User Guide

TRANSIL

Microsomal Binding Kit

TMP-0120-2096

Version 3, Revision 03

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Contents

1 Quick Protocol

1. Plate Thawing and preparation

- Thaw plate or individual tube units for 3h at room temperature (alternatively overnight).
- Spin plate quickly for 5 seconds at 750 g.
- Make sure the plate has a working temperature between 20°C and 25°C when starting the experiment.
- Leave caps closed while preparing the test compound.

2. Drug Candidate Preparation

- Prepare 16x stock solutions of each compound in 32% solvent (e.g. DMSO) - yields a final solvent conc. of 2%.
- 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 80 µM stock solutions.
- Since each compound is added in an aliquot of 15 µl to each well of an 8-well tube unit, at least 120 µl stock solution are required for each compound. Allow an additional 80 µl for accurate pipetting.

3. Drug Candidate Addition

- Open wells with supplied decapper.
- Mix the stock solutions carefully.
- Transfer 15 µl of the 16x stock solution to a column of 8 wells of the TRANSIL assay plate proceeding column by column. Change tips after each transfer step to avoid carryover of beads.
- Close tube wells and make sure that the capband is oriented in the same direction as before.

4. Incubation and Supernatant Sampling

- Incubate the plates on a plate shaker at 1000 rpm for 12 minutes.
- Spin the plate in a swing-out centrifuge for 10 minutes at 750 g.
- Transfer 50 – 100 µl supernatant in a standard 96 well plate for analytical quantification. Make sure that no beads are carried along.

5. 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 complex, costly, and often uncertain outcome of the drug discovery and development process requires the simultaneous optimization of several properties. It has now long been recognized that favorable potency and selectivity characteristics are not the sole hallmarks of a successful drug discovery program, nor is the safety profile considered to be the only hurdle to be overcome, although it is of paramount importance.

The ability to prospectively predict the pharmacokinetics of new chemical entities in humans is a powerful means by which scientists involved in the discovery of new drugs can select for further development only those compounds with the potential to be successful therapeutic agents.

The half-life of a drug is a major contributor to the dosing regimen, and it is a function of the clearance and apparent volume of distribution (VD), each of which can be predicted and combined to predict the half-life. Drugs with short half-lives are more likely to be required to be administered more frequently than those with long half-lives. Dosing regimen is also intrinsically linked to other factors such as the pharmacodynamics of the drug and the difference between systemic concentrations associated with side effects vs those minimally required for efficacy. However, these latter attributes are much more difficult to predict from in vitro or animal data and will be different for each therapeutic target. Thus, a great deal of focus has been placed on the prediction of human half-life. While methods using allometric scaling or correlative methods exist for prediction of half-life, greater success is attained if the two major components of half-life, clearance and volume of distribution (note that the TRANSIL Intestinal Absorption Kit is well suited for VD predictions), are predicted separately and combined to generate a half-life prediction (Obach et al 1997).

When estimating intrinsic clearance in metabolic stability incubations with microsomes or hepatocytes, the disappearance of the parent compound is measured over a time course. The clearance rate derived from these experiments is only reflecting the intrinsic clearance rate, if the test compound is available to interact freely with the CYP enzymes at the assumed target concentration. However, this is frequently not the case, because compounds tend to bind quantitatively to microsomal membranes, which can significantly reduce the available concentration – even to 1/100 or less of the total concentration in the incubation (Obach, 1999; McLure et al., 2000; Austin et al., 2002; Hallifax and Houston, 2006). Consequently, it is important to estimate microsomal binding in these in vitro incubations. Often, the binding to microsomal membranes is estimated as the free fraction of drug in incubations of microsomes (without cofactor) in dialysis rigs. As equilibration of microsomal binding in dialysis systems tends to require overnight incubations and may suffer more than other experiments from compound instability through hydrolysis and precipitation of poorly soluble drugs. Moreover, the experimentally determined free fraction relates to the experimental concentration of microsomes which will in many cases differ from the incubations of metabolic stability experiments.

Microsomal binding not only reduces the concentration of free drug available to be metabolised by CYP enzymes, it also reduces the concentration which is available to inhibit the enzymes. It has been demonstrated that non-specific microsomal binding can account for underestimation of inhibitor potency (i.e., overestimation of IC50 or Ki values) when dealing with lipophilic basic drugs. This in turn can lead to an underestimation of risk of drug-drug interactions. In particular, mechanism based inhibitor studies can be affected to a large extent by microsomal binding, because of the high microsome concentrations that a typically employed in these experiments. Hence, the fraction of drug bound to microsomes is also an important correction of experiments assessing the inhibition potential.

Applications of TRANSIL Microsomal Binding Assay

The TRANSIL Microsomal Binding assay assesses the affinity of drugs to microsomal membranes. This affinity is defined as the distribution coefficient between drug in the lipid phase versus drug in the buffer phase. This distribution coefficient is a physical chemistry constant of each drug and thus allows great flexibility in calculating the free fractions of drug in any experimental microsome incubation and can be used to estimate the intracellular in vivo free fraction of drugs in the liver, when the concentration of membrane can be determined. Besides the versatility of the measured unit of microsomal binding the assay is very fast, requires only 12 minutes incubation, and can be fully automated.

3 Basic assay principle

The principle of the TRANSIL Microsomal Binding assay is to assess the affinity of test compounds to microsomal membranes (Figure 1). The membrane affinity is determined by incubating a fixed concentration of the drug candidate with varying concentrations of membrane surface area immobilized on the silica beads. A total of 8 wells of a tube unit/plate are used to determine the microsomal membrane affinity for each compound (Figure 2). Six wells contain microsomal membrane silica beads while two serve as references to account for non-specific binding and contain buffer only. Using the spreadsheet and algorithms supplied with the assay, the affinity to the microsomal membranes is calculated from remaining free compound concentration in the supernatant of each well with membrane beads. Any of the available detection systems, such as HPLC-UV, LC-MS/MS, scintillation counting, etc. can be used for quantification, as long as it can quantify µM concentrations in volumes of 50 µl or less.

