



User Guide

**TRANSIL<sup>XL</sup>**

**Membrane Affinity**

**Assay**

TMP-0410-2096

Version 3, Revision 00

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# 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 50x stock solutions of each compound in pure solvent (e.g. DMSO) - yields a final solvent conc. of 2%.
- The final compound concentration in the assay depends on the compound's solubility, analytical method and instrumentation: If permitted by compound solubility use 5 µM final assay concentration. This requires 250 µM stock solutions.
- Since each compound is added in an aliquot of 10 µl to each well of an 8-well tube unit, at least 80 µl stock solution are required for each compound. Allow an additional 40 µl for accurate pipetting.

## 3. Drug Candidate Addition

- Open wells with supplied decapper.
- Mix the stock solutions carefully.
- Transfer 10 µl of the 50x 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 or mix by aspirating and suspending 10 times a volume of 300 µl in each well.
- 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.

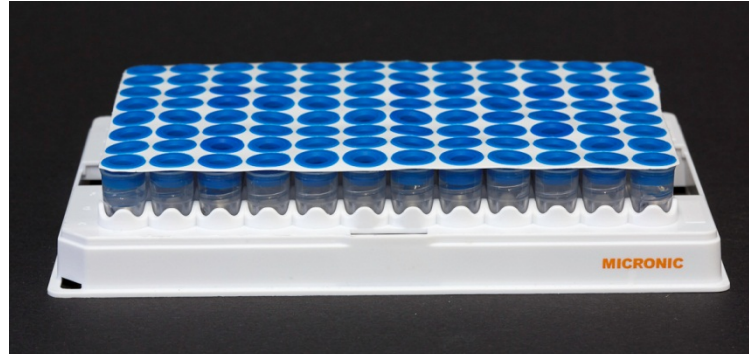
## 2 Applications of TRANSIL<sup>XL</sup> Membrane Affinity Assay

The TRANSIL<sup>XL</sup> Membrane Affinity kit enables researchers to measure the interaction of drugs with a membrane. This in vitro assay is designed to measure the distribution coefficient of test compounds between phosphatidylcholine membranes and an aqueous buffer. We refer to this distribution coefficient as membrane affinity.

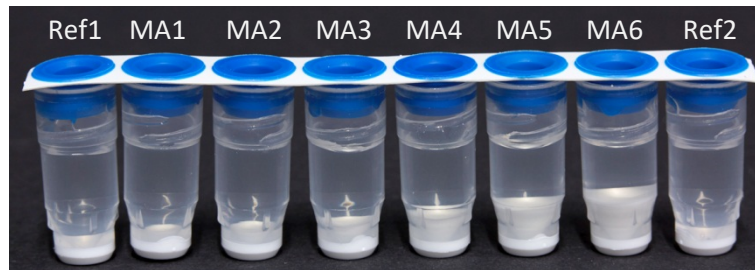
## 3 Basic assay principle

The principle of the TRANSIL<sup>XL</sup> Membrane Affinity kit is to assess the affinity of test compounds to phosphatidylcholine membranes. 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 (Figure 1). A total of 8 wells of a tube unit/plate are used to determine the membrane affinity for each compound (Figure 1). Six wells contain 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 phosphatidylcholine 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.

a)



b)



c)

