User Guide

TRANSIL

MSA Binding Kit

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Version 2, Revision 02

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Contents

1 Quick Protocol

1. Plate Thawing and preparation

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

2. Drug Candidate Preparation

- Prepare 16x stock solutions of each compound in 32% solvent (e.g. DMSO) - yields a final solvent conc. of 2%.
- The final compound concentration in the assay depends on the compounds solubility, analytical method and instrumentation: If permitted by compound solubility use 5 µM final assay concentration. This requires 80 µM stock solutions.
- Since each compound is added in an aliquot of 15 µl to each well of an 8-well tube unit, at least 120 µl stock solution are required for each compound. Allow an additional 80 µl for accurate pipetting.

3. Drug Candidate Addition

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

4. Incubation and Supernatant Sampling

- Incubate the plate for 12 minutes by inverting on a rotator or manual rotation.
- 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

According to the free drug hypothesis, only the unbound fraction (fu) of a drug can cross biological membranes, interact with pharmacological targets, or undergo metabolic elimination. Many drugs – particularly lipophilic compounds – bind extensively to plasma proteins, which can substantially reduce the circulating free drug concentration. Under equilibrium conditions, the unbound drug concentration is expected to be similar in plasma and tissues; however, active transport processes and intracellular binding can lead to deviations from this relationship.

In preclinical pharmacokinetic studies, plasma proteins from the respective species, such as mouse, play an analogous role in drug binding. The most important plasma proteins involved in drug binding are serum albumin (in mouse: MSA) and α1-acid glycoprotein (AGP). Serum albumin is the most abundant plasma protein in mouse plasma (typically 20–40 g/L) and accounts for the majority of drug binding. It contains multiple drug-binding regions, including the well-characterized Sudlow site I (warfarin site) and Sudlow site II (benzodiazepine site), which accommodate a wide range of structurally diverse compounds. Differences in albumin binding between species can lead to differences in free drug exposure and pharmacokinetics, making species-specific binding data important for translational studies.

AGP is present at much lower baseline concentrations in mouse plasma (typically 0.05–0.3 g/L), but as an acute-phase protein its levels can increase several-fold during inflammation, infection, or stress. Variations in AGP concentration can therefore alter the free fraction of drugs and influence pharmacokinetic behavior. Plasma protein binding data are therefore routinely integrated into pharmacokinetic and pharmacodynamic models used during drug discovery and development.

Because plasma protein binding strongly influences drug exposure, clearance, distribution, and drug–drug interactions, it is routinely assessed during early drug discovery and development. Conventional techniques used to determine plasma protein binding include equilibrium dialysis, ultrafiltration, and ultracentrifugation. Although widely used, these approaches can be time-consuming, analytically demanding, and difficult to apply in high-throughput settings. In particular, accurate determination of very low free fractions often requires highly sensitive analytical methods such as LC-MS/MS or radiolabeled compounds.

Many modern test compounds, including lipophilic small molecules, macrocycles, and targeted protein degraders, exhibit very high plasma protein binding, often resulting in free fractions below 1%. Accurate characterization of such compounds can be analytically challenging using conventional dialysis-based techniques.

Conventional plasma protein binding methods such as equilibrium dialysis may also be affected by membrane adsorption, slow equilibration, compound instability, or non-specific binding to experimental materials, which can complicate the interpretation of binding measurements.

Plasma protein concentrations may vary substantially between individuals and disease states, particularly for AGP, which is an acute-phase protein. Such variability can alter the free fraction of drugs and contribute to interindividual variability in pharmacokinetics.

Assays based on purified proteins provide a well-defined experimental system in which protein concentration and composition are controlled, enabling reproducible and mechanistically interpretable measurement of binding parameters and facilitating mechanistic interpretation.

The increasing diversity of therapeutic modalities, including peptides, macrocycles, and targeted protein degraders, further emphasizes the need for robust and scalable methods for characterizing protein binding across a wide range of molecular properties.

In contrast to direct measurement of plasma free fraction, determining the dissociation constant (K_D) of drug–protein interactions provides a mechanistic description of binding that can be used to predict plasma protein binding under different protein concentrations and physiological conditions. This allows binding estimates to be adjusted for varying albumin or AGP concentrations, which frequently occur in disease states or different patient populations.