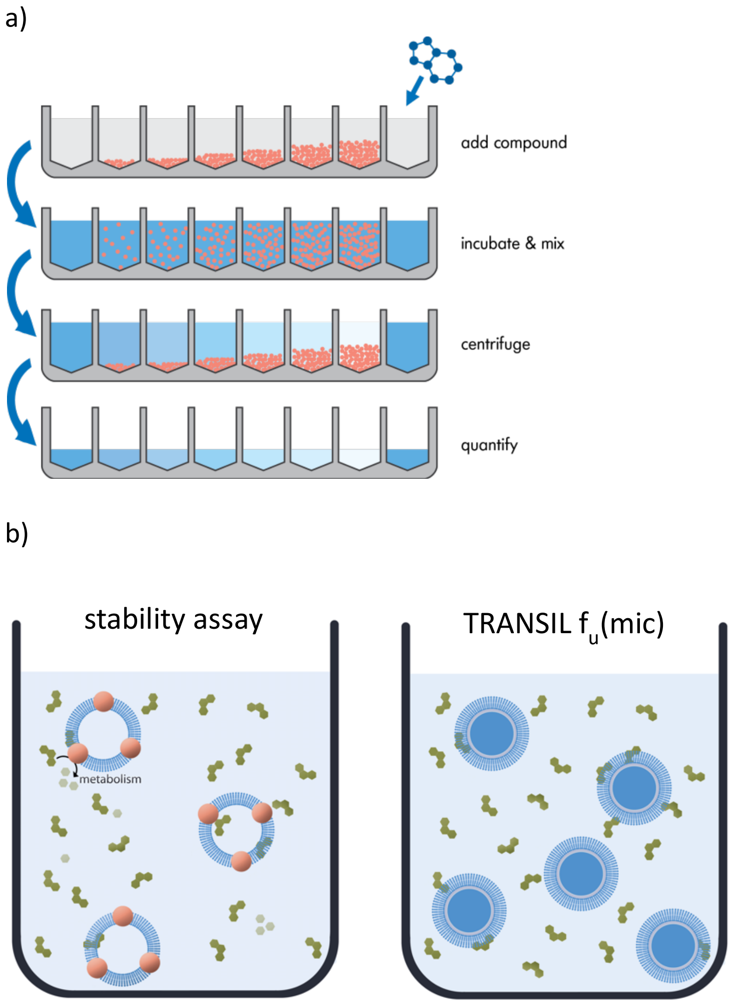


Figure 1: TRANSIL Microsomal Binding Assay principle. a) Illustration of the assay workflow: The same amount of drug is added to all wells followed by a mixing step. After 12 minutes incubation, beads are separated from the buffer by centrifugation and the remaining supernatant is sampled for quantification. b) Illustration of a typical metabolic stability assay setup (left panel) compared to a TRANSIL incubation (right panel) containing beads with immobilized microsomal membranes (blue spheres associated with membranes). Only the proportion of compound freely available in the buffer phase can be metabolized by liver microsomal enzymes (metabolism enzymes are illustrated by the red spherical objects located in the blue membrane vesicles). Depending on the affinity of the test compound to microsomal membranes a certain proportion of the compound is distributed within the membrane phase and thus, lowering the free concentration. The f_u(mic) obtained from the TRANSIL experiment can be used to correct the metabolism rates in the stability setup.

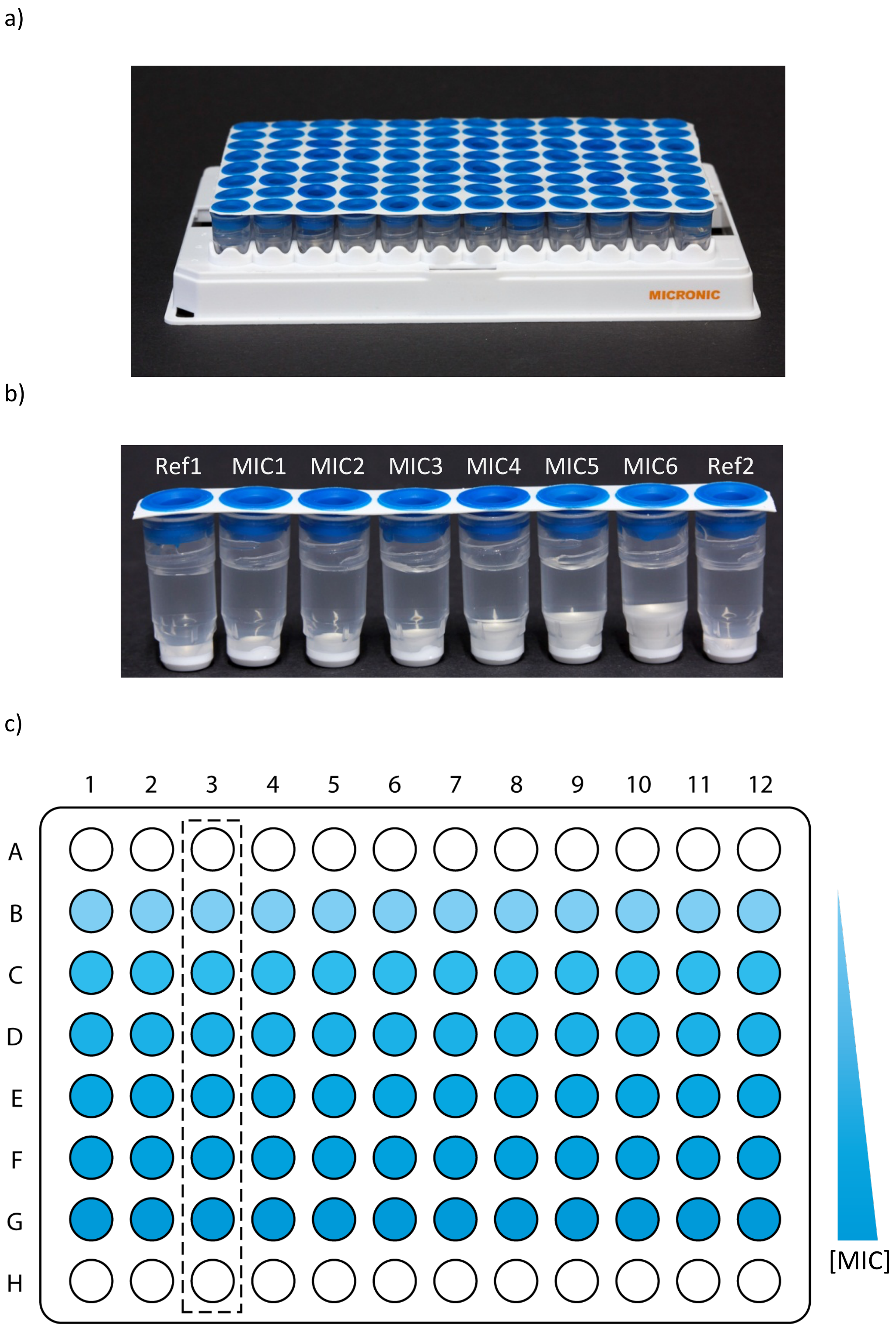


Figure 2: The TRANSIL Microsomal Binding Kit uses a column of 8 wells to determine the affinity to microsomal membranes (MIC). a) Photography of the assay plate and b) the annotated tube units supplied. c) Illustration of the assay plate showing the reference rows A and H (white wells) as well as the increasing microsomal membrane concentration from wells B to G (blue). The dashed line indicates the row orientation of the plate: the same amount of drug is added to all tube wells in one column. The plate can be used for 12 compounds.

4 Kit components

A TRANSIL Microsomal Binding Kit is composed of the following items:

No.	Qty.	Item
1	1	A 96 well plate with twelve units of 8 tubes filled with a suspension of TRANSIL Microsomal Binding Beads suspended in 10 mM phosphate buffered saline adjusted to pH 7.4. Tube units are locked in the assay plate for optimal handling with liquid handlers. Tube units can be de-locked easily from the lower side of the plate. This allows the flexibility to run less than 12 test compounds per experiment if required.
2	1	Decapper-8
3	1	Instruction manual
4	1	CD with spreadsheet calculation

5 Abbreviations

cmp	Compound
conc	Concentration
DMSO	Dimethyl sulfoxide
f_u(mic)	Unbound fraction of drug in metabolism experiment
MA	Membrane affinity defined as the concentration of drug in membrane (lipid) over concentration of drug in buffer: $MA = \frac{c_l}{c_b}$. The mass balance equation is used to calculate membrane affinity from experimental data.
PBS	Dulbecco's Phosphate buffered saline used in 1x concentration
TQI	TRANSIL Quality Index
r^2	Correlation coefficient

V_b	Buffer volume
V_l	Lipid volume

6 Equipment

The following equipment is required to run the TRANSIL Microsomal Binding kit:

No.	Instrument	Specification
1	Plate shaker	For high speed mixing (min. 800 rpm), i.e. MixMate (Eppendorf).  Alternatively, a vortexer with a plateholder can be used.
2	Centrifuge	Including rotor for SBS standard assay plates

7 Assay preparation

Upon receipt the kit should be stored at -20°C (-4°F).

