SOVICELL
DRUG DISCOVERY TOOLS

User Guide

TRANSIL

Brain Absorption Kit

TMP-0110-2096

TMP-0110-2196

Version 3, Revision 06

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Part No. TMP-0110-2096M

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 1 µM final assay concentration. This requires 16 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

Early assessment of drug candidate availability in the Central Nervous System (CNS) is essential for CNS drugs development and useful for optimizing the toxicity profile of non-CNS drugs (Hitchcock & Pennington, 2006; Di et al., 2008). Designing pharmaceutical agents, so that they pass the blood-brain barrier and are freely available to interact with receptors, is one of the great challenges in CNS drug development (Alavijeh et al., 2005). This is because more than 98% of all new candidates do not cross the blood-brain barrier efficiently (Teresaki & Pardridge, 2000). Hence, one of the significant challenges in treating CNS conditions is drug passage across the blood-brain barrier (Pardridge, 1997, Tamai & Tsuji, 2000). However, at least equally important is the extent of brain tissue binding (Maurer et al., 2005; Summerfield et al., 2007). The stronger the binding of the drug candidate to the brain tissue, the lower will be the unbound fraction of the drug that can freely interact with the target receptors. The unbound fraction greatly influences the extent of the free drug concentration in brain which ultimately interacts with the target receptors. Therefore, investigation of drug disposition into brain as well as brain tissue binding will improve efforts in drug discovery.

2.1 Rate and Extend of Brain Penetration

Rate and extent of drug absorption into the brain are considered key parameters for the evaluation of CNS drug likeness. Several end-points have been used to estimate brain disposition in terms of rate and extent. The rate of drug absorption from blood to brain can be of direct relevance, in particular for indications such as antiepileptic agents where a fast action is desired. However, the rate is not directly related to the time a drug reaches its equilibrium distribution between blood and brain. It has been noted that drugs with low permeability rate can reach very rapid equilibrium in vivo (Hammarlund-Udenaes et al., 1997). Notwithstanding these difficulties in the direct interpretation of rate measurements the rate of drug uptake into brain is often considered proportional to the extent of brain penetration. Thus, rate measurements frequently replace extent measurements based on the distribution coefficient between brain and plasma (B/P ratio or on the log scale logBB), because it became apparent that the distribution coefficient varies over a wide range, even for highly efficacious CNS drugs.

Estimating extent of brain penetration from the rate of brain penetration requires several assumptions about influx, efflux, clearance rates, capillary surface areas, and transporter density that render such estimates somewhat inaccurate. Moreover, it has been

shown that the key problem with the extent estimate based on the brain-to-plasma distribution coefficient is its deficiency in predicting the unbound drug concentration that is freely available to interact with receptors in the brain's interstitial fluid (Maurer et al., 2005, Summerfield et al., 2006). Extent estimates based on permeability rates fall short of the same problem. Hence, it is more promising to combine a high quality estimate of the extent of drug absorption with an estimate of brain tissue binding, because tissue binding affects the free concentration in brain more effectively than the total concentration in brain (Lui et al., 2008). In fact, the better a drug is able to penetrate the brain, the greater the likelihood that it will be tightly bound to the brain tissue. Drugs entering the brain more easily tend to be more lipophilic, and since the brain's dry mass is dominated by lipids they will bind to the lipid fraction of the brain to a greater extent than less lipophilic compounds.

2.2 Brain Availability

Relative differences in drug candidates' free concentration can be predicted by the drug's brain-to-plasma distribution (B/P ratio) and it's unbound fraction in the brain. In fact, brain availability, *A*, defined as the product of the B/P ratio and the free fraction fu of the drug in brain is proportional to the free concentration in brain:

$$A = \frac{[D]_{brain}}{[D]_{plasma}} \cdot f_u(D)_{brain} \propto c_u(D)_{brain} \qquad \text{(I)}$$

where $[D]_{brain}$ denotes the total concentration of drug in brain and $[D]_{plasma}$ denotes the total concentration of drug in plasma, while $c_u(D)_{brain}$ denotes the free concentration of drug in brain. Hence, the availability of a drug in brain represents the fraction of drug entering the brain and being unbound and thus freely available to interact with receptors. This availability scale can be used to rank compounds by their potential effect at the receptor site.

The free concentration $c_u(D)_{brain}$ further depends upon the drug's dosage which is related to its pharmacodynamic properties. Consequently, the availability scale can be further improved by relating the fraction of drug entering the brain and being unbound to the drug's potency, i.e. it's inhibition constant. This yields the efficacy, *E*, of drugs in brain and can be obtained by dividing the candidates' availability by their inhibition constant K_i:

$$E = \frac{A}{K_i} \qquad \text{(II)}$$

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This efficacy scale, *E*, expresses the dosage independent effect of a drug. A low *E* value requires a high drug dosage while a large *E* value requires only a small dosage. Hence, this scale is well suited for ranking compounds based on their efficacy and provides a means of relating data from toxicological studies to the drug's potency.