The TRANSIL MSA and TRANSIL AGP Binding Assays were developed to exploit this principle. The assays determine K_D values by titrating defined concentrations of purified plasma proteins against a constant drug concentration. By measuring binding to MSA and AGP separately under controlled conditions, the method enables rapid and reproducible estimation of plasma protein binding without the long equilibration times required by traditional dialysis-based techniques.

Because the assay measures binding to purified proteins under defined conditions, it isolates the intrinsic affinity of the drug–protein interaction, whereas plasma-based

measurements reflect a mixture of binding processes that depend on plasma composition and experimental conditions.

3 Applications of the TRANSIL MSA Binding Kit

The TRANSIL MSA Binding Kit enables researchers to predict the extent of plasma protein binding of molecules in preclinical species such as mouse. Measurement of albumin binding in mouse supports interpretation of pharmacokinetic data in preclinical studies and enables translation between preclinical and human exposure. The innovative TRANSIL assay is an *in vitro* kit designed to assess the affinity of drugs to mouse serum albumin. This enables the researcher to predict the fraction bound to plasma proteins in a highly defined and pH stable assay system.

3.1 Simulation of plasma binding in various disease states

In conjunction with the TRANSIL AGP Binding kit you obtain the dissociation constants K_D of your test items for both albumin and AGP. This allows you to assess plasma protein binding in populations with non-standard plasma composition and in preclinical models and disease states with altered plasma protein composition.

3.2 High-Throughput Lead Optimization

Because the assay provides results in 12 minutes and is fully automatable, it is uniquely positioned for early-stage lead optimization.

- **Rapid SAR Support:** Use the kit to quickly rank-order large chemical libraries based on their albumin binding affinity to guide medicinal chemistry efforts. Albumin binding data can also be used to interpret structure–property relationships, since small structural changes often lead to measurable changes in protein affinity.
- **Efficiency:** Unlike dialysis, the minimal labor requirements allow for the screening of dozens of compounds per day by a single operator.

3.3 Potency Normalization in Cell-Based and Enzyme Assays

The presence of albumin in assays can mask the true potency of a test compound.

- **"True" IC_{50} Determination:** Use the MSA binding data to calculate the effective free concentration of a compound in serum-containing media.

- **In Vitro to In Vivo Translation:** Correcting for MSA binding allows for a more accurate prediction of how a compound will behave in preclinical in vivo studies where protein binding is a major factor.

3.4 Pharmacokinetic (PK) Parameter Prediction

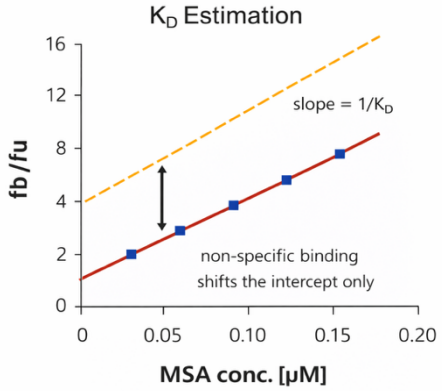
Albumin binding is an important determinant of a drug's pharmacokinetic profile.

- **Volume of Distribution V_d:** MSA binding together with lipophilicity and membrane affinity affect a test item's volume of distribution.
- **Half-life $t_{1/2}$:** Use the K_D values determined by the TRANSIL kit to estimate potential drug half-life, as highly bound drugs often exhibit slower clearance.

3.5 Working with Sticky or Low-Recovery Compounds

Traditional methods like equilibrium dialysis often fail when compounds stick to plasticware or membranes.

- **Robustness:** The TRANSIL algorithm is specifically designed to be robust against low recovery, providing reliable affinity estimates even when recovery is low. The TRANSIL algorithm determines binding from the slope of the graph f_b/f_u versus the albumin concentration, making the method largely insensitive to absolute compound recovery as shown below.