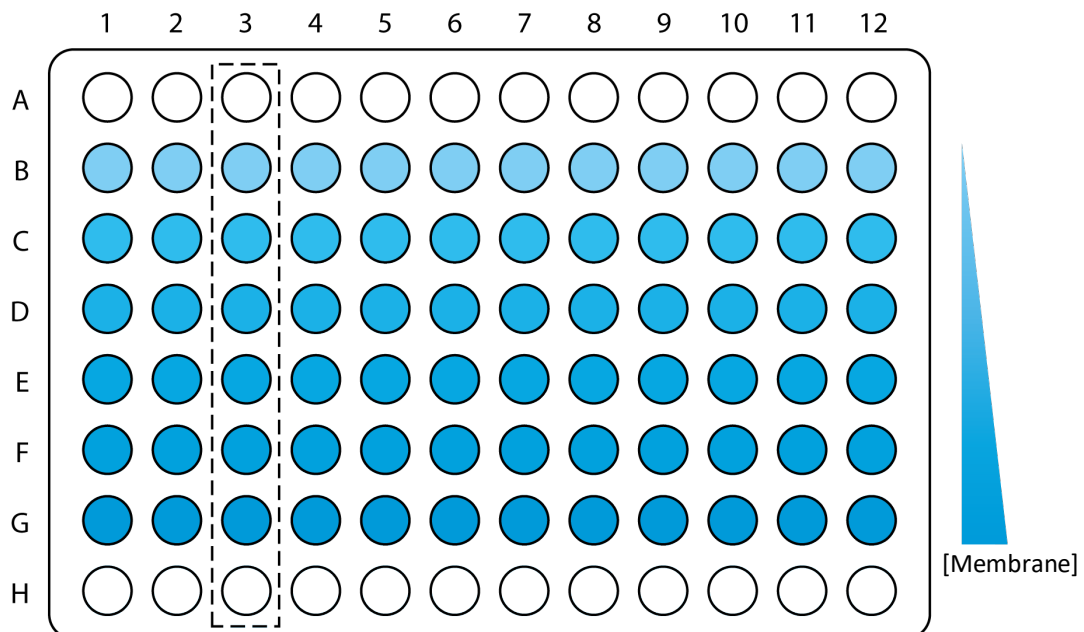


Figure 1: The TRANSIL<sup>XL</sup> Membrane Affinity Assay uses a column of 8 wells to determine the affinity to phosphatidylcholine membranes. a) Photograph of the assay plate and b) the annotated tube units supplied. c) Illustration of the assay plate showing the reference rows A and H (white wells) as well as the increasing

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<sup>XL</sup> Membrane Affinity Assay 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 Membrane Affinity Beads suspended in 10 mM phosphate buffered saline adjusted to pH 7.4. The TRANSIL Membrane Affinity beads are membrane vesicles reconstituted from egg yolk and stabilized on porous silica beads.
2	1	Decapper-8
3	1	Instruction manual
4	1	CD with spreadsheet calculation

## 5 Abbreviations

cmp	Compound
conc	Concentration
DMSO	Dimethyl sulfoxide
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 <sup>2</sup>	Correlation coefficient
VD	Volume of distribution

V <sub>b</sub>	Buffer volume
V <sub>l</sub>	Lipid volume


## 6 Reagents

The following reagents are required to run the TRANSIL<sup>XL</sup> Membrane Affinity Assay:

No.	Reagent	Specification
1	DMSO	For preparation of drug candidate stock solution
2	Dulbecco's PBS (1x)	For preparation of drug candidate stock solution

## 7 Equipment

The following equipment is required to run the TRANSIL<sup>XL</sup> Membrane Affinity Assay:

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

## 8 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.

## 9 Drug candidate preparation

Prepare a 50x stock solution for each drug candidate in DMSO yielding a final DMSO concentration of 2%. If other solvents are preferred, please contact Sovicell's technical support to ensure assay compatibility.

Please consider the following:

Concentration: The TRANSIL<sup>XL</sup> Membrane Affinity Assay 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 compound's 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 120  $\mu\text{l}$  per compound. It is necessary to have at least 80  $\mu\text{l}$  of the stock solution for each compound drug candidate since to each of the 8 tube wells 10  $\mu\text{l}$  of the compound is added.

## 10 Replicates

The TRANSIL<sup>XL</sup> Membrane Affinity 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.

## 11 Assay procedure

The workflow of the TRANSIL<sup>XL</sup> Membrane Affinity Assay is illustrated in Figure 2.

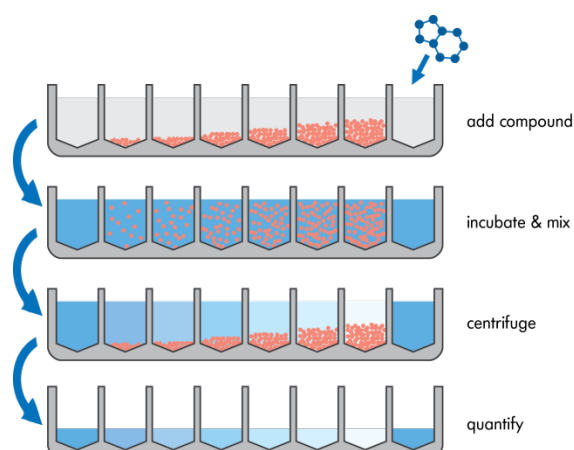


Figure 2: TRANSIL<sup>XL</sup> Membrane Affinity Assay workflow: The same amount of drug is added to all wells followed by a mixing step. After 12 minutes incubation, beads are separated from the buffer by centrifugation and the remaining supernatant is sampled for quantification.