2.3 Estimation of $K_{p,uu,brain}$

The total brain-plasma concentration ratio denoted by $K_{p,b}$ or logBB has been the most widely used parameter for in-silico prediction of brain exposure. It is calculated as logarithm of the ratio of the concentration of the drug molecule in the brain to that in blood, at equilibrium. It basically measures the way the drug molecule partitions itself between the brain and the blood. Fridén et al (2009) define it as the concentration ratio of total drug in brain ($A_{t,b}$) over total drug in plasma (C_p):

$$K_{p,b} = \frac{A_{t,b}}{C_p} = 10^{logBB}. \quad \text{(III)}$$

While logBB gives an indication of how much drug enters the brain, it has little bearing on how much drug will be available at the receptor sites. Thus, correcting this ratio for the fraction of drug unbound yields a better understanding of the potential drug action.

Hence, the unbound brain-to-plasma concentration ratio $K_{p,uu,brain}$ is a more frequently used parameter for estimating the amount of free drug in brain ISF. It is defined as

$$K_{p,uu,b} = \frac{C_{u,brainISF}}{C_{u,p}} = \frac{K_{p,b}}{V_{u,b} \cdot f_{u,p}} \quad \text{(IV)}$$

Where $V_{u,b}$ is the unbound volume of distribution in brain and $f_{u,p}$ the unbound fraction of drug in plasma. Hammarlund-Udenaes et al (2007) defines $V_{u,b}$ as:

$$V_{u,b} = \frac{A_{t,b} - V_p \cdot C_{t,p}}{C_{u,b}} \quad \text{(V)}$$

Which can be simplified for blood free perfused brains ($V_{t,p}$=0):

$$V_{u,b} = \frac{A_{t,b}}{C_{u,b}} = \frac{C_b \cdot V_b}{C_{u,b}} = \frac{C_b \cdot V_b}{C_b \cdot f_{u,b}} = \frac{V_b}{f_{u,b}} \quad \text{(VI)}$$

Inserting (VI) in (IV) yields:

$$K_{p,uu,b} = \frac{1}{V_b} \cdot \frac{f_{u,b}}{f_{u,p}} \cdot K_{p,b} \quad \text{(VII)}$$

and inserting (III) in (VII) yields an expression for $K_{p,uu,brain}$ that can be computed from the kit's estimates:

$$K_{p,uu,b} = \frac{1}{V_b} \cdot \frac{f_{u,b}}{f_{u,p}} \cdot 10^{logBB} \quad \text{(VIII)}$$

Along with a species specific assumption about the brain volume V_b.

2.4 Estimating Brain Tissue Binding

Brain tissue binding is a key parameter for determining brain availability of drugs. Several methods are available for estimating tissue binding. Brain microdialysis evaluates tissue binding in situ. It is the most realistic and accurate method to measure the free concentration in brain, however, it requires extensive resources, cannot be easily applied to lipophilic compounds, and has a very low throughput.

The brain slice method is the second most realistic approach as it maintains the tissue integrity and thus minimizes binding to structures that may become exposed while preparing crude extracts as for dialysis with brain extract (Becker & Liu, 2006). However, this approach is also resource demanding and cannot be performed in high throughput format.

A more rapid alternative is dialysis with rat brain homogenate (Maurer et al., 2005). This approach allows fast assessment of brain tissue binding and is less labor intensive than the brain slice method. The main caveats of this method are the preparation of rat brains and the downstream analytical processes requiring quantification of compound concentrations in the lipid phase. The latter may lead to matrix effects such as ion suppression in mass spectrometry and consequently to unreliable results.

The TRANSIL Brain Absorption assay kit is a matrix free method that yields results comparable to dialysis with brain homogenate, while it is simple to perform, requires fewer resources and is therefore more economical and is easily adoptable to automation for high throughput screening.

2.5 Estimating Brain-to-Plasma Distribution

The B/P ratio measures how much drug crosses the blood-brain barrier. Taken alone this measure is difficult to interpret. As mentioned above, compounds which cross the blood-brain barrier more readily than others have a high tendency to bind to brain tissue. Marketed CNS drugs span at least a range of B/P ratios from 0.1 to 40. Thus, the marketed CNS drugs span a 400-fold range of B/P ratios while they span only a 5-fold range in free concentration in brain

(Lui et al. 2008). This indicates that while the B/P ratio alone is less helpful for predicting CNS druggability, the free fraction as well as the availability is a good indicator for CNS drug likeness.

Besides measuring the B/P ratio in vivo it can be predicted by the ratio of the free fraction of drug in plasma over the free fraction of drug in brain (Friden et al., 2007) or by a prediction model based on the drugs' polar surface area, plasma protein binding and affinity to brain membrane using TRANSIL Brain Absorption kit. Both predictions assume absence of active transport processes. Hence, significant influence of efflux transporters on brain-to-plasma distribution will result in overestimation of total brain concentration using either approach. For practical purposes this overestimation may not be critical, since successful CNS drugs on the market are not substrates for efflux transporters, and because in vitro evidence for efflux transport doesn't always manifest itself in vivo (Summerfield et al., 2006).