- **Internal Validation** of a TRANSIL assay result is summarized in the TRANSIL Quality Index (TQI). The TQI evaluates the reliability of the calculated protein binding constant using five independent quality metrics derived from the experimental dataset. Each metric receives a partial score from 0 (poor) to 10 (excellent). The final TQI is calculated as a weighted average of these scores. The components are:

1. Model Fit: Evaluates how well the experimental data follow the expected binding model. The estimated intercept should be close to zero, since an unbiased slope (K_D) implies a zero intercept.
2. Correlation Coefficient to quantify how closely the data follow the expected linear relationship.
3. Number of valid data points: Scores the dataset based on how many protein concentration points remain after excluding outliers or missing values.
4. Data consistency: Checks whether binding increases steadily with increasing protein concentration, as expected for non-cooperative binding.
5. Measured vs. Predicted Reference Signal: Compares the observed LC-MS peak area in reference wells with the value predicted from the TRANSIL wells by linear regression. Large deviations may indicate nonspecific binding or analytical non-linearity.

4 How the TRANSIL MSA Binding Kit works

4.1 Assay Principle overview

The TRANSIL MSA Binding Assay streamlines the quantification of protein-binding kinetics by replacing time-consuming traditional methods with a robust, bead-based technology. Unlike equilibrium dialysis, which can take 24 to 48 hours to reach a steady state, the TRANSIL assay provides accurate results in just 12 minutes or less. The process is designed for maximum efficiency with minimal labor requirements, as the rapid binding to the TRANSIL beads eliminates the need for lengthy incubation periods. The high surface area of the bead-bound proteins enables rapid equilibration of the drug–protein interaction, allowing equilibrium binding conditions to be reached within minutes.

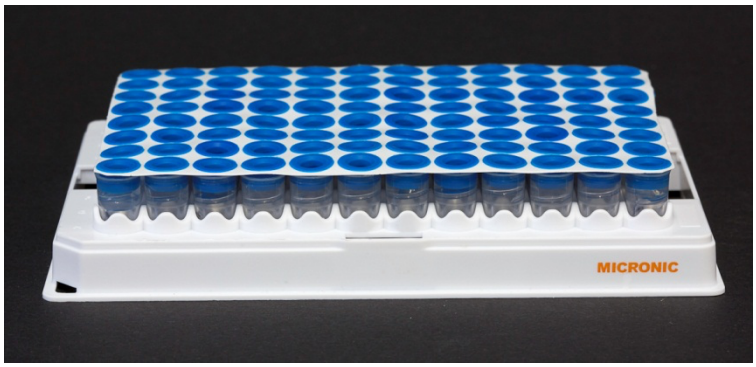
Furthermore, the assay includes integrated internal quality controls, ensuring that recovery and results are validated within the primary run without the need for additional experiments. The measurement remains robust against low recovery, providing reliable data even for challenging compounds. Because the biological phase is immobilized on beads, downstream analytical processes are significantly faster. Additionally, the assay maintains a stable pH environment, preventing the measurement errors and "pH drift" frequently encountered in dialysis. For high-throughput environments, the entire workflow

is fully automatable, allowing for seamless integration into existing robotic liquid handling systems.

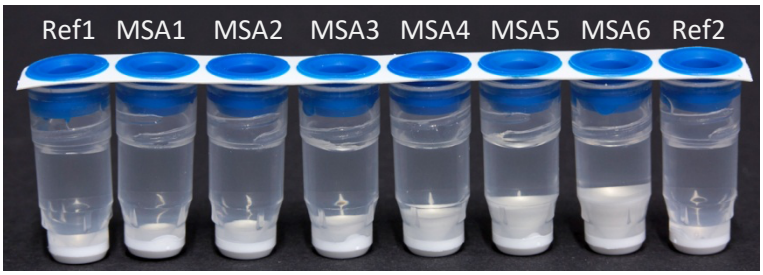
4.2 Technical Assay Principle

The TRANSIL MSA Binding Kit utilizes a bead-based approach to determine the binding affinity of test compounds to mouse serum albumin. The core of the assay involves incubating a fixed concentration of the test compound with varying concentrations of albumin that has been immobilized on silica beads. This is typically performed using 8 wells for each compound (Figure 1): six wells contain the MSA-coated silica beads to measure binding, while two wells contain only buffer to serve as references for non-specific binding. After the rapid incubation, the affinity is calculated based on the remaining free compound concentration found in the supernatant of each well using the specialized spreadsheets and algorithms supplied with the kit. The system is highly flexible regarding detection, as any standard system - such as HPLC or LCMS - can be used for quantification, provided it can measure micromolar concentrations in volumes of 50 µl or less.

a)



b)



c)

