User Guide

TRANSIL

Membrane Affinity Kit

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1 Applications of TRANSIL Membrane Affinity kit

The TRANSIL 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.

2 Basic assay principle

The principle of the TRANSIL 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). The assay consists of 8 glass vials: Six vials 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 kit, the affinity of test compounds to the membrane is calculated from remaining free compound concentration in the supernatants. Any of the available detection systems, such as LC-MS/MS, scintillation counting, etc. can be used for quantification, as long as it can quantify µM concentrations in volumes of 200 µl or less.

Figure 1: Photograph of vials supplied with the TRANSIL Membrane Affinity kit. The assay uses 8 vials to determine the affinity of drug candidates to membranes.

3 Kit components

A TRANSIL Membrane Affinity Kit is composed of the following items:

No.	Qty.	Item
1	8	Eight glass vials 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	Instruction manual
3	1	CD with spreadsheet calculation

The product is shipped on dry ice to ensure that the beads remain in suspension and that the buffer volume remains constant for accurate quantitative analysis.

4 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

5 Reagents

The following reagents are required to run the TRANSIL Membrane Affinity assay:

No.	Reagent	Specification
1	DMSO	For preparation of 10x drug candidate stock solution
2	Dulbecco's PBS (1x)	For preparation of 10x drug candidate stock solution

6 Equipment

The following equipment is required to run the TRANSIL Membrane Affinity assay:

No.	Instrument	Specification
1	Vortexer	Supplied with a platform
2	Centrifuge	Including rotor adapters for glass vials

7 Vial preparation

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

Before use, thaw the glass vials at 4°C for a period of 12 hours (overnight) or, at 20°C for a period of 3 hours. Make sure the glass vials have reached room temperature (between 20° and 25°C) prior to assay. Alternatively, the glass vials can be placed in a water bath for 15 minutes to reach a temperature of 20°C to 25°C.

The caps from the TRANSIL Membrane Affinity assay vials should only be removed, immediately before adding the compounds for testing and the vials should be capped again after adding the test compounds.

8 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:

8.1 Drug candidate stock Solutions

Concentration: The TRANSIL 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 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 120 µl per compound. It is necessary to have at least 80 µl of the stock solution for each compound drug candidate since to each of the 8 vials 10 µl of the compound is added.

8.2 DMSO concentration

The final assay concentration of DMSO should not exceed 5%.

9 Replicates

The TRANSIL Membrane Affinity kit assay is designed such that one compound utilizes 8 vials – two references and six vials 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

When the TRANSIL Membrane Affinity assay has reached room temperature and the test compounds are prepared, add 10 µl of test compound to each of the 8 vials.

10.2 Mixing

Mix the content of each vial by vortexing for 15 seconds. Make sure that the vials are closed tightly.

10.3 Incubation

Incubate the beads and compound suspension for 12 minutes while rotating vials. Alternatively, mix the vials every 3 minutes by quickly vortexing (< 5 sec).

10.4 Separation of beads and buffer

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

10.5 Sampling of supernatant

Sample supernatant for quantification. Vials may be placed directly in an autosampler for analysis.

11 Sample quantification

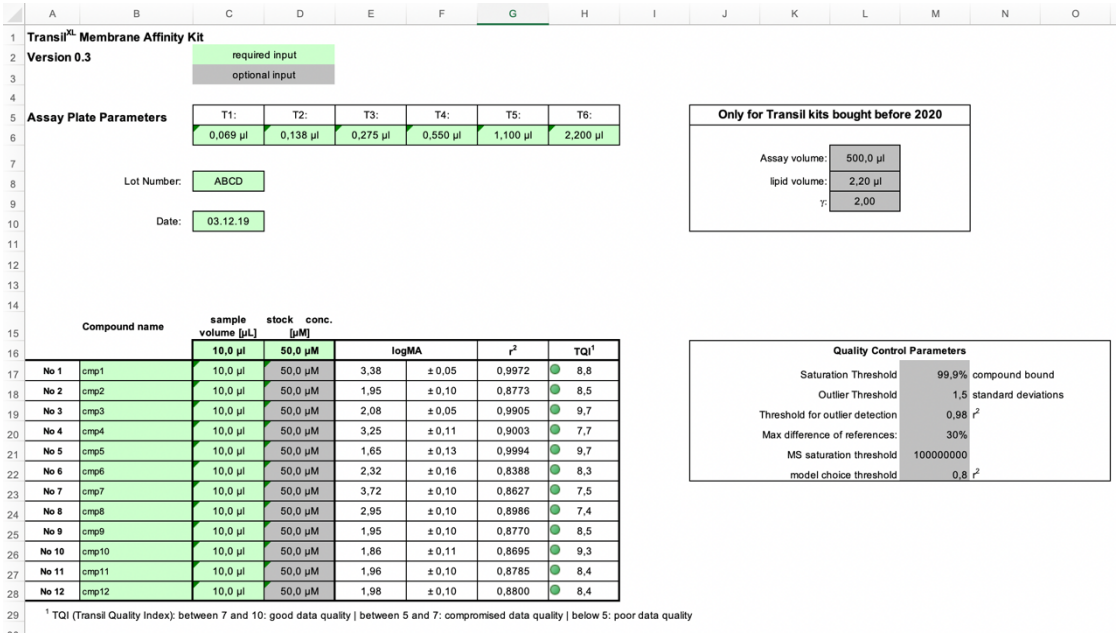
Use your analytical technique of choice for quantifying the compound concentration in the supernatant obtained in the last assay step.