2.6 Active Transport

The pharmacokinetics of brain delivery is further complicated by active influx and efflux transport at the BBB. The mechanisms by which efflux transporters act will influence the brain pharmacokinetics in different ways (Begley, 2004). It has been suggested that the permeability limiting glycoprotein (Pgp), the best known and, according to the current understanding, the most important efflux transporter for exogenous substances, lowers brain drug concentrations through two mechanisms: a gate-keeper function that prevents molecules from entering the brain and an extrusion mechanism via which molecules already present in the cytoplasm of the BBB endothelial cells are transported back to the blood (Higgins & Linton, 2004). The effect on the brain concentration time profiles will depend on which of these two functions is predominant. It can also be speculated that some compounds are transported from the brain ISF into the endothelial cells by abluminal transporters and thereafter by luminal transporters from the BBB to the blood. Hence, transporters located at the two membranes of the BBB may work together to reduce or increase brain concentrations of certain compounds.

2.7 Lead Compound Prioritization

Several competing and complementary methods exist to assess active and passive brain disposition as well as brain tissue binding of drug candidates. Recent developments have provided new rationales and methods to assess crucial ADME properties of CNS drugs. Methods assessing drug disposition into the brain based on passive diffusion are generally much less resourced demanding than methods investigating the active transport components. If the active transport component is an efflux process, then its relevance depends upon the degree of passive influx and brain tissue binding. Only if the drug's availability is reasonably high will the efflux process significantly reduce the net disposition into the brain. Hence, for lead candidate selection of CNS drugs it will be most economic to estimate the availability or efficacy of drugs in brain based on passive permeability processes first and later focus on more labor intensive investigations of active transport processes and only on those drug candidates that have reasonably high CNS efficacies. This approach will save considerable amount of resources and significantly accelerate the ADME screening of lead optimization.

However, screening for CNS availability will not only be instrumental for CNS drug development. Also, drug discovery and development projects for other indication areas may benefit from inclusion of CNS availability in the lead optimization assay portfolio as this parameter is tightly linked to CNS side-effects. Non-CNS drugs that are highly available in brain will have high free concentrations in brain. Hence, the likelihood that those drugs will non-specifically interact with receptors and may cause typical CNS side effects such as nausea, headache, insomnia, etc. is substantially increased.

3 Applications of TRANSIL Brain Absorption assay kit

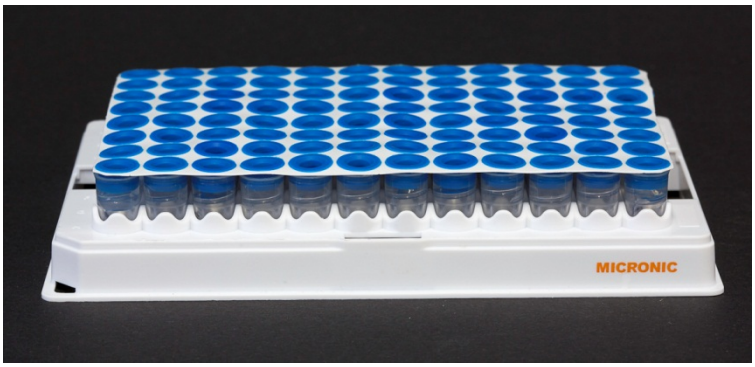
The TRANSIL Brain Absorption Assay kit will enable researchers to predict the extent of brain penetration of molecules. The innovative TRANSIL is an in-vitro assay designed to assess how such compounds interact with brain lipid membranes and estimates

i. the extent of brain penetration
ii. the extent of brain tissues binding and thus how much compound will be freely available in brain to bind to target receptors

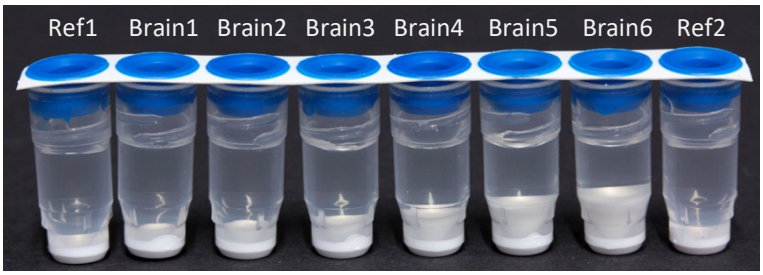
4 Basic assay principle

The principle of the TRANSIL Brain Absorption assay is to assess the affinity of test compounds to brain 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. A total of 8 wells of a tube unit/plate are used to determine the brain membrane affinity for each compound (Figure 1). Six wells contain brain 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 brain 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.

a)



b)



c)