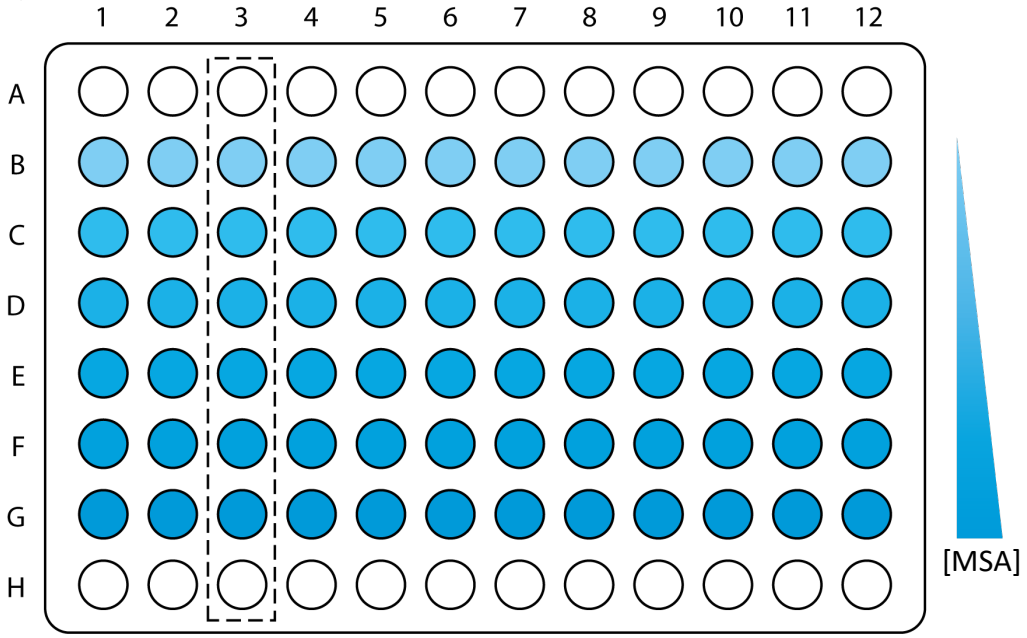


Figure 1: The TRANSIL MSA Binding Kit uses a column of 8 wells to determine the affinity to mouse serum albumin (MSA). a) Photography of the assay plate and b) the annotated tube units supplied. c) Illustration of the assay plate showing the reference rows A and H (white wells) as well as the increasing MSA 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 MSA Binding Kit is composed of the following items:

No.	Qty.	Item
1	1	A 96 well plate with twelve units of 8 tubes filled with a suspension of TRANSIL Plasma Protein Binding Beads (MSA) 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

AGP	α_1 acid glycoprotein, synonymous to AAG
cmp	Compound
conc	Concentration
DMSO	Dimethyl sulfoxide
K_D	Dissociation constant
MSA	Mouse Serum Albumin
$logK_{b/f}$	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.
PBS	Dulbecco's Phosphate buffered saline used in 1x concentration
PPB	Plasma protein binding

7 Reagents

The following reagents are required to run the TRANSIL MSA Binding 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 MSA Binding kit:

No.	Instrument	Specification
1	Rotator	For rotation of the plate along one of its axes.
2	Centrifuge	Including rotor for SBS standard assay plates

9 Assay preparation

Upon receipt the kit should be stored at +4°C.

Before use, equilibrate the assay plate to room temperature. Make sure the tubes have reached room temperature (between 20° and 25°C) prior to assay. Spin the 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 MSA Binding Kit can be used in conjunction with different analytical methods and instruments. These include LC/MS/MS, as well as other methods such as scintillation counting. Please note that the lower limit of the compound concentration in the assay is only limited by the detection limit and dynamic range of the analytical system used. However, we advise to choose a compound concentration high enough to assure that the quantification is fully within the linear range of the instrument. Alternatively, it is advised to prepare a detailed calibration curve to account for non-linearities. Please contact the customer service for further advice on the best approach to the particular compound and situation.

The upper limit of the compound concentration in the assay is limited by the compounds solubility as well as the saturation of individual beads or the entire bead suspension with the test compound. Therefore, we recommend using final assay concentrations of 5 µM or less.

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

11 Replicates

The TRANSIL MSA Binding assay is designed such that one compound utilizes 8 wells – two references and 6 wells with increasing MSA concentration. 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 MSA Binding assay is illustrated in Figure 2.