12 Data analysis

Open the supplied spreadsheet for data analysis and follow the steps below to obtain the results for the TRANSIL Membrane Affinity 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 2).

12.1 Assay parameters

Open the “main” tab and enter the assay parameters in the row C6 to H6. Enter the lot specific parameters from the certificate of analysis that came with the assay. Also, enter the lot number and the assay date.



Transil^{XL} Membrane Affinity Kit

Version 0.3

required input

optional input

Assay Plate Parameters

T1:	T2:	T3:	T4:	T5:	T6:
0,069 µl	0,138 µl	0,275 µl	0,550 µl	1,100 µl	2,200 µl

Lot Number: ABCD

Date: 03.12.19

Only for Transil kits bought before 2020

Assay volume:	500,0 µl
lipid volume:	2,20 µl
γ:	2,00

Compound name		sample volume [µL]	stock conc. [µM]	logMA		r^2	TQI[1]
		10,0 µl	50,0 µM				
No 1	cmp1	10,0 µl	50,0 µM	3,38	± 0,05	0,9972	8,8
No 2	cmp2	10,0 µl	50,0 µM	1,95	± 0,10	0,8773	8,5
No 3	cmp3	10,0 µl	50,0 µM	2,08	± 0,05	0,9905	9,7
No 4	cmp4	10,0 µl	50,0 µM	3,25	± 0,11	0,9003	7,7
No 5	cmp5	10,0 µl	50,0 µM	1,65	± 0,13	0,9994	9,7
No 6	cmp6	10,0 µl	50,0 µM	2,32	± 0,16	0,8388	8,3
No 7	cmp7	10,0 µl	50,0 µM	3,72	± 0,10	0,8627	7,5
No 8	cmp8	10,0 µl	50,0 µM	2,95	± 0,10	0,8986	7,4
No 9	cmp9	10,0 µl	50,0 µM	1,95	± 0,10	0,8770	8,5
No 10	cmp10	10,0 µl	50,0 µM	1,86	± 0,11	0,8695	9,3
No 11	cmp11	10,0 µl	50,0 µM	1,96	± 0,10	0,8785	8,4
No 12	cmp12	10,0 µl	50,0 µM	1,98	± 0,10	0,8800	8,4

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

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

Figure 2: Screen shot of the “main” tab of the spreadsheet for analyzing data from the TRANSIL Membrane Affinity kit. The “main” tab is used to enter lot specific data as well as for reporting final results, the TRANSIL Quality Index (TQI).

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 vial 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 in the stock solution).