Follow the following 5 steps for the assay procedure:

### 11.1 Compound addition

Mix the compound stock solution carefully by vortexing. When the TRANSIL<sup>XL</sup> Membrane Affinity Assay 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 10  $\mu\text{l}$  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.

### 11.2 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.

Alternatively, the plate can be incubated and mixed via aspiration and suspension for 10 times in each well using an electronic multichannel pipette or robot with a volume of 300 µl.

### 11.3 Separation of beads and buffer

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

### 11.4 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.

## 12 Sample quantification

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

## 13 Data analysis

Open the supplied spreadsheet for data analysis and follow the steps below to obtain the results for the TRANSIL<sup>XL</sup> Membrane Affinity Assay. 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).

### 13.1 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.

	A	B	C	D	E	F	G	H	I	J	K	
1	<b>Transil<sup>XL</sup> Membrane Affinity Assay</b>											
2												
3			required input									
4			optional input									
5												
6	<b>Assay Plate Parameters</b>											
7												
8		Lot Number:	ABCD									
9		Date:	dd.mm.yyyy									
10		Assay volume IA:	500.0 µl									
11		Lipid content:	30.00 µl									
12		γ:	1.80									
13												
14												
15		Compound name	sample volume [µL]	stock conc. [µM]								
16			10.0 µl	250.0 µM		logMA	r <sup>2</sup>	TQI <sup>1</sup>				
17	No 1	cmp1	10.0 µl	250.0 µM	2.26	± 0.07	0.9946	● 8.7				
18	No 2	cmp2	10.0 µl	250.0 µM	0.89	± 0.07	0.9101	● 9.5				
19	No 3	cmp3	10.0 µl	250.0 µM	1.00	± 0.02	0.9970	● 9.7				
20	No 4	cmp4	10.0 µl	250.0 µM	2.14	± 0.08	0.9298	● 7.7				
21	No 5	cmp5	10.0 µl	250.0 µM	0.64	± 0.09	0.9984	● 9.6				
22	No 6	cmp6	10.0 µl	250.0 µM	1.23	± 0.18	0.8744	● 8.3				
23	No 7	cmp7	10.0 µl	250.0 µM	2.61	± 0.07	0.8977	● 7.5				
24	No 8	cmp8	10.0 µl	250.0 µM	1.84	± 0.07	0.9292	● 7.6				
25	No 9	cmp9	10.0 µl	250.0 µM	0.89	± 0.07	0.9098	● 9.5				
26	No 10	cmp10	10.0 µl	250.0 µM	0.81	± 0.08	0.9033	● 9.5				
27	No 11	cmp11	10.0 µl	250.0 µM	0.90	± 0.07	0.9111	● 9.5				
28	No 12	cmp12	10.0 µl	250.0 µM	0.92	± 0.07	0.9124	● 9.5				
29	<sup>1</sup> TQI (Transil Quality Index): between 7 and 10: good data quality   between 5 and 7: compromised data quality   below 5: poor data quality											
30												

Figure 3: Screen shot of the “main” tab of the spreadsheet for analyzing data from the TRANSIL<sup>XL</sup> Membrane Affinity Assay. 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 VD and Pint.