Before use, thaw the assay at 4°C for a period of 12 hours (overnight) or, at room temperature for a period of 3 hours. Make sure the tubes have reached room temperature (between 20° and 25°C) prior to starting the assay. After thawing, spin plate quickly for 5 seconds at 750 g to collect all liquid at the bottom.

If it is desired to analyze less than 12 compounds at the same time, it is possible to remove columns of 8 tubes, interlocked by the lid-strip. We advise to remove the strips which shall be saved for future experiments and leave the tubes for current use on the rack. Remove tube strips by carefully pushing the individual tubes up from the bottom of the plate rack. Always keep lids closed when removing tubes.

8 Drug candidate preparation

Prepare a 16x stock solution for each drug candidate in DMSO. The final assay DMSO concentration can range from 2% to 6%. A 2% DMSO concentration is recommended (requires 32% DMSO in 16x compound stock) as higher DMSO concentrations may result in slight underestimation of binding.

Please consider the following:

Concentration: The TRANSIL Microsomal Binding Kit can be used in conjunction with different analytical methods and instruments. These include 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 a detailed calibration curve 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.

Volume: We recommend preparing a volume of at least 200 µl per compound. It is necessary to have at least 120 µl of the stock solution for each compound drug candidate since to each of the 8 tube wells 15 µl of the compound is added.

9 Replicates

The TRANSIL Microsomal Binding assay is designed such that one compound utilizes 8 wells – two references and 6 wells with increasing immobilized biological phase (membrane surface area). Therefore, the assay provides 6-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.

10 Assay procedure

Follow the following 5 steps for the assay procedure:

10.1 Compound addition

Mix the compound stock solution carefully by vortexing. When the TRANSIL Microsomal Binding kit has reached room temperature and the plate has been centrifuged briefly, remove the capbands with the decapper only immediately before compound addition. Make sure to maintain the original capband direction so that lids will be returned to the original wells to avoid any cross-contamination of beads etc. Add 15 µl of test compound to each well of a tube unit of 8 wells. Use one tube unit per compound (for example wells A1 to H1) so that twelve compounds can be analyzed using one kit. Change tips after each compound transfer step to avoid carryover of beads.

10.2 Incubation

Incubate the plates for twelve minutes on a plate shaker at 1000 rpm at RT.

NB: The first time a plate shaker is used for TRANSIL assays it is essential to determine that all the beads are resuspended in solution. To ensure beads are resuspended, visually inspect the plate after 1 min. If necessary increase the mixing speed until all beads are resuspended. Alternatively, manually invert plate to ensure all beads are resuspended.

10.3 Separation of beads and buffer

Spin the plate for 10 minutes at up to 750 g to sediment the beads from the suspension.

10.4 Sampling of supernatant

Take 50 – 100µl samples from the supernatants for analysis. Handling tips:

- Make sure that no beads are carried along when transferring the supernatant to the quantification plate.
- For supernatant sampling we advise not to remove the tubes from the rack. However, it may be convenient to remove and discard closed tube strips after supernatant sampling for easier access to the remaining tubes on the rack. Make sure to close the tubes after sampling and before discarding.
- When manually sampling supernatants we advise to guide the pipette tips along the tube walls.

11 Sample quantification

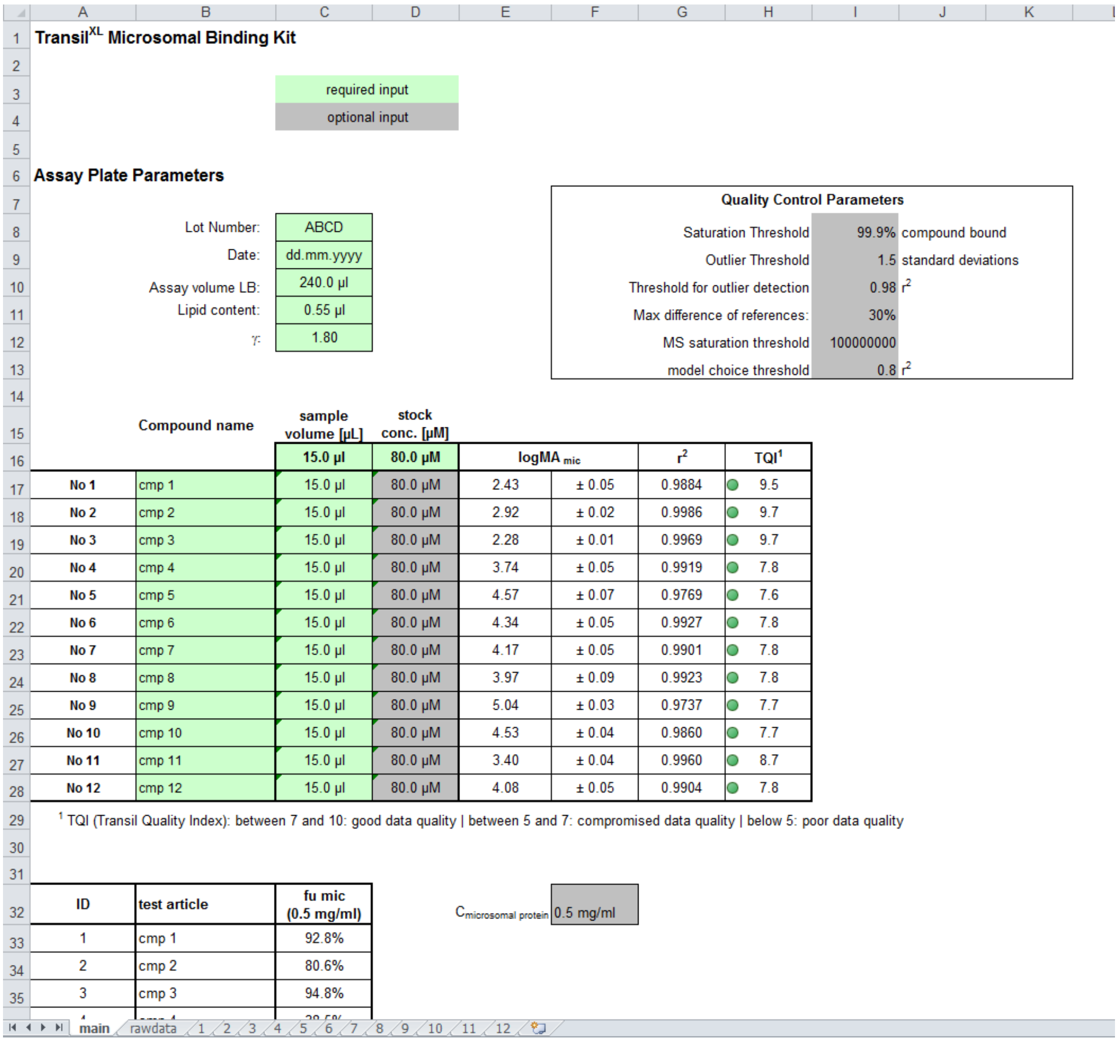
Use your analytical technique of choice for quantifying the compound concentration in the supernatant obtained in the last assay step.

12 Data analysis

Open the supplied spreadsheet for data analysis and follow the steps below to obtain the results for the TRANSIL Microsomal Binding kit. Only the fields marked in green require user input. Cells marked with gray background contain default values which may need to be adjusted (Figure 3).

12.1 Assay parameters

Open the “main” tab and enter the assay parameters in the column C8 to C12. Enter the lot specific parameters from the certificate of analysis that came with the assay plate. Also, enter the lot number and the assay date.