12.3 Raw data from sample quantification

Open the tab “raw data” and enter the peak areas or heights for each vial in column G (Figure 3). Note that column A lists the name of the compound used in each vial. Caution: Make sure to begin data entry in field G6 for the first vial (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	**Please enter the peak area or concentration data in column G below**									
2										
3		**Please leave missing data fields blank**								
4										
5	**test article**				**Well**	**Sample**	**Area / height**	**nm / amu**	**Note**	
6	**cmp1**				A-1	Ref 1	666700	376.2 / 165.2		
7	**cmp1**				B-1	Well 1	129610			
8	**cmp1**				C-1	Well 2	61477			
9	**cmp1**				D-1	Well 3	32845			
10	**cmp1**				E-1	Well 4	16497			
11	**cmp1**				F-1	Well 5	8005.3			
12	**cmp1**				G-1	Well 6	5755.3			
13	**cmp1**				H-1	Ref 2	756790			
14	**cmp2**				A-2	Ref 1	320380	285.2 / 154.1		
15	**cmp2**				B-2	Well 1	207800			
16	**cmp2**				C-2	Well 2	141330			
17	**cmp2**				D-2	Well 3	94802			
18	**cmp2**				E-2	Well 4	56409			
19	**cmp2**				F-2	Well 5	19827			
20	**cmp2**				G-2	Well 6	10118			
21	**cmp2**				H-2	Ref 2	344770			
22	**cmp3**				A-3	Ref 1	220390	319.3 / 200.2		
23	**cmp3**				B-3	Well 1	37780			
24	**cmp3**				C-3	Well 2	17251			
25	**cmp3**				D-3	Well 3	8882			
26	**cmp3**				E-3	Well 4	1529.1			
27	**cmp3**				F-3	Well 5	2531.6			
28	**cmp3**				G-3	Well 6	1632.9			
29	**cmp3**				H-3	Ref 2	241230			
30	**cmp4**				A-4	Ref 1	1982300	375.4 / 201.2		
31	**cmp4**				B-4	Well 1	672010			
32	**cmp4**				C-4	Well 2	352130			
33	**cmp4**				D-4	Well 3	201500			
34	**cmp4**				E-4	Well 4	113540			
35	**cmp4**				F-4	Well 5	43277			
36	**cmp4**				G-4	Well 6	20438			
37	**cmp4**				H-4	Ref 2	2166300			
38	**cmp5**				A-5	Ref 1	168130	276.4 / 231.2		
39	**cmp5**				B-5	Well 1	24980			
40	**cmp5**				C-5	Well 2	10945			
41	**cmp5**				D-5	Well 3	5829.5			
42	**cmp5**				E-5	Well 4	3192.7			

Figure 3: Screen shot of the "rawdata" tab of the spreadsheet for analyzing data from the TRANSIL Membrane Affinity kit. The "rawdata" tab is used to enter peak area or concentration data from the supernatants of the assay 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:

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

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 vial:

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

The results for the membrane affinity are reported in column E17 to E28 along with the TRANSIL Quality Index in column H17 to H28.

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 vial 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 tab "1" with the index from 1 to 8 for each respective drug. Figure 4 illustrates the information reported on each individual drug tested.

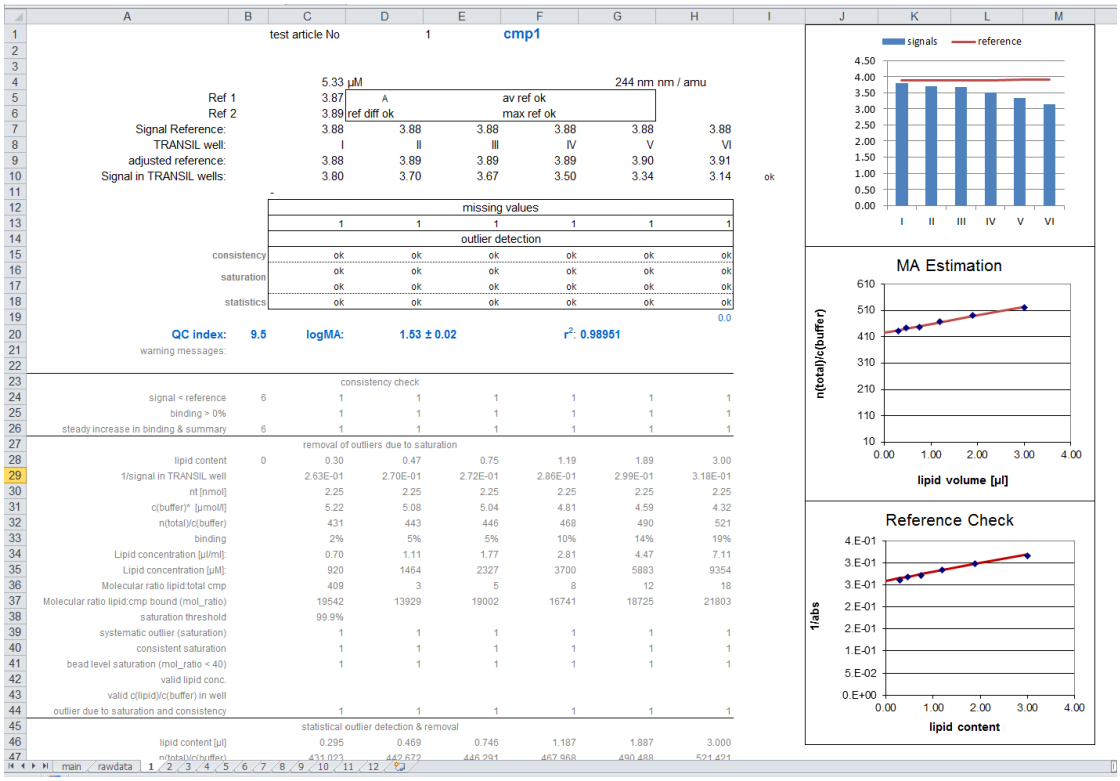


Figure 4: Screen shot of the details “1” tab of the spreadsheet for analyzing data from the TRANSIL Membrane Affinity kit. The “1” tab shows calculated concentrations in each vial and all calculations performed to derive the affinity to membranes as well as three plots indicating the experiment performance.