### 13.2 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).

### 13.3 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.

	A	B	C	D	E	F	G	H	I	J
1	<b>Please enter the peak area or concentration data in column G below</b>									
2										
3	Please leave missing data fields blank									
4										
5	test article			Well	Sample	Area / height	nm / amu	Note		
6	cmp 1			A-1	Ref 1	199880	278.4 / 121.1			
7	cmp 1			B-1	Well 1	202710				
8	cmp 1			C-1	Well 2	193380				
9	cmp 1			D-1	Well 3	184270				
10	cmp 1			E-1	Well 4	166290				
11	cmp 1			F-1	Well 5	155210				
12	cmp 1			G-1	Well 6	127620				
13	cmp 1			H-1	Ref 2	213680				
14	cmp 2			A-2	Ref 1	157880	399.1 / 119.1			
15	cmp 2			B-2	Well 1	150350				
16	cmp 2			C-2	Well 2	143490				
17	cmp 2			D-2	Well 3	131270				
18	cmp 2			E-2	Well 4	108580				
19	cmp 2			F-2	Well 5	83639				
20	cmp 2			G-2	Well 6	58063				
21	cmp 2			H-2	Ref 2	179420				
22	cmp 3			A-3	Ref 1	304310	837.6 / 158.1			
23	cmp 3			B-3	Well 1	318260				
24	cmp 3			C-3	Well 2	314560				
25	cmp 3			D-3	Well 3	303540				
26	cmp 3			E-3	Well 4	289720				
27	cmp 3			F-3	Well 5	267220				
28	cmp 3			G-3	Well 6	227120				
29	cmp 3			H-3	Ref 2	346400				
30	cmp 4			A-4	Ref 1	257950	329.3 / 162.1			
31	cmp 4			B-4	Well 1	101910				
32	cmp 4			C-4	Well 2	76804				
33	cmp 4			D-4	Well 3	56021				
34	cmp 4			E-4	Well 4	37631				
35	cmp 4			F-4	Well 5	28307				
36	cmp 4			G-4	Well 6	17816				
37	cmp 4			H-4	Ref 2	269860				
38	cmp 5			A-5	Ref 1	19699	285.2 / 152.2			
39	cmp 5			B-5	Well 1	1932.1				
40	cmp 5			C-5	Well 2	1332.5				
41	cmp 5			D-5	Well 3	849.27				
42	cmp 5			E-5	Well 4	613.04				
43	cmp 5			F-5	Well 5	455.1				
44	cmp 5			G-5	Well 6	306.7				
45	cmp 5			H-5	Ref 2	30682				
46	cmp 6			A-6	Ref 1	155480	267.2 / 193.3			
47	cmp 6			B-6	Well 1	22414				
48	cmp 6			C-6	Well 2	15924				
49	cmp 6			D-6	Well 3	10316				
50	cmp 6			E-6	Well 4	6649.4				
51	cmp 6			F-6	Well 5	4452.1				
52	cmp 6			G-6	Well 6	2859.6				
53	cmp 6			H-6	Ref 2	156490				
54	cmp 7			A-7	Ref 1	58074	319.3 / 200.2			
55	cmp 7			B-7	Well 1	11758				

Figure 4: Screen shot of the "rawdata" tab of the spreadsheet for analyzing data from the TRANSIL<sup>XL</sup> Membrane Affinity Assay. The "rawdata" tab is used to enter peak area or concentration data from the supernatants of the assay plate after incubation and centrifugation.



## 13.4 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.

### 13.4.1 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:

$$MA = \frac{c_l}{c_b} \quad (1)$$

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

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

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:

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

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 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. Please refer to the trouble shooting section if this occurs.