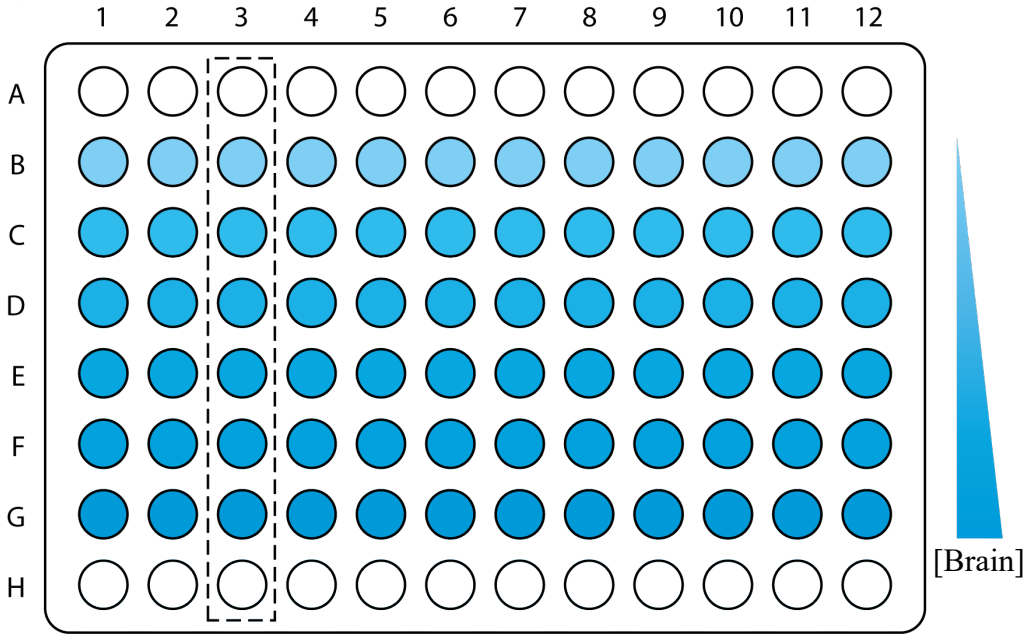


Figure 1: The TRANSIL Brain Absorption Kit uses a column of 8 wells to determine the affinity to brain membranes (Brain). a) Photography of the assay plate and b) the annotated tube units supplied. c) Illustration of the assay plate showing the reference rows A and H (white wells) as well as the increasing brain 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.

5 Kit components

A TRANSIL Brain Absorption 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 Brain Absorption 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

6 Abbreviations/ Glossary

A	Availability defined as $A = \frac{[D]_{brain}}{[D]_{plasma}} \cdot f_u(D)_{brain}$ is proportional to the free concentration in brain.
AGP	Human α_1 acid glycoprotein, synonymous to AAG. The physiological concentration of AGP varies between 0.36 and 1.46 g/L. In this assay a molar ratio of 1:24 is assumed between AGP and HSA.
B/P ratio	Brain to plasma distribution coefficient defined as $B/P = \frac{[D]_{brain}}{[D]_{plasma}}$. This ratio has been traditionally used to optimize the brain penetration of CNS drugs. However, the ratio varies more than 400-fold for highly efficacious CNS drugs. Therefore, its use for ranking drug candidates is limited. More meaningful is the brain availability (see also Availability) in conjunction with the potency of the drug (see also Efficacy).

Brain tissue binding	Brain tissue binding affects how much drug will be freely available to interact with receptors in the brain's interstitial fluid. Binding to brain tissue is primarily influence by interaction with the brain lipid membranes.
cmp	Compound
conc	Concentration
DMSO	Dimethyl sulfoxide
E	Efficacy defined as $E = \frac{A}{K_i}$ where A denotes the availability and K_i the drug's inhibition constant.
f_u(brain)	Unbound fraction of drug in brain tissue. The free concentration of drugs in brain has been demonstrated to correlate strongly with the drugs' pharmacodynamics. The estimate of the free fraction of drug in brain relates the total concentration of drug in brain to the free concentration in brain. The brain free fraction is determined by brain tissue binding.
HSA	Human Serum Albumin. The physiological concentration of HSA varies between 35 and 60 g/L with an average of 42 g/L.
ISF	Interstitial fluid of the brain. Compartment of the brain where most drug receptors are located.
K_D	Dissociation constant
K_i	Inhibition constant
HSA	Human Serum Albumin
logBB	Brain to plasma distribution coefficient on logarithmic scale. Defined as $\log BB = \log\left(\frac{[D]_{brain}}{[D]_{plasma}}\right)$. Refer to the B/P ratio for further details.

log$K_{b/f}$	Logit transformed plasma protein binding defined as the log of the ratio of bound fraction of the drug over the unbound fraction of the drug.
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
PPB	Plasma protein binding
PSA	Polar surface area used to indicate computed total polar surface area
TQI	TRANSIL Quality Index
r^2	Correlation coefficient
V_b	Buffer volume
V_l	Lipid volume

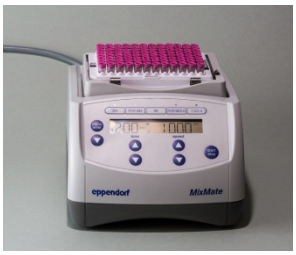
7 Reagents

The following reagents are required to run the TRANSIL Brain Absorption kit:

No.	Reagent	Specification
1	DMSO	For preparation of 16x drug candidate stock solution
2	Dulbecco's PBS (1x)	For preparation of 16x drug candidate stock solution

8 Equipment

The following equipment is required to run the TRANSIL Brain Absorption 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

9 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.

10 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 Brain Absorption 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 1 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.

11 Replicates

The TRANSIL Brain Absorption 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.

12 Assay procedure

The workflow of the TRANSIL Brain Absorption assay is illustrated in Figure 2.