Transil^{XL} Microsomal Binding Kit

🟩 required input
▨ optional input

Assay Plate Parameters

Lot Number:	ABCD
Date:	dd.mm.yyyy
Assay volume LB:	240.0 µl
Lipid content:	0.55 µl
γ:	1.80

Quality Control Parameters

Saturation Threshold	99.9%	compound bound
Outlier Threshold	1.5	standard deviations
Threshold for outlier detection	0.98	r^2
Max difference of references:	30%	
MS saturation threshold	100000000	
model choice threshold	0.8	r^2

	Compound name	sample volume [µL]	stock conc. [µM]					
		15.0 µl	80.0 µM	logMA $_{mic}$		r^2		TQI[1]
No 1	cmp 1	15.0 µl	80.0 µM	2.43	± 0.05	0.9884	●	9.5
No 2	cmp 2	15.0 µl	80.0 µM	2.92	± 0.02	0.9986	●	9.7
No 3	cmp 3	15.0 µl	80.0 µM	2.28	± 0.01	0.9969	●	9.7
No 4	cmp 4	15.0 µl	80.0 µM	3.74	± 0.05	0.9919	●	7.8
No 5	cmp 5	15.0 µl	80.0 µM	4.57	± 0.07	0.9769	●	7.6
No 6	cmp 6	15.0 µl	80.0 µM	4.34	± 0.05	0.9927	●	7.8
No 7	cmp 7	15.0 µl	80.0 µM	4.17	± 0.05	0.9901	●	7.8
No 8	cmp 8	15.0 µl	80.0 µM	3.97	± 0.09	0.9923	●	7.8
No 9	cmp 9	15.0 µl	80.0 µM	5.04	± 0.03	0.9737	●	7.7
No 10	cmp 10	15.0 µl	80.0 µM	4.53	± 0.04	0.9860	●	7.7
No 11	cmp 11	15.0 µl	80.0 µM	3.40	± 0.04	0.9960	●	8.7
No 12	cmp 12	15.0 µl	80.0 µM	4.08	± 0.05	0.9904	●	7.8

[1] TQI (Transil Quality Index): between 7 and 10: good data quality | between 5 and 7: compromised data quality | below 5: poor data quality

ID	test article	fu mic (0.5 mg/ml)
1	cmp 1	92.8%
2	cmp 2	80.6%
3	cmp 3	94.8%

$C_{microsomal\ protein}$ 0.5 mg/ml

Figure 3: Screen shot of the “main” tab of the spreadsheet for analyzing data from the TRANSIL Microsomal Binding Kit. The “main” tab is used to enter lot specific data as well as for reporting final results, the TRANSIL Quality Index (TQI) and predictions of the unbound fraction in microsomal incubations.

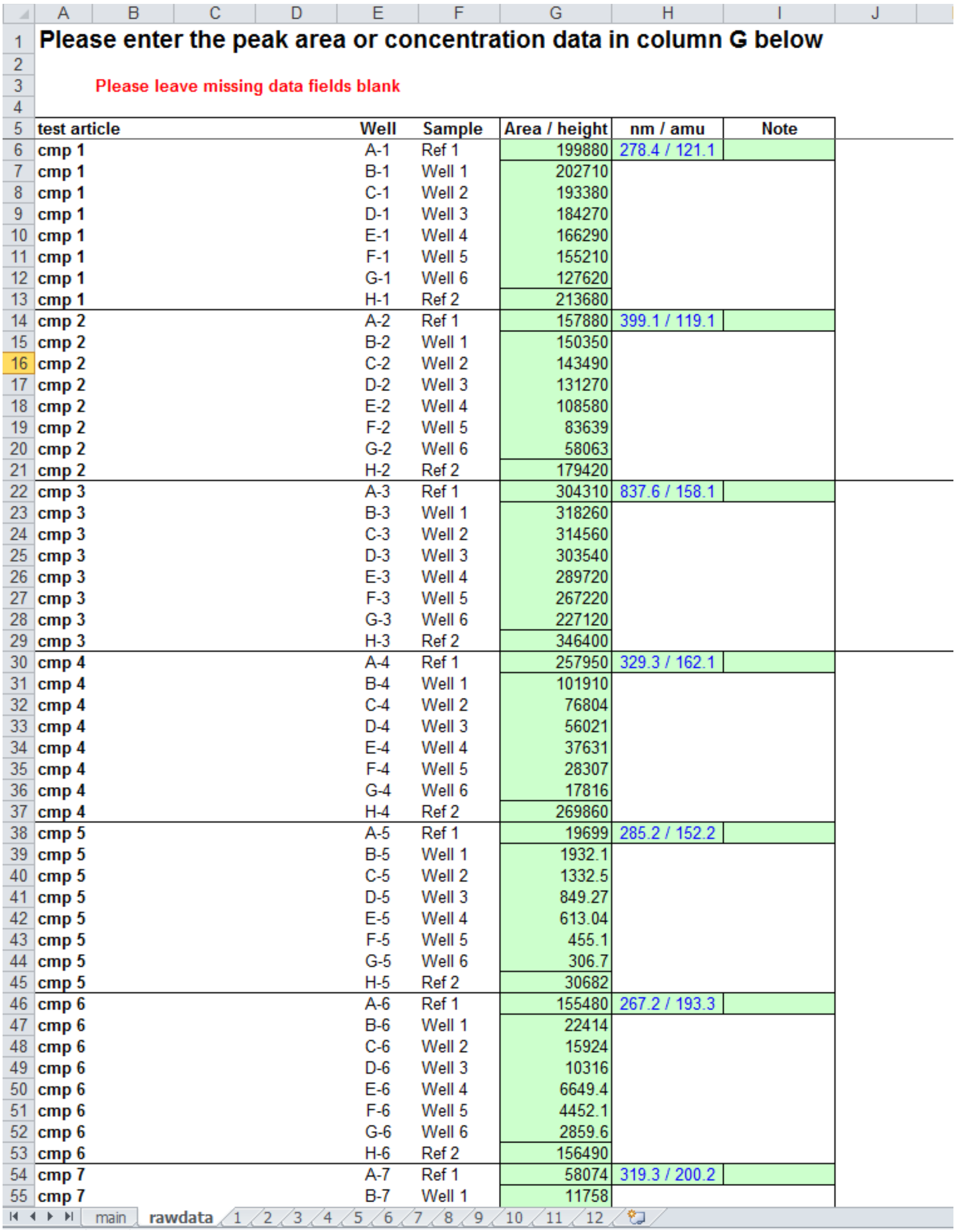
12.2 Compound information

Please enter the compound names in the column B17 to B28 of the “main” tab. Enter the sample volume added to each well in field C16. If a different sample volume was used for each drug, enter the sample specific volumes in the column C17 to C28.

Enter the concentration of the samples’ stock solutions in field D16. If a different sample concentration was used for each drug, enter the sample specific concentrations in the column D17 to D28 (remember this is the concentration of the stock solution).

12.3 Raw data from sample quantification

Open the tab “raw data” and enter the peak areas or heights for each well in column G (Figure 4). Note that column A lists the name of the compound used in each well. Caution: Make sure to begin data entry in field G6 for the first well of the plate (A1). When scrolling through the spreadsheet the header line in row 5 remains in place, while the fields for peak area entry move up.