### 13.4.2 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.

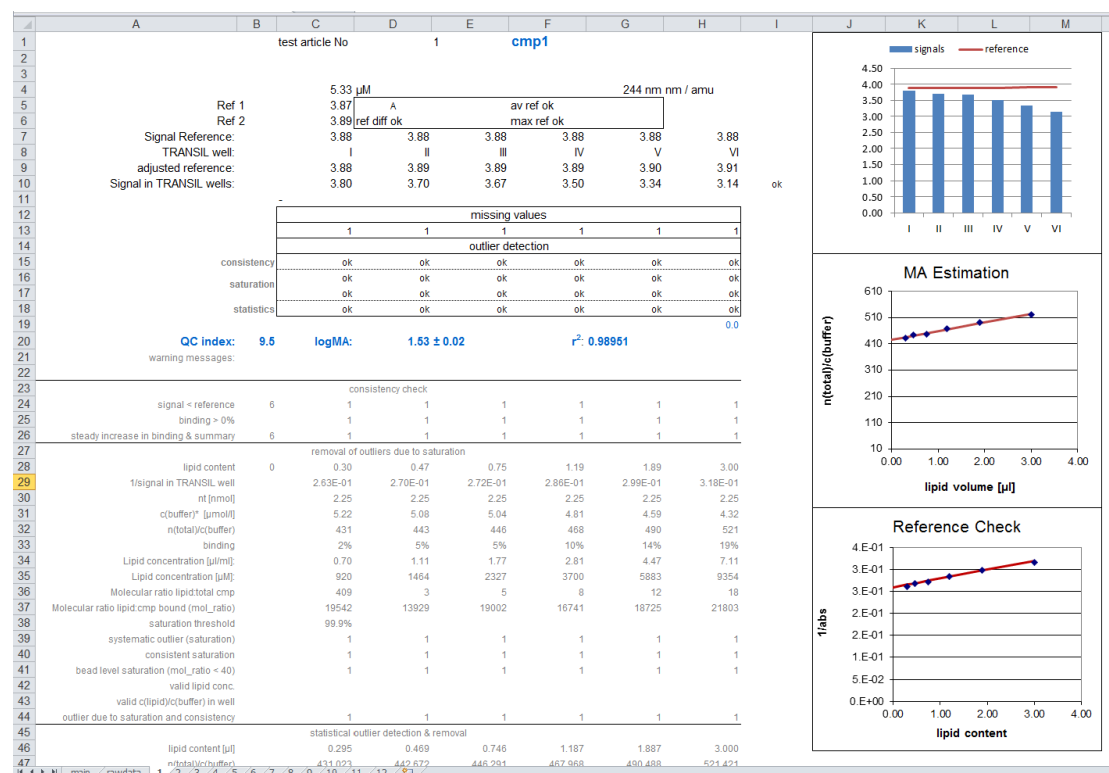


Figure 5: Screen shot of the details “1” tab of the spreadsheet for analyzing data from the TRANSIL<sup>XL</sup> Membrane Affinity Assay. The “1” tab shows calculated concentrations in each well and all calculations performed to derive the affinity to membranes as well as three plots indicating the experiment performance.

### 13.5 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.

#### 13.5.1 Model fit (intercept)

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

$$\frac{n_t}{c_b} = MA \cdot V_1 + V_b \quad (3)$$

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  $\pm 10\%$  around the true value a partial quality score of 10 is attributed. If the estimated buffer volume is within an interval  $\pm 50\%$  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.

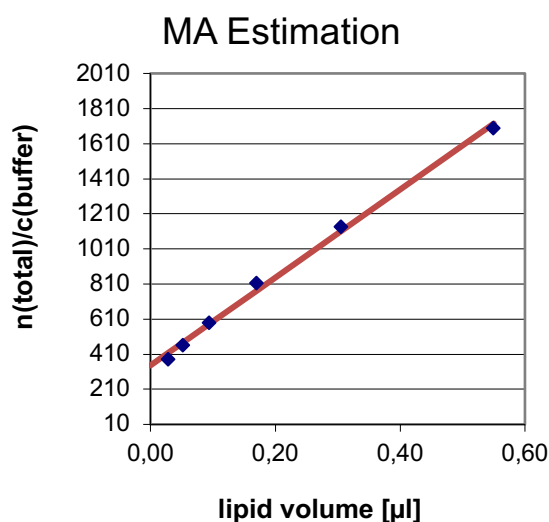


Figure 6: Illustration of fitting experimental data to equation (3) to determine the membrane affinity. A buffer volume of 500 µL has been used in the experiment, the intercept was estimated to be 346 µL, hence a quality score of 5 was attributed to the model fit.

#### 13.5.2 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.

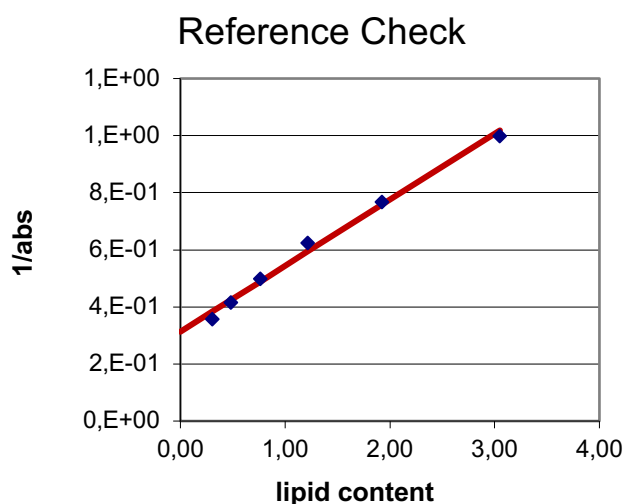


Figure 7: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the lipid content. The inverse of the intercept represents the expected peak area of the references.

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

### 13.5.3 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

---

#### 13.5.4 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

#### 13.5.5 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 of data points.

Table 4: Partial quality scores for the number of consistent 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

---

### 13.5.6 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.

### 13.5.7 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.

### 13.5.8 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

## 14 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.

## 15 Trouble shooting

### 15.1 Poor recovery

#### 15.1.1 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.

### 15.1.2 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.

## 15.2 Non-linearity of the response

### 15.2.1 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.

### 15.2.2 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 can provide an appropriate assay for high affinity compounds.
- 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 from 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.