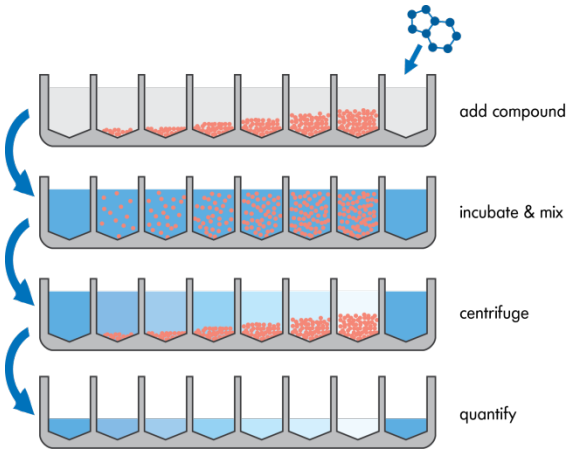


Figure 2: TRANSIL Brain Absorption Assay workflow: The same amount of drug is added to all wells followed by a mixing step. After 12 minutes incubation, beads are separated from the buffer by centrifugation and the remaining supernatant is sampled for quantification.

Follow the following 5 steps for the assay procedure:

12.1 Compound addition

Mix the compound stock solution carefully by vortexing. When the TRANSIL Brain Absorption 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.

12.2 Incubation

Incubate the plate with repeated aspiration and suspension to ensure proper mixing:

- Small molecules: 20 cycles
- Large molecules: 120 cycles

Alternatively, a plate shaker may be used @ 1000 RPM for 12 minutes at RT. 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 Separation of beads and buffer.

12.3 Separation of beads and buffer

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

12.4 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.

13 Sample quantification

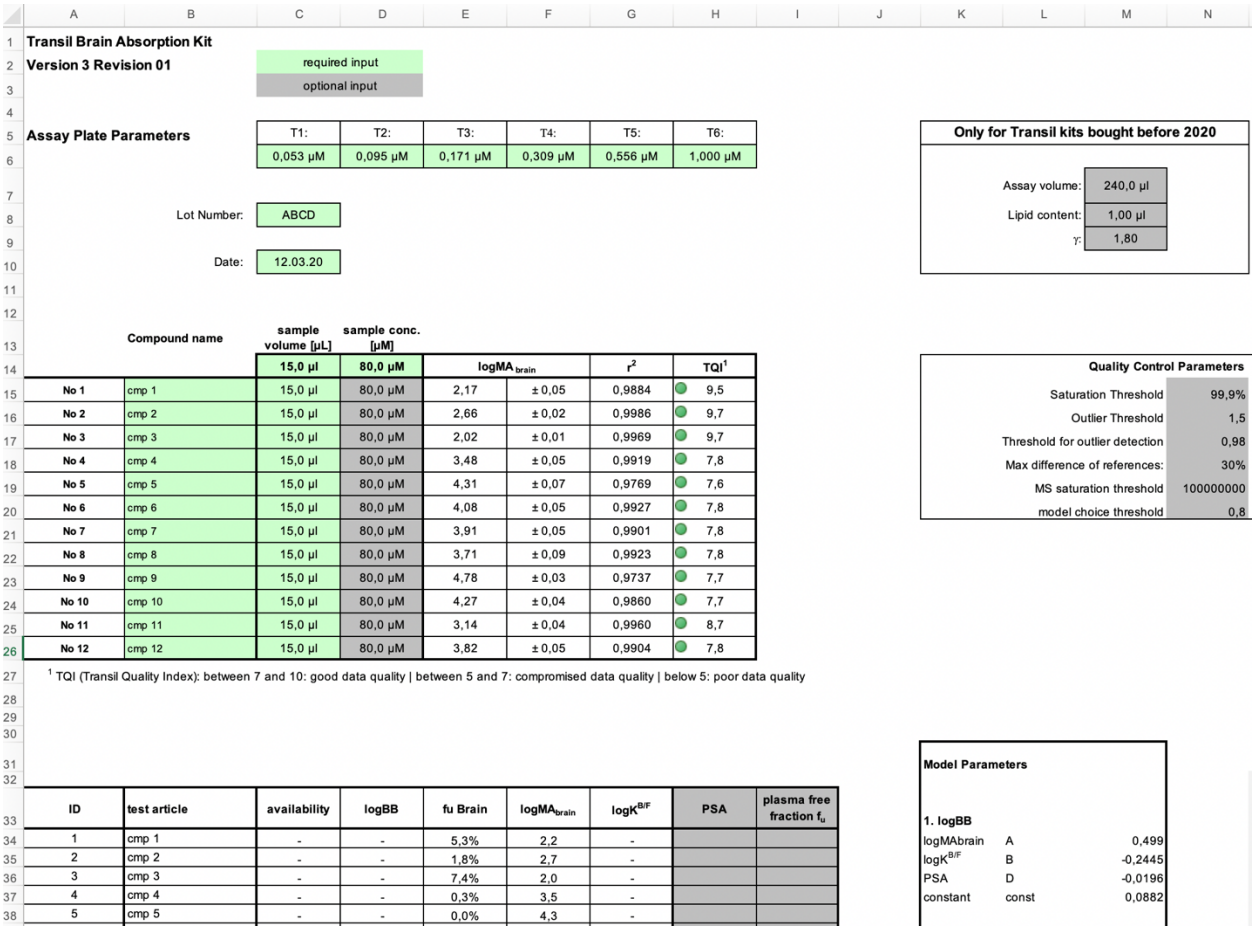
Use your analytical technique of choice for quantifying the compound concentration in the supernatant obtained in the last assay step.