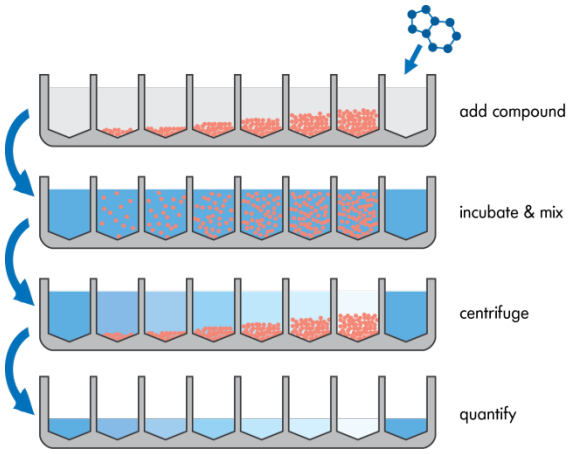


Figure 2: TRANSIL MSA Binding Assay workflow: The same amount of drug is added to all wells followed by a mixing step. After 12 minutes incubation, beads are separated from the buffer by centrifugation and the remaining supernatant is sampled for quantification.

Follow the following 5 steps for the assay procedure:

12.1 Compound addition

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

12.2 Incubation

Incubate the plate for twelve minutes by rotating it along one axes using a rotator or by manual rotation.

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

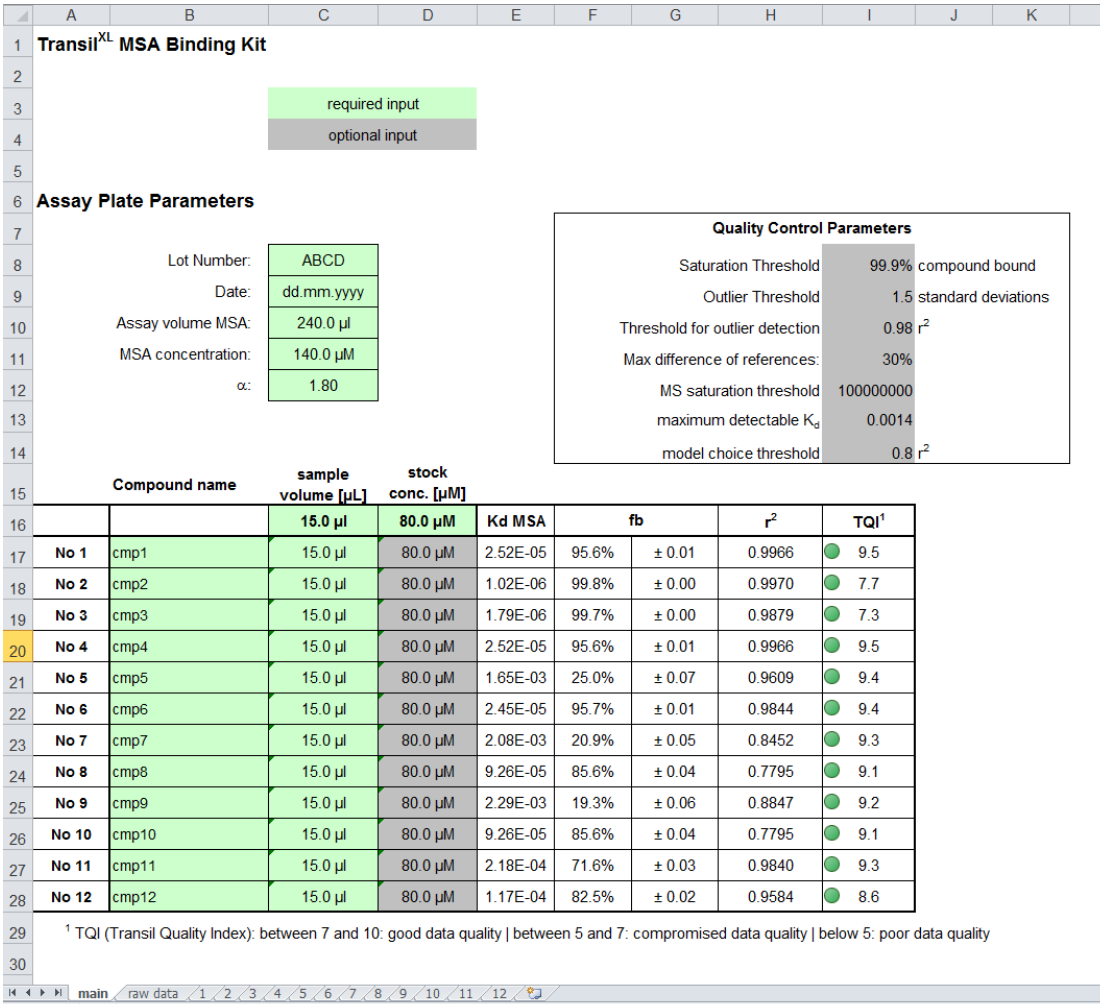
Use your analytical technique of choice for quantifying the compound concentration in the supernatant obtained in the last assay step.