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:

$$\frac{n_t}{c_b} = MA \cdot V_l + V_b \qquad (4)$$

where MA denotes the membrane affinity, which is defined as the ratio $MA = c_l / c_b$. Fitting optimal data to equation (4) 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.

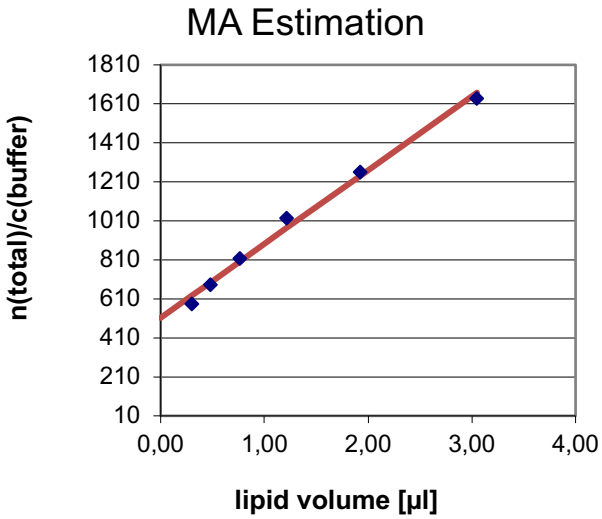


Figure 4: Illustration of fitting experimental data to equation (4) to determine the membrane affinity. A buffer volume of 422 µL has been used in the experiment, the intercept was estimated to 512 µL, hence a quality score of 5 was attributed to the model fit.

12.5.2 Match of measured versus predicted reference signal (ref)

When determining the membrane affinity via 5 or 6 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 vials by linear regression, since lipid binding can be assumed to be a non-cooperative process (Figure 5). This score has a weight of 3 in the TQI.

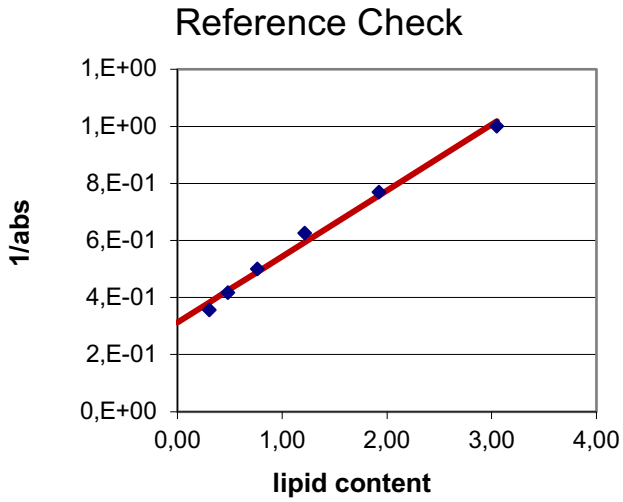


Figure 5: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the lipid content. The inverse of the intercept represents the expected peak area of the references.

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 vials. 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 (4) 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 (4).

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 (4).

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 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 vial 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 vial, 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 (4).

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

12.5.6 Slopes of binding

Data fitted to equation (4), plotted in Figure 4 as well as the percentage binding shall increase with increasing membrane surface area (Figure 5). Hence, the slopes of these graphs must all be positive. Most critical of all is the relationship of equation (4), if it has a positive slope it receives a vote of 10 points, otherwise zero. If the relationship plotted in Figure 5 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 5% and have a higher peak area than the measurements in the TRANSIL vials, the average references is computed and a partial score 10 is attributed. If the reference peak areas are higher than those of the TRANSIL vials, but differences between the two measurements exceed 5%, the maximum of the measurements is chosen. However, if the reference peak areas do not exceed the peak areas from the TRANSIL vials 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 vial. 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

13 Storage and shelf life

The plates 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, the vials should be used within 24h.

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 14.2).

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 6) may indicate non-linear response issues.