Please enter the peak area or concentration data in column G below

Please leave missing data fields blank

test article	Well	Sample	Area / height	nm / amu	Note
cmp 1	A-1	Ref 1	199880	278.4 / 121.1	
cmp 1	B-1	Well 1	202710		
cmp 1	C-1	Well 2	193380		
cmp 1	D-1	Well 3	184270		
cmp 1	E-1	Well 4	166290		
cmp 1	F-1	Well 5	155210		
cmp 1	G-1	Well 6	127620		
cmp 1	H-1	Ref 2	213680		
cmp 2	A-2	Ref 1	157880	399.1 / 119.1	
cmp 2	B-2	Well 1	150350		
cmp 2	C-2	Well 2	143490		
cmp 2	D-2	Well 3	131270		
cmp 2	E-2	Well 4	108580		
cmp 2	F-2	Well 5	83639		
cmp 2	G-2	Well 6	58063		
cmp 2	H-2	Ref 2	179420		
cmp 3	A-3	Ref 1	304310	837.6 / 158.1	
cmp 3	B-3	Well 1	318260		
cmp 3	C-3	Well 2	314560		
cmp 3	D-3	Well 3	303540		
cmp 3	E-3	Well 4	289720		
cmp 3	F-3	Well 5	267220		
cmp 3	G-3	Well 6	227120		
cmp 3	H-3	Ref 2	346400		
cmp 4	A-4	Ref 1	257950	329.3 / 162.1	
cmp 4	B-4	Well 1	101910		
cmp 4	C-4	Well 2	76804		
cmp 4	D-4	Well 3	56021		
cmp 4	E-4	Well 4	37631		
cmp 4	F-4	Well 5	28307		
cmp 4	G-4	Well 6	17816		
cmp 4	H-4	Ref 2	269860		
cmp 5	A-5	Ref 1	19699	285.2 / 152.2	
cmp 5	B-5	Well 1	1932.1		
cmp 5	C-5	Well 2	1332.5		
cmp 5	D-5	Well 3	849.27		
cmp 5	E-5	Well 4	613.04		
cmp 5	F-5	Well 5	455.1		
cmp 5	G-5	Well 6	306.7		
cmp 5	H-5	Ref 2	30682		
cmp 6	A-6	Ref 1	155480	267.2 / 193.3	
cmp 6	B-6	Well 1	22414		
cmp 6	C-6	Well 2	15924		
cmp 6	D-6	Well 3	10316		
cmp 6	E-6	Well 4	6649.4		
cmp 6	F-6	Well 5	4452.1		
cmp 6	G-6	Well 6	2859.6		
cmp 6	H-6	Ref 2	156490		
cmp 7	A-7	Ref 1	58074	319.3 / 200.2	
cmp 7	B-7	Well 1	11758		

Figure 4: Screen shot of the “rawdata” tab of the spreadsheet for analyzing data from the TRANSIL Microsomal Binding Kit. The “rawdata” tab is used to enter peak area or concentration data from the supernatants of the assay plate after incubation and centrifugation.

12.4 Results

The spreadsheet calculates membrane affinities and QC parameters immediately after entering the lot specific information, compound names and concentrations, as well as the raw data from quantification.

12.4.1 Membrane affinity

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:

$$\mathrm{MA} = \frac{c_l}{c_b} . \qquad (1)$$

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

$$n_t = c_b \cdot V_b + c_l \cdot V_l \qquad (2)$$

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:

$$\frac{n_t}{c_b} = \frac{c_l}{c_b} \cdot V_l + V_b = \mathrm{MA} \cdot V_l + V_b \qquad (3)$$

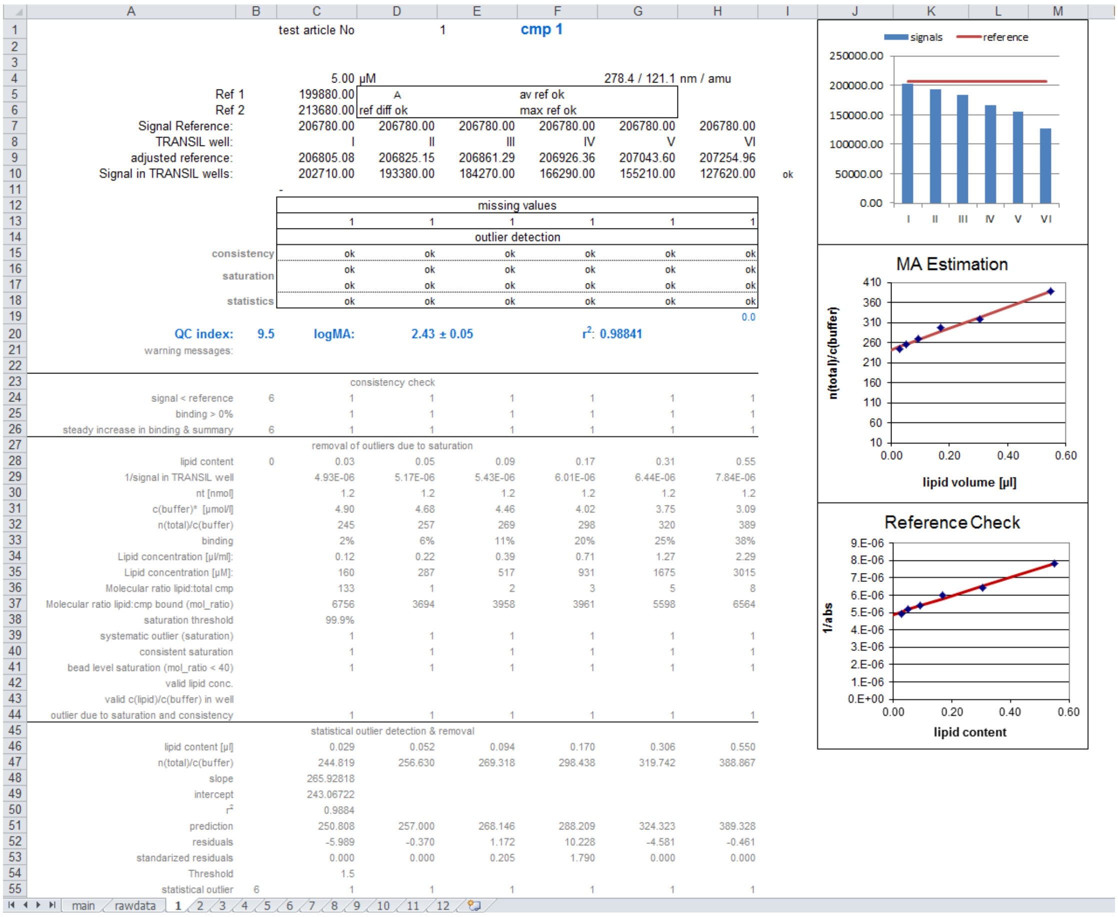
The results for the membrane affinity are reported in column E17 to E28 along with the TRANSIL Quality Index.

Results with an index greater than 7 are of good quality, results with an index between 5 and 7 are compromised, but may be reasonably accurate, while results with an index below 5 are poor and should be reported with caution.

The default requirement for good references is that both measurements will not deviate more than 30%. This assumption can be changed by setting the margin in cell I11 to a different value. If the references differ more than this threshold of 30% the spreadsheet uses the highest reference value. However, if the highest reference value is lower than the concentration determined in the TRASNSIL 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. Please refer to the trouble shooting section if this occurs.

12.4.2 Detailed measurement results –membrane affinity

Detailed measurement results can be found for each drug can be found on the spreadsheet's detail tabs with the indices from 1 to 12 for each respective drug. Figure 5 illustrates the information reported on each individual drug tested.



