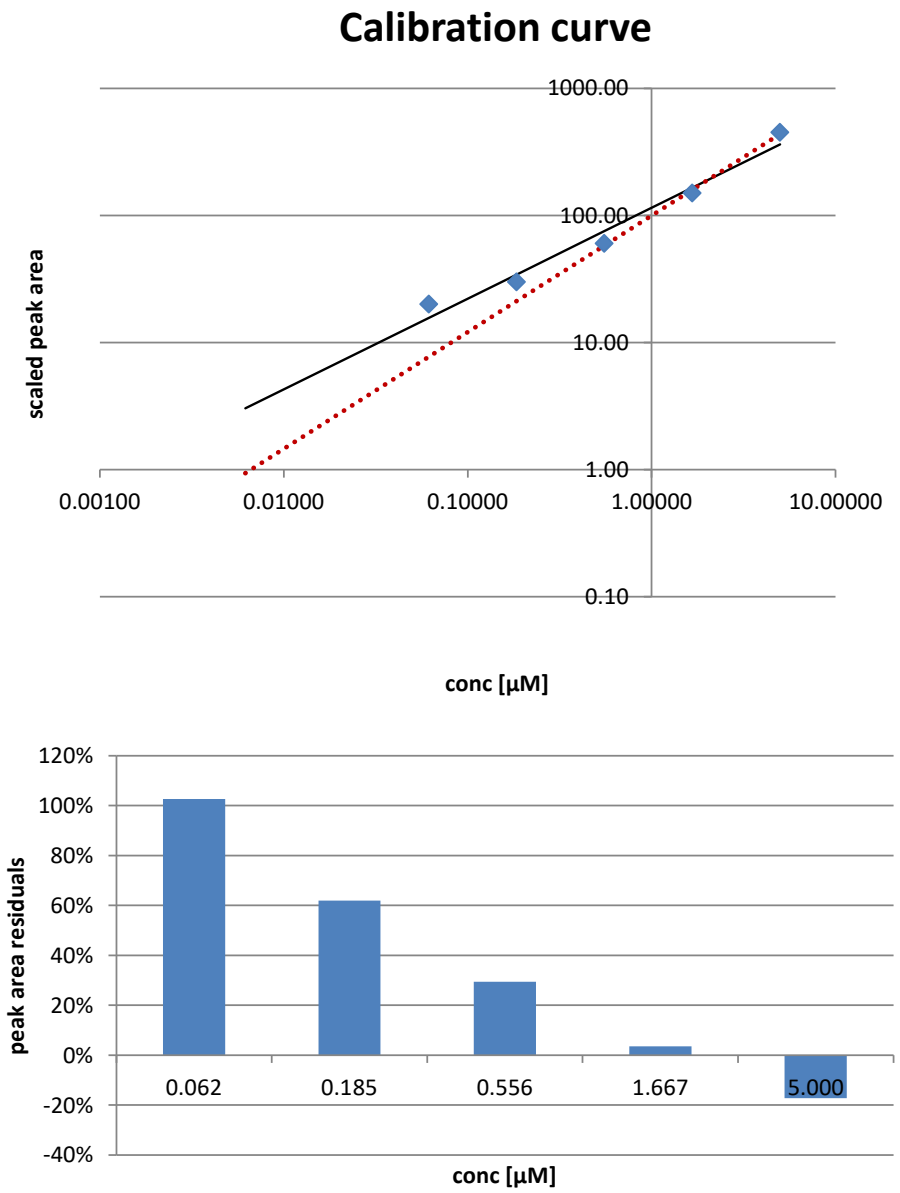


Figure 19: The example of the calibration curve plot on the raw data tab (upper panel) illustrates the non-linear response problem at low test compound concentrations. The dotted red line represents the expected ideal regression line (assuming that the three highest concentrations exhibit a linear concentration- peak area response), while the actual regression fitted to the whole data set is shown in black. The deviations of the regressions are visualized in the bar plot (lower panel) showing the peak area residuals in percent for the five calibration samples. Deviations exceeding 40% provide some indication for the occurrence of non-linearity phenomena in the analytical response.

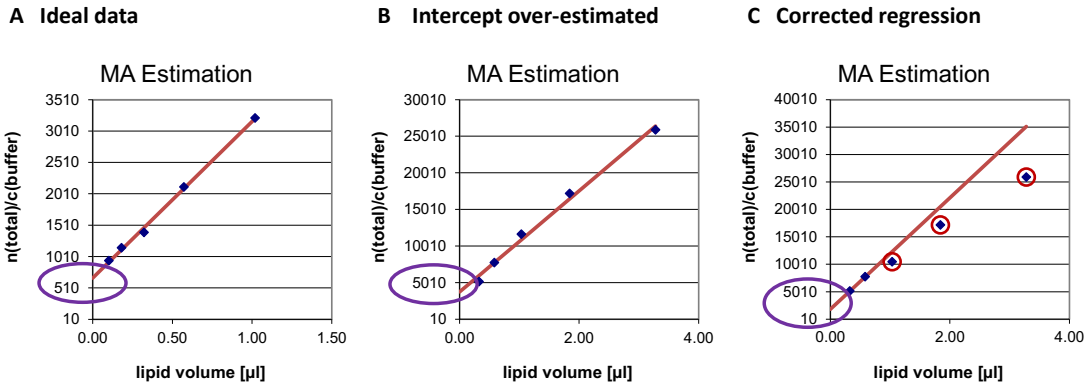


Figure 20: "MA estimation" plots of the individual data analysis tabs MAd1 to Mad5 of the spreadsheet. The intercept of the regression which represents the assay buffer volume of 500µl as well as the curve shape are good indicators for non-linear response problems. **A** Ideal data with an intercept of 595 µl. **B** Strongly over-estimated intercept of 3992 µl. **C** Data points from wells with high lipid contents (data points at low analytical concentrations) which run into the non-linear response were removed from figure B resulting in the regression line shown in C here. Removing the affected data points underlines the non-linear curve shape which is hardly visible in figure B.

18.4.2 Problem-solving approaches

Adapting the fu weights of the model fit (c.f. section 3) or increasing the test compound concentration helps to circumvent such problems. Alternatively, increasing plasma content in the assay will also increase supernatant concentrations and help to solve the problems. The latter approach is generally only necessary for special test compounds which are highly challenging from the analytical point of view. Custom assay designs are recommended for this type of test compounds. Please contact Sovicell support for assistance.

19 References

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