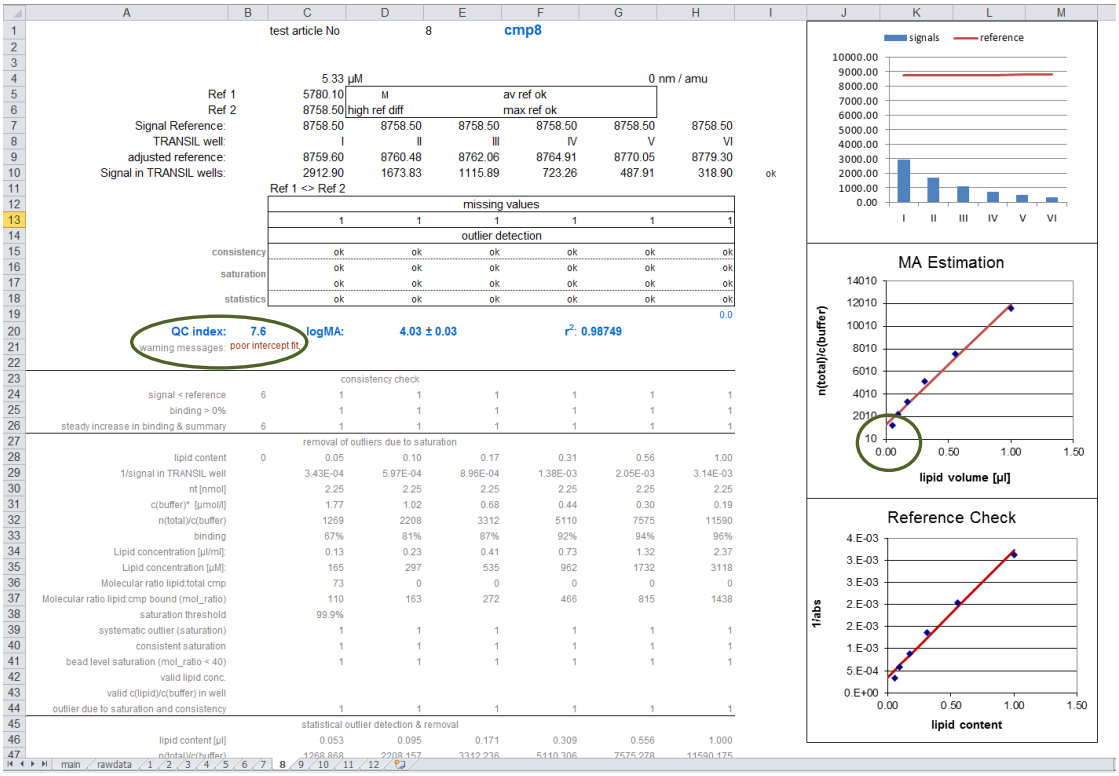


Figure 6: Illustration of the non-linear response issue which can be recognized by a poor intercept fit (green circles) which usually comes along with a curved plot of nt/cb versus lipid volume plot for the MA estimation. Both the poor intercept fit and the deviation from linearity in this plot are a good indication of the non-linear instrument response to decreasing compound concentrations.

14.2.2 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. 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.

14.3 Low Membrane affinity

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 7).

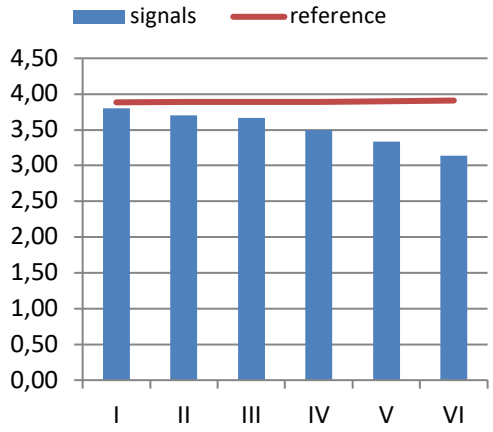


Figure 7: Illustration of a bar plot of a compound exhibiting low membrane affinity (c.f. individual data analysis tabs of the spreadsheet). The blue bars show the detected signals in the supernatants of TRANSIL wells I to VI. As the compound distribute only weekly into the membranes, supernatant concentrations 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 High TRANSIL Content Kit version of the TRANSIL High Sensitivity Binding kit. Hence, it is generally not necessary to obtain more accurate membrane affinity estimates when the affinity falls below the accuracy threshold of the standard TRANSIL Membrane Affinity kit. However, should a more accurate membrane affinity estimate be required contact Sovicell's support to receive a TRANSIL Membrane Affinity kit with adjusted TRANSIL content (higher than the standard kit).

Technical Support

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