	A	B	C	D	E	F	G	H	I
1			test article No	1		cmp 1			
2									
3									
4			5.00 µM				278.4 / 121.1 nm / amu		
5	Ref 1		199880.00	A		av ref ok			
6	Ref 2		213680.00	ref diff ok		max ref ok			
7	Signal Reference:		206780.00	206780.00	206780.00	206780.00	206780.00	206780.00	
8	TRANSIL well:		I	II	III	IV	V	VI	
9	adjusted reference:		206805.08	206825.15	206861.29	206926.36	207043.60	207254.96	
10	Signal in TRANSIL wells:		202710.00	193380.00	184270.00	166290.00	155210.00	127620.00	ok
11			-						
12			missing values						
13			1	1	1	1	1	1	
14			outlier detection						
15		consistency	ok	ok	ok	ok	ok	ok	
16		saturation	ok	ok	ok	ok	ok	ok	
17			ok	ok	ok	ok	ok	ok	
18		statistics	ok	ok	ok	ok	ok	ok	
19								0.0	
20	QC index:	9.5	logMA:	2.43 ± 0.05		r^2: 0.98841			
21	warning messages:								
22									
23				consistency check					
24	signal < reference	6	1	1	1	1	1	1	
25	binding > 0%		1	1	1	1	1	1	
26	steady increase in binding & summary	6	1	1	1	1	1	1	
27				removal of outliers due to saturation					
28	lipid content	0	0.03	0.05	0.09	0.17	0.31	0.55	
29	1/signal in TRANSIL well		4.93E-06	5.17E-06	5.43E-06	6.01E-06	6.44E-06	7.84E-06	
30	nt [nmol]		1.2	1.2	1.2	1.2	1.2	1.2	
31	c(buffer)* [µmol/l]		4.90	4.68	4.46	4.02	3.75	3.09	
32	n(total)/c(buffer)		245	257	269	298	320	389	
33	binding		2%	6%	11%	20%	25%	38%	
34	Lipid concentration [µl/ml]:		0.12	0.22	0.39	0.71	1.27	2.29	
35	Lipid concentration [µM]:		160	287	517	931	1675	3015	
36	Molecular ratio lipid:total cmp		133	1	2	3	5	8	
37	Molecular ratio lipid:cmp bound (mol_ratio)		6756	3694	3958	3961	5598	6564	
38	saturation threshold		99.9%						
39	systematic outlier (saturation)		1	1	1	1	1	1	
40	consistent saturation		1	1	1	1	1	1	
41	bead level saturation (mol_ratio < 40)		1	1	1	1	1	1	
42	valid lipid conc.								
43	valid c(lipid)/c(buffer) in well								
44	outlier due to saturation and consistency		1	1	1	1	1	1	
45				statistical outlier detection & removal					
46	lipid content [µl]		0.029	0.052	0.094	0.170	0.306	0.550	
47	n(total)/c(buffer)		244.819	256.630	269.318	298.438	319.742	388.867	
48	slope		265.92818						
49	intercept		243.06722						
50	r^2		0.9884						
51	prediction		250.808	257.000	268.146	288.209	324.323	389.328	
52	residuals		-5.989	-0.370	1.172	10.228	-4.581	-0.461	
53	standarized residuals		0.000	0.000	0.205	1.790	0.000	0.000	
54	Threshold		1.5						
55	statistical outlier	6	1	1	1	1	1	1	

Figure 5: Screen shot of the details "1" tab of the spreadsheet for analyzing data from the TRANSIL Microsomal Binding Assay kit. The "1" 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.

12.5 TRANSIL Quality Index

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.

12.5.1 Model fit (intercept)

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

$$\frac{n_t}{c_b} = MA \cdot V_l + V_b \tag{3}$$

Fitting optimal data to equation (3) 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.

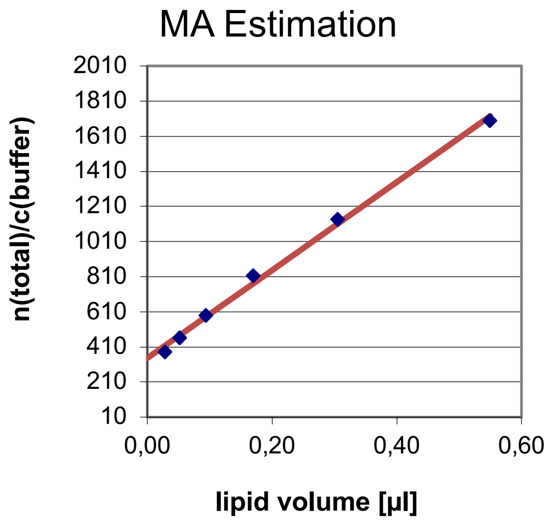


Figure 6: Illustration of fitting experimental data to equation (3) to determine the membrane affinity. A buffer volume of 240 µL has been used in the experiment, the intercept was estimated to 346 µL, hence a quality score of 5 was attributed to the model fit.

12.5.2 Match of measured versus predicted reference signal (ref)

When determining the membrane affinity via the six 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 7). This score has a weight of 3 in the TQI.

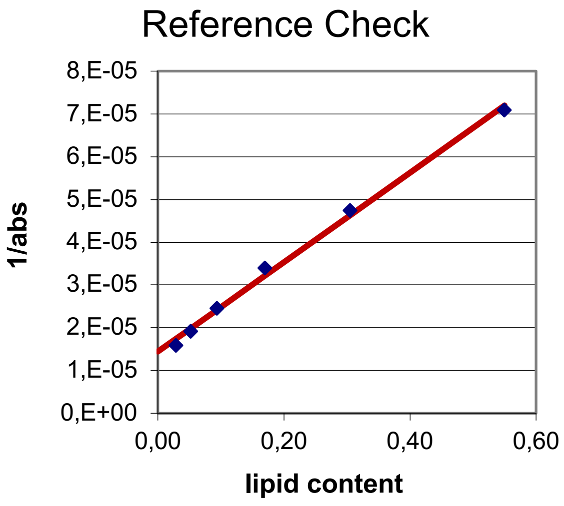


Figure 7: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the lipid content. The inverse of the intercept represents the expected peak area of the references.

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

Table 1: 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

12.5.3 *Correlation coefficient (r^2)*

The correlation coefficient from fitting the experimental data to equation (3) also contributes as a partial quality score (Table 2). This score has a weight of 3 in the TQI.

Table 2: Partial quality scores for the least square model fit of the experimental data to equation (3).

r^2	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

12.5.4 Number of outliers or missing data (DP)

The number of data points used to calculate the membrane affinity is also used as partial quality score (Table 3). This score has a weight of 2 in the TQI.

Table 3: Partial quality scores for the number of data points used in the model fit of the experimental data to equation (3).

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

12.5.5 Data consistency (C)

With increasing lipid volume, i.e. increasing lipid membrane surface, the binding of 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 4. This score has a weight of only 1 in the TQI as it may affect also the number or data points.

Table 4: Partial quality scores for the number of consitent data points used in the model fit of the experimental data to equation (3).

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

12.5.6 Slopes of binding

Data fitted to equation (3), plotted in Figure 6 as well as the percentage binding shall increase with increasing membrane surface area (Figure 7). Hence, the slopes of these graphs must all be positive. Most critical of all is the relationship of equation (3), if it has a positive slope it receives a vote of 10 points, otherwise zero. If the relationship plotted in Figure 7 has a positive slope, a vote of 5 points is granted. If the binding curve has a positive slope, a vote of 5 points is granted, otherwise zero. If the total count of votes is 20, a partial score of 10 will be attributed, if the total vote is 10 a score of 5 is attributed, and if the total vote is 0, a partial score of 0 is attributed to the data set.