14 Data analysis

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

14.1 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 plate. Also, enter the lot number and the assay date.



Transil Brain Absorption Kit
Version 3 Revision 01

required input
optional input

Assay Plate Parameters

T1:	T2:	T3:	T4:	T5:	T6:
0,053 µM	0,095 µM	0,171 µM	0,309 µM	0,556 µM	1,000 µM

Lot Number: ABCD

Date: 12.03.20

Only for Transil kits bought before 2020

Assay volume:	240,0 µl
Lipid content:	1,00 µl
γ:	1,80

	Compound name	sample volume [µL]	sample conc. [µM]	logMA brain		r^2	TQI[1]
		15,0 µl	80,0 µM				
No 1	cmp 1	15,0 µl	80,0 µM	2,17	± 0,05	0,9884	9,5
No 2	cmp 2	15,0 µl	80,0 µM	2,66	± 0,02	0,9986	9,7
No 3	cmp 3	15,0 µl	80,0 µM	2,02	± 0,01	0,9969	9,7
No 4	cmp 4	15,0 µl	80,0 µM	3,48	± 0,05	0,9919	7,8
No 5	cmp 5	15,0 µl	80,0 µM	4,31	± 0,07	0,9769	7,6
No 6	cmp 6	15,0 µl	80,0 µM	4,08	± 0,05	0,9927	7,8
No 7	cmp 7	15,0 µl	80,0 µM	3,91	± 0,05	0,9901	7,8
No 8	cmp 8	15,0 µl	80,0 µM	3,71	± 0,09	0,9923	7,8
No 9	cmp 9	15,0 µl	80,0 µM	4,78	± 0,03	0,9737	7,7
No 10	cmp 10	15,0 µl	80,0 µM	4,27	± 0,04	0,9860	7,7
No 11	cmp 11	15,0 µl	80,0 µM	3,14	± 0,04	0,9960	8,7
No 12	cmp 12	15,0 µl	80,0 µM	3,82	± 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

Quality Control Parameters

Saturation Threshold	99,9%
Outlier Threshold	1,5
Threshold for outlier detection	0,98
Max difference of references:	30%
MS saturation threshold	100000000
model choice threshold	0,8

ID	test article	availability	logBB	fu Brain	$logMA_{brain}$	$logK^{B/F}$	PSA	plasma free fraction f_u
1	cmp 1	-	-	5,3%	2,2	-		
2	cmp 2	-	-	1,8%	2,7	-		
3	cmp 3	-	-	7,4%	2,0	-		
4	cmp 4	-	-	0,3%	3,5	-		
5	cmp 5	-	-	0,0%	4,3	-		

Model Parameters

1. logBB		
logMAbrain	A	0,499
$logK^{B/F}$	B	-0,2445
PSA	D	-0,0196
constant	const	0,0882

Figure 3: Screen shot of the “main” tab of the spreadsheet for analyzing data from the TRANSIL Brain Absorption 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 brain.

14.2 Compound information

Please enter the compound names in the column B17 to B28 of the “main” tab. Enter the sample volume added (15µl) 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).

14.3 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		

main rawdata 1 2 3 4 5 6 7 8 9 10 11 12

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

14.4 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.

14.4.1 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.

14.4.2 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.