14 Data analysis

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

14.1 Assay parameters

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



Transil^{XL} MSA Binding Kit

required input
optional input

Assay Plate Parameters

Lot Number:	ABCD
Date:	dd.mm.yyyy
Assay volume MSA:	240.0 µl
MSA concentration:	140.0 µM
α:	1.80

Quality Control Parameters

Saturation Threshold	99.9%	compound bound
Outlier Threshold	1.5	standard deviations
Threshold for outlier detection	0.98	r^2
Max difference of references:	30%	
MS saturation threshold	100000000	
maximum detectable K_d	0.0014	
model choice threshold	0.8	r^2

	Compound name	sample volume [µL]	stock conc. [µM]	Kd MSA	fb		r^2	TQI[1]
		15.0 µl	80.0 µM					
No 1	cmp1	15.0 µl	80.0 µM	2.52E-05	95.6%	± 0.01	0.9966	9.5
No 2	cmp2	15.0 µl	80.0 µM	1.02E-06	99.8%	± 0.00	0.9970	7.7
No 3	cmp3	15.0 µl	80.0 µM	1.79E-06	99.7%	± 0.00	0.9879	7.3
No 4	cmp4	15.0 µl	80.0 µM	2.52E-05	95.6%	± 0.01	0.9966	9.5
No 5	cmp5	15.0 µl	80.0 µM	1.65E-03	25.0%	± 0.07	0.9609	9.4
No 6	cmp6	15.0 µl	80.0 µM	2.45E-05	95.7%	± 0.01	0.9844	9.4
No 7	cmp7	15.0 µl	80.0 µM	2.08E-03	20.9%	± 0.05	0.8452	9.3
No 8	cmp8	15.0 µl	80.0 µM	9.26E-05	85.6%	± 0.04	0.7795	9.1
No 9	cmp9	15.0 µl	80.0 µM	2.29E-03	19.3%	± 0.06	0.8847	9.2
No 10	cmp10	15.0 µl	80.0 µM	9.26E-05	85.6%	± 0.04	0.7795	9.1
No 11	cmp11	15.0 µl	80.0 µM	2.18E-04	71.6%	± 0.03	0.9840	9.3
No 12	cmp12	15.0 µl	80.0 µM	1.17E-04	82.5%	± 0.02	0.9584	8.6