12.5.7 Reference treatment

For each compound two references are measured in the assay kit. If the references vary by no more than 30% and have a higher peak area than the measurements in the TRANSIL wells, the average references is computed and a partial score 10 is attributed. If the reference peak areas are higher than those of the TRANSIL wells, but differences between the two measurements exceed 30%, the maximum of the measurements is chosen. However, if the reference peak areas do not exceed the peak areas from the TRANSIL wells the reference measurements are discarded and the first TRANSIL measurement is taken as reference. In this case a partial score of 6 is attributed.

12.5.8 TRANSIL peak areas exceed reference peak areas

The reference peak areas should always exceed the peak areas from the TRANSIL well. If not, the stability or solubility of the compound is compromised. Therefore, the fewer TRANSIL measurements meet this criterion, the lower the partial score attributed to the data set (Table 5).

Table 5: Partial quality scores for the number of data TRANSIL peak areas being higher than reference peak areas.

No. of TRANSIL peak areas higher than reference peak areas	Score
5	10
4	7
3	4
2	2
1	1
0	0

12.6 Calculation of f_u(mic) using membrane affinity data

The free fraction (f_u) in the presence of liver microsomes is defined in a microsomal stability experiment as the amount of drug in the buffer (n_b) over the total amount of drug (n_t):

$$f_u = \frac{n_b}{n_t} = \frac{c_b \cdot (V_t - V_l)}{n_t} \tag{4}$$

V_t is the total assay volume while V_l defines the lipid volume in the microsomal preparation. The total amount of drug in the experiment (n_t) can be described by the mass balance equation (cf. section 12.4.1):

$$n_t = c_b \cdot V_b + c_l \cdot V_l = c_b \cdot (V_t - V_l) + c_l \cdot V_l \tag{5}$$

The membrane affinity is defined as the concentration of drug in membrane over the concentration of drug in buffer:

$$MA = \frac{c_l}{c_b}$$

and can be rearranged to

$$c_l = c_b \cdot MA \tag{6}$$

Inserting equation (5) and (6) into equation (4) provides the relation between the membrane affinity and the free fraction of the test compound after simplification and rearrangement:

$$f_u(\mathrm{mic}) = \frac{1}{1 + \mathrm{MA} \cdot \frac{V_l}{V_t - V_l}} \tag{7}$$

The lipid to protein concentration ratio, denoted as CLP, has been experimentally determined for human liver microsomes:

$$\mathrm{CLP}_{\text{human mic}} = \frac{c_l}{c_P} = 0.581 \frac{\mu l_{\text{lipid}}}{mg_{\text{protein}}} \tag{8}$$

This ratio is used to calculate the lipid content in the human microsomal incubations given the assay volume V_t and the protein content c_p of the microsomes:

$$V_l = \mathrm{CLP} \cdot c_P \cdot V_t \tag{9}$$

The experimental constant CLP can be used for scaling the f_u(mic) to all desired protein concentrations. Spreadsheet default settings compute the f_u(mic) at a microsomal concentration of 0.5 mg protein/ ml. The spreadsheet automatically converts the f_u(mic) after inserting the preferred protein concentration in field F32 of the “main” tab.

12.7 Influence of the f_u(mic) on clearance estimates

Since only the unbound fraction of drug is available for metabolic decay the clearance estimated in the microsomal incubation Cl_{inc} is lower than the theoretical intrinsic clearance Cl_{int} that would be observed if the drug would not bind to microsomal membranes. The equation

$$Cl_{int} = \frac{Cl_{inc}}{f_u(\mathrm{mic})} \tag{9}$$

describes the relationship between the clearance rate measured in the incubation and the intrinsic clearance. However, one should note that the in vivo clearance will also be influenced by reduction of the free fraction of drug in the liver tissue due to binding to cell and golgi membranes.

13 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.

14 Trouble shooting

14.1 Poor recovery

14.1.1 Challenges and problem identification

Poor data quality such as low TQI's, poor regression fits, or strong variation in duplicate measurements of references may indicate reduced recovery due to poor solubility or stickiness of the test compound. This can result in lower compound concentrations in the reference wells than in the TRANSIL wells. The spreadsheet detects if reference measurements are lower than the signal in the first TRANSIL well. In this case, the spreadsheet replaces the reference value with the measurement from the first TRANSIL well. Consequently, the first TRANSIL well is discarded from the data analysis. Treatment of the references is reported on each compound page in cell D5. The letter "A" (=average) refers to normal treatment as before, "M" (=maximum) is chosen when the difference between references exceeds the value specified in cell I11 of the summary page, and "R" denotes the replacement with the signal in the first TRANSIL well.

For evaluation of recovery issues, include a separate control vial with pure organic solvent (e.g. DMSO) and the test compound in the same concentration as the final assay concentration. Comparison of the peak areas or counts from this organic solvent control and the peak areas from the according calibration signal or the assay references yields a good indication of compound losses through incomplete solubility in the aqueous buffer system or through unspecific binding. Please note that comparing the absolute peak area should be done with caution because of matrix effects.

14.1.2 Problem-solving approaches

i. Sovicell support team can assist you in checking the plausibility of the data if solubility/non-specific binding 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. DMSO content can be increased. The assay tolerates up to 10% DMSO.
iii. Test compound concentration can be reduced, however, it has to be considered that running the assay with lower compound concentrations increases the likelihood of measurements outside the linear range of the instruments (c.f. section 15.2).

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

14.2 Non-linearity of the response

14.2.1 Challenges and problem identification

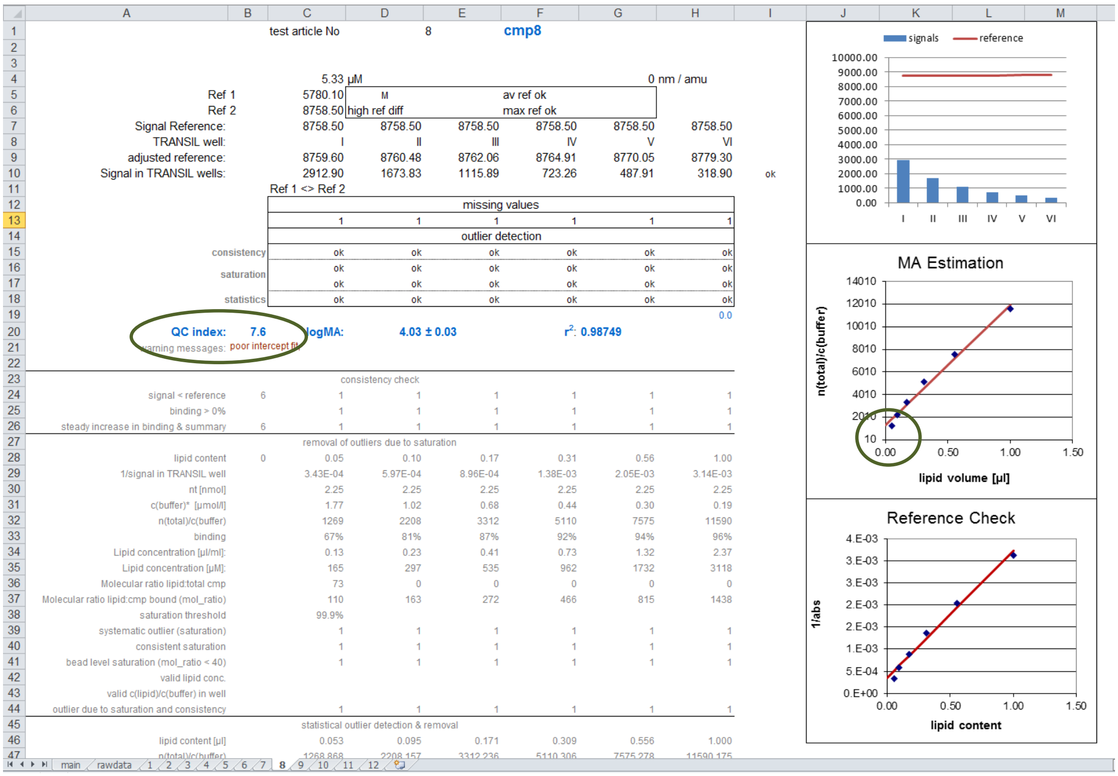
Frequently, it is observed that mass spectrometers exhibit a non-linear response even in concentration ranges up to 100x above the detection limit. Likewise, impurities of radiolabelled compounds can lead to similar effects when the impurity exhibits different binding properties from the parent compound.