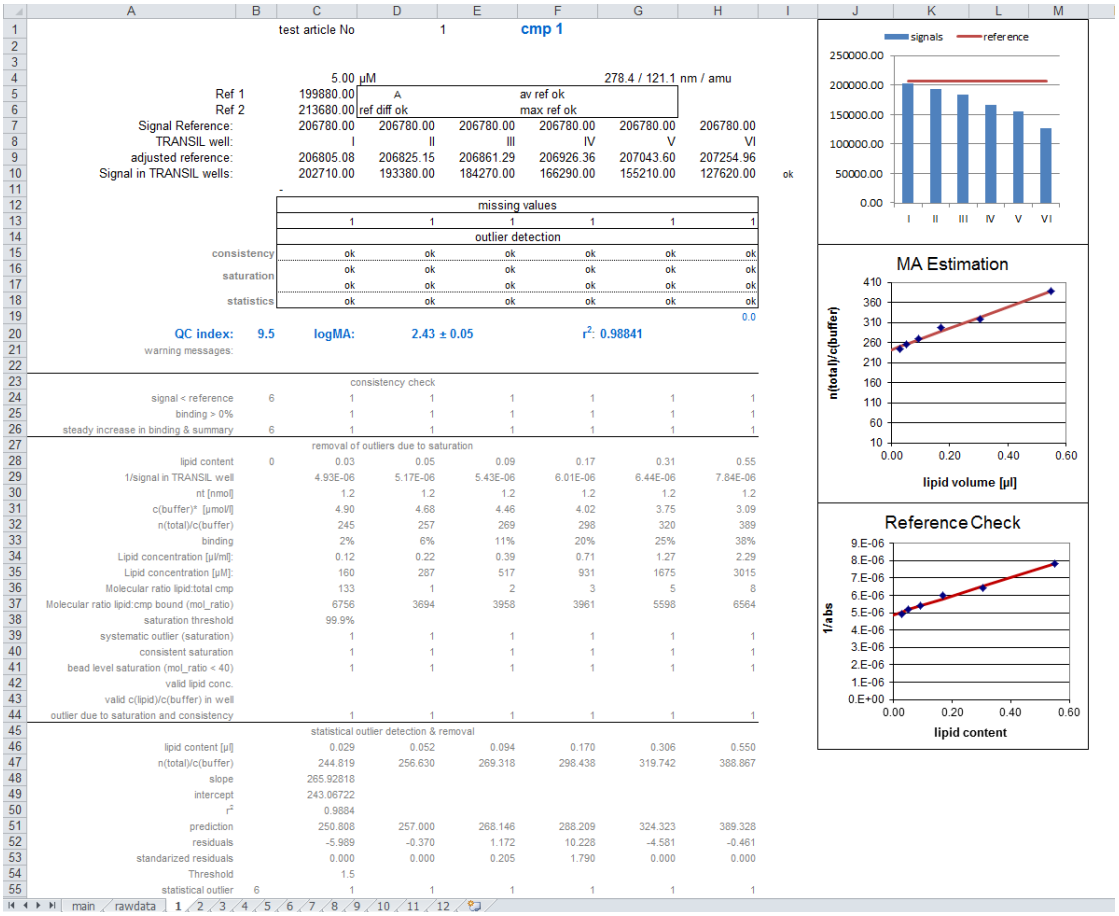


Figure 5: Screen shot of the details "1" tab of the spreadsheet for analyzing data from the TRANSIL Brain Absorption Assay kit. The "1" tab shows calculated concentrations in each well and all calculations performed to derive the affinity to membranes as well as three plots indicating the experiment performance.

14.4.3 Prediction of logBB and the brain free fraction

Hybrid models are used to predict the brain free fraction and the distribution coefficient between brain and plasma. The free fraction of drug in brain is calculated according to the following equation involving the measurement of the brain membrane affinity:

$$f_{u,brain} = 10^{b_1 \cdot \log MA_{brain} + b_2}. \quad (4)$$

The free fraction of drug in brain is reported in column E36 to E47 of the "main" tab of the supplied spreadsheet.

The distribution coefficient between brain and plasma calculated according to a hybrid model based on the brain membrane affinity, the logit transformed plasma protein binding, and the polar surface area:

$$\log BB = a_1 \cdot \log MA_{brain} + a_2 \cdot \log K_{b/f} + a_3 \cdot PSA + a_4 \quad (5)$$

The brain-to-plasma distribution coefficient logBB is reported in column D36 to D47 of the "main" tab of the spreadsheet. For the calibration of the model we used an algorithm for predicting the polar surface area provided by Molinspiration (http://www.molinspiration.com/services/psa.html).

For predicting the brain free fraction and the brain-to-plasma distribution coefficient the spreadsheet uses the parameters listed in Table 1. The parameters are given in the area K33 to M43 of the spreadsheet and can be adjusted if necessary.

Table 1: Model parameters for predicting the brain free fraction and the brain-to-plasma distribution coefficient.

Parameter	Value
a_1	0.4990
a_2	-0.2445
a_3	-0.0196
a_4	0.0882
b_1	-0.9586
b_2	0.8054

14.5 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.

14.5.1 Model fit (intercept)

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

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

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.

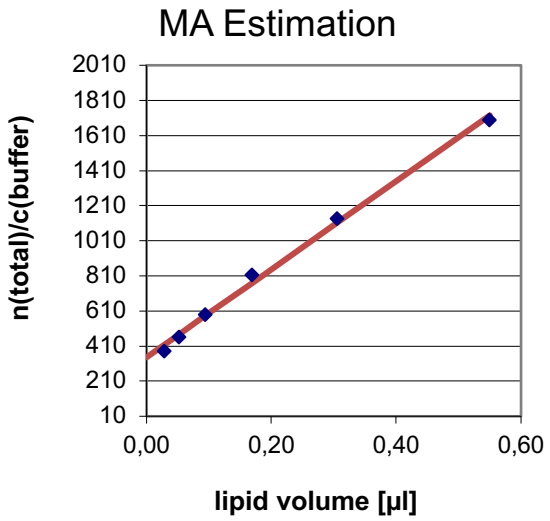


Figure 6: Illustration of fitting experimental data to equation (3) to determine the membrane affinity. A buffer volume of 240 µL has been used in the experiment, the intercept was estimated to 346 µL, hence a quality score of 5 was attributed to the model fit.

14.5.2 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.

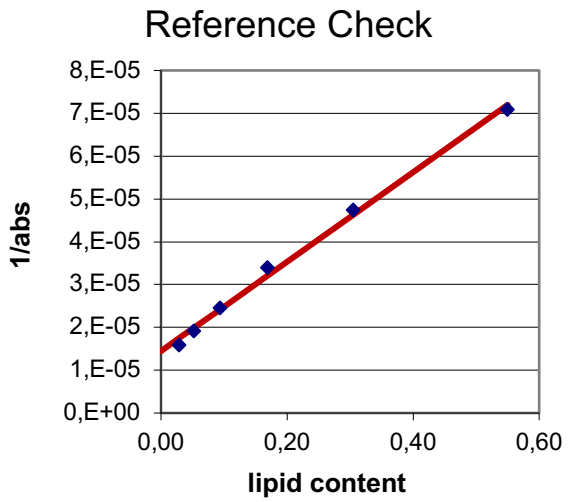


Figure 7: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the lipid content. The inverse of the intercept represents the expected peak area of the references.