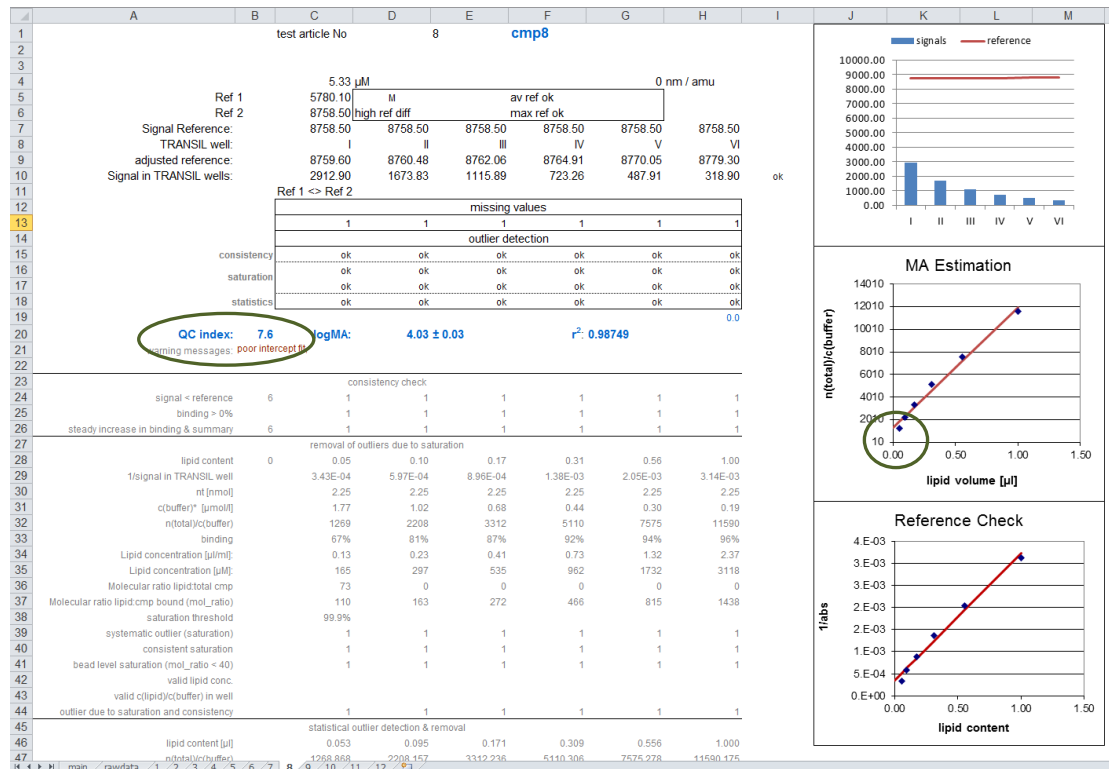


Figure 8: Illustration of the non-linear response issue which can be recognized by a poor intercept fit (green circles) which usually comes along with a curved plot of  $n(\text{total})/c(\text{buffer})$  versus lipid volume plot for the MA estimation. Both the poor intercept fit and the deviation from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

### 15.3 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.

#### 15.3.1 Challenges and problem identification

Low affinity compounds yield supernatant concentrations in the assay that deviate only marginally from the reference signals (Figure 9).

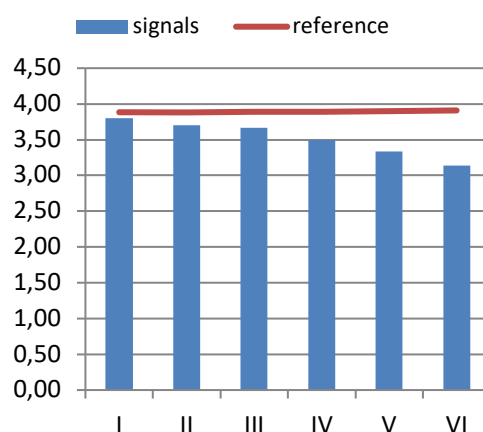


Figure 9: Illustration of a bar plot of a compound exhibiting low membrane affinity (c.f. individual data analysis tabs of the spreadsheet). The blue bars show the detected signals in the supernatants of TRANSIL wells I to VI. As the compound distributes only weakly into the membranes, supernatant concentrations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

#### Technical Support

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