The warning message poor intercept fit or a non-linear shape of the regression (visualized by the “MA Estimation plot” in the individual data analysis tabs of the spreadsheet; see Figure 8) may indicate non-linear response issues.

14.2.2 Problem-solving approaches

i. Increasing the test compound concentration will increase supernatant concentrations and help to eliminate the non-linear instrument response at low concentrations.
ii. Non-linear response issue is primarily observed with high affinity compounds. A kit with lower lipid content will increase supernatant concentrations and help to eliminate the non-linear instrument response. To further improve the measurement accuracy of compounds with high membrane affinities we offer the TRANSIL Microsomal Binding Kit for high affinity compounds (TMP-0120-2296).
iii. If test compound concentration is limited by poor compound solubility, a detailed calibration curve covering the non-linear response can be recorded and used to calculate test compound concentrations. The concentrations calculated form the non-linear calibration curve can then be entered in the spreadsheet’s raw data tab instead of peak areas. 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.



	A	B	C	D	E	F	G	H	I
1			test article No	8		**cmp8**			
2									
3									
4			5.33 µM					0 nm / amu	
5	Ref 1		5780.10	M		av ref ok			
6	Ref 2		8758.50	high ref diff		max ref ok			
7	Signal Reference:		8758.50	8758.50	8758.50	8758.50	8758.50	8758.50	
8	TRANSIL well:		I	II	III	IV	V	VI	
9	adjusted reference:		8759.60	8760.48	8762.06	8764.91	8770.05	8779.30	
10	Signal in TRANSIL wells:		2912.90	1673.83	1115.89	723.26	487.91	318.90	ok
11			Ref 1 <> Ref 2						
12			missing values						
13			1	1	1	1	1	1	
14			outlier detection						
15	consistency		ok	ok	ok	ok	ok	ok	
16	saturation		ok	ok	ok	ok	ok	ok	
17			ok	ok	ok	ok	ok	ok	
18	statistics		ok	ok	ok	ok	ok	ok	
19								0.0	
20	**QC index:**	**7.6**	**logMA:**	**4.03 ± 0.03**		**r^2: 0.98749**			
21	warning messages:	poor intercept fit							
22									
23			consistency check						
24	signal < reference	6	1	1	1	1	1	1	
25	binding > 0%		1	1	1	1	1	1	
26	steady increase in binding & summary	6	1	1	1	1	1	1	
27			removal of outliers due to saturation						
28	lipid content	0	0.05	0.10	0.17	0.31	0.56	1.00	
29	1/signal in TRANSIL well		3.43E-04	5.97E-04	8.96E-04	1.38E-03	2.05E-03	3.14E-03	
30	nt [nmol]		2.25	2.25	2.25	2.25	2.25	2.25	
31	c(buffer)* [µmol/l]		1.77	1.02	0.68	0.44	0.30	0.19	
32	n(total)/c(buffer)		1269	2208	3312	5110	7575	11590	
33	binding		67%	81%	87%	92%	94%	96%	
34	Lipid concentration [µl/ml]:		0.13	0.23	0.41	0.73	1.32	2.37	
35	Lipid concentration [µM]:		165	297	535	962	1732	3118	
36	Molecular ratio lipid:total cmp		73	0	0	0	0	0	
37	Molecular ratio lipid:cmp bound (mol_ratio)		110	163	272	466	815	1438	
38	saturation threshold		99.9%						
39	systematic outlier (saturation)		1	1	1	1	1	1	
40	consistent saturation		1	1	1	1	1	1	
41	bead level saturation (mol_ratio < 40)		1	1	1	1	1	1	
42	valid lipid conc.								
43	valid c(lipid)/c(buffer) in well								
44	outlier due to saturation and consistency		1	1	1	1	1	1	
45			statistical outlier detection & removal						
46	lipid content [µl]		0.053	0.095	0.171	0.309	0.556	1.000	
47	n(total)/c(buffer)		1268.868	2208.157	3312.236	5110.306	7575.278	11590.175	

Figure 8: Illustration of the non-linear response issue which can be recognized by a poor intercept fit (green circles) which usually comes along with a curved plot of nt/cb versus lipid volume plot for the MA estimation. Both the poor intercept fit and the deviation from linearity in this plot are a good indication of the non-linear instrument response to decreasing compound concentrations.

14.3 Low Membrane affinity

If compound binding to TRANSIL is not increasing with increasing TRANSIL concentration, then the compounds exhibit very low affinity to the TRANSIL lipid membrane. This means their membrane affinity is very low. The spreadsheet will automatically use an appropriate alternative approach for the calculation of the membrane affinity if such problems occur.

14.3.1 Challenges and problem identification

Compounds with very low membrane affinity (logMA < 2) are not accurately measured. Low affinity compounds yield supernatant concentrations in the assay that deviate only marginally from the reference signals (Figure 9).

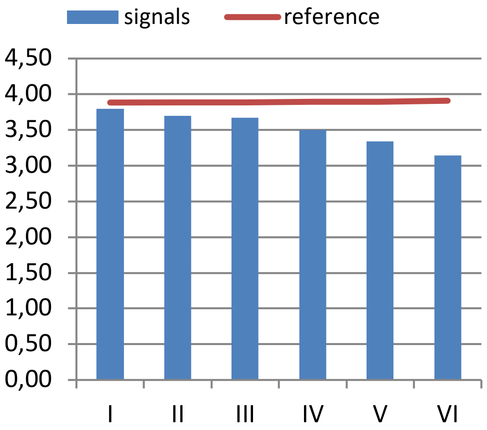


Figure 9: Illustration of a bar plot of a compound exhibiting low membrane affinity (c.f. individual data analysis tabs of the spreadsheet). The blue bars show the detected signals in the supernatants of TRANSIL wells I to VI. As the compound distributes only weekly into the membranes, supernatant concentrations differ only marginally from the reference signals (red line).

14.3.2 Problem-solving approaches

In case the membrane affinity turns out to be below 300 (logMA < 2.5) we recommend using the TRANSIL Microsomal Binding Kit - low affinity compounds (Product No. TMP-0120-2196). This kit contains an adjusted TRANSIL content (higher than the standard kit) and hence, provides more accurate results.

Technical Support

Phone: +49 341 52044-0

Email: contact@sovicell.com

15 References

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Hallifax, D., and Houston, J. B. (2006). Binding of drugs to hepatic microsomes: comment and assessment of current prediction methodology with recommendation for improvement. *Drug Metab Dispos* **34**, 724-726; author reply 727.

McLure, J. A., Miners, J. O., and Birkett, D. J. (2000). Nonspecific binding of drugs to human liver microsomes. *Br J Clin Pharmacol* **49**, 453-461.

Obach, R. S. (1999). Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab Dispos* **27**, 1350-1359.