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

Table 2: 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

14.5.3 Correlation coefficient (r^2)

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

Table 3: 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

14.5.4 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 4). This score has a weight of 2 in the TQI.

Table 4: 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

14.5.5 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 5. This score has a weight of only 1 in the TQI as it may affect also the number or data points.

Table 5: 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

14.5.6 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.

14.5.7 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.

14.5.8 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 6).

Table 6: 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

15 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.

16 Trouble shooting

16.1 Poor recovery

16.1.1 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.

16.1.2 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 16.2).

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.

16.2 Non-linearity of the response

16.2.1 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.

16.2.2 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 Brain Absorption Kit for high affinity compounds (TMP-0110-2296).

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.

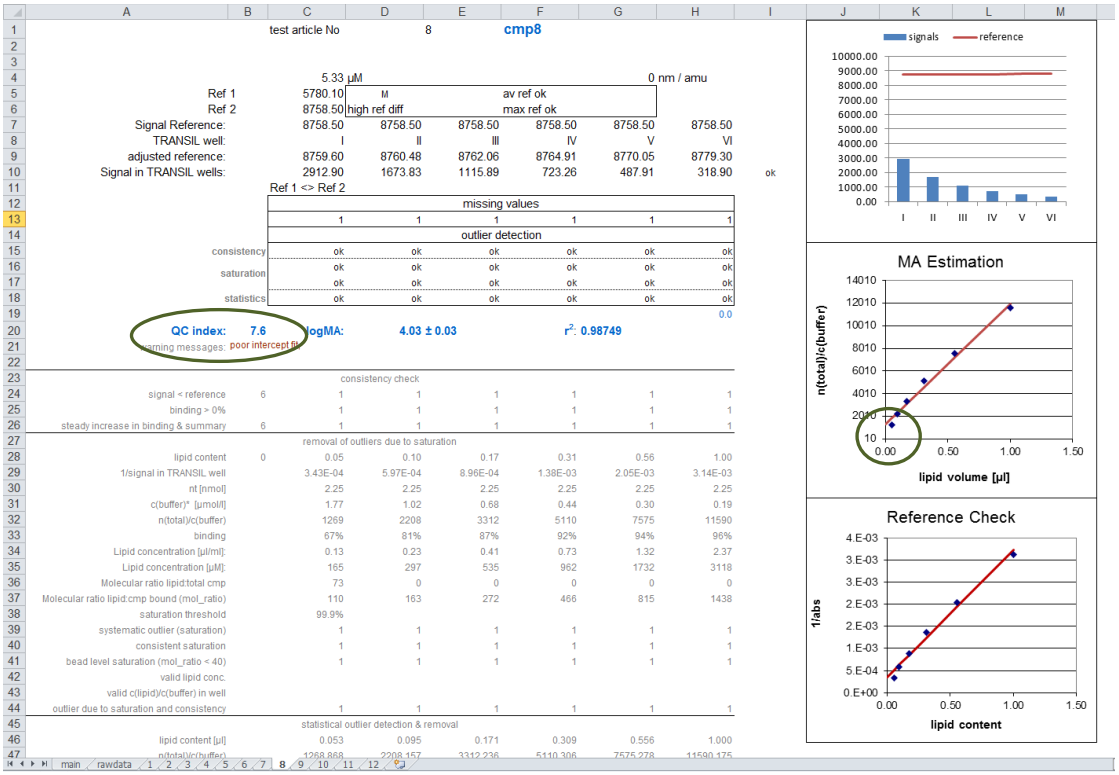


Figure 8: Illustration of the non-linear response issue which can be recognized by a poor intercept fit (green circles) which usually comes along with a curved plot of nt/cb versus lipid volume plot for the MA estimation. Both the poor intercept fit and the deviation from linearity in this plot are a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

16.3 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.

16.3.1 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).

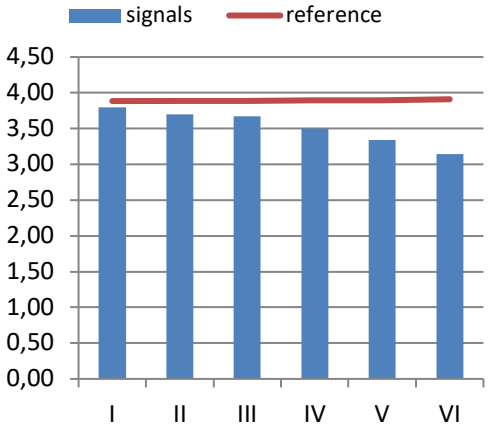


Figure 9: Illustration of a bar plot of a compound exhibiting low membrane affinity (c.f. individual data analysis tabs of the spreadsheet). The blue bars show the detected signals in the supernatants of TRANSIL wells I to VI. As the compound distributes only weekly into the membranes, supernatant concentrations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

16.3.2 Problem-solving approaches

In case the membrane affinity turns out to be below 100 (logMA < 2) we recommend using the TRANSIL Brain Absorption Kit - low affinity compounds (Product No. TMP-0110-2196). 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

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