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

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

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

14.2 Compound information

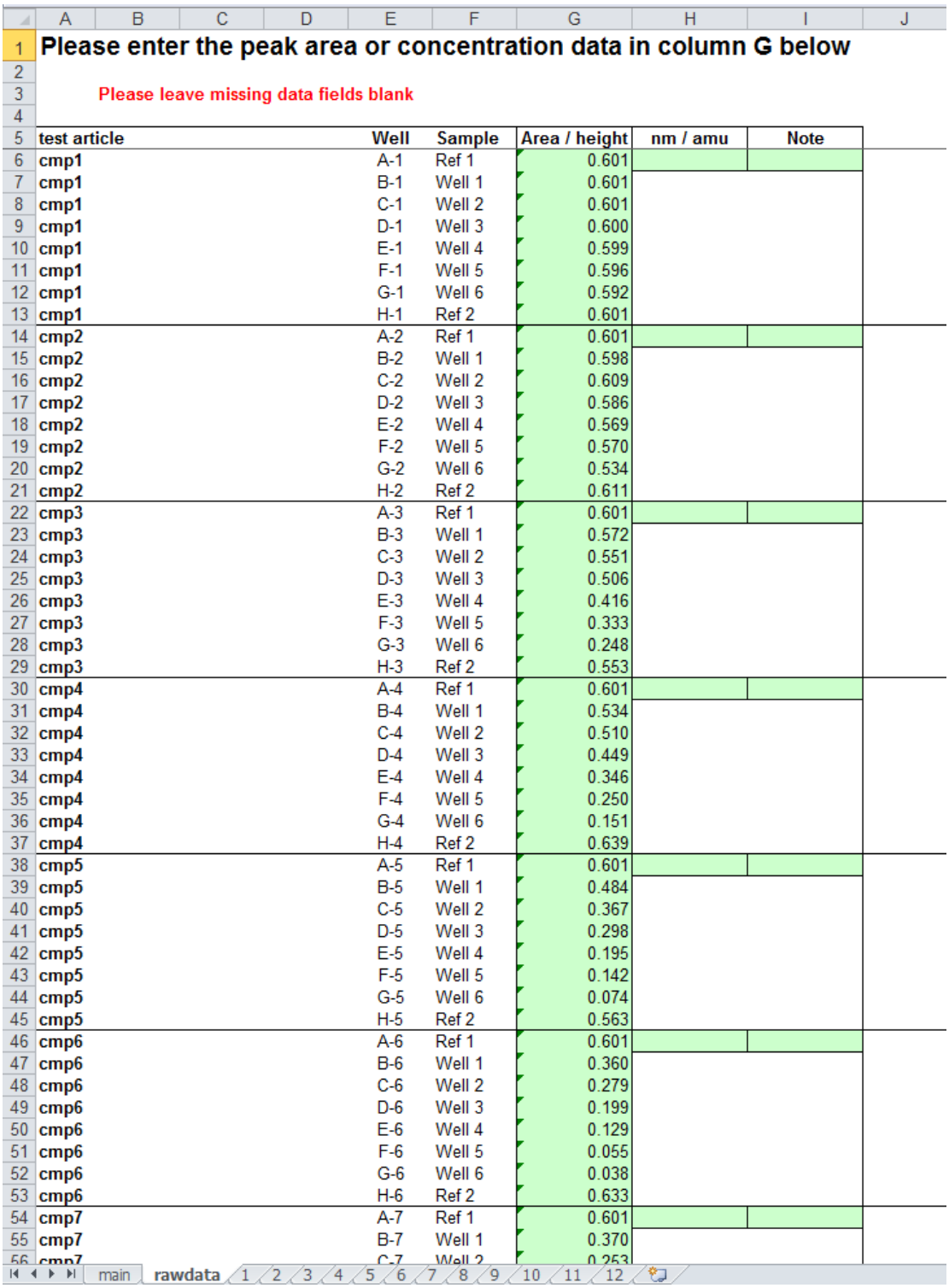
Please enter the compound names in the column B17 to B28 of the “main” tab. Enter the sample volume added to each well in field C16. If a different sample volume was used for each drug, enter the sample specific volumes in the column C17 to C28.

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 in 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
cmp1	A-1	Ref 1	0.601		
cmp1	B-1	Well 1	0.601		
cmp1	C-1	Well 2	0.601		
cmp1	D-1	Well 3	0.600		
cmp1	E-1	Well 4	0.599		
cmp1	F-1	Well 5	0.596		
cmp1	G-1	Well 6	0.592		
cmp1	H-1	Ref 2	0.601		
cmp2	A-2	Ref 1	0.601		
cmp2	B-2	Well 1	0.598		
cmp2	C-2	Well 2	0.609		
cmp2	D-2	Well 3	0.586		
cmp2	E-2	Well 4	0.569		
cmp2	F-2	Well 5	0.570		
cmp2	G-2	Well 6	0.534		
cmp2	H-2	Ref 2	0.611		
cmp3	A-3	Ref 1	0.601		
cmp3	B-3	Well 1	0.572		
cmp3	C-3	Well 2	0.551		
cmp3	D-3	Well 3	0.506		
cmp3	E-3	Well 4	0.416		
cmp3	F-3	Well 5	0.333		
cmp3	G-3	Well 6	0.248		
cmp3	H-3	Ref 2	0.553		
cmp4	A-4	Ref 1	0.601		
cmp4	B-4	Well 1	0.534		
cmp4	C-4	Well 2	0.510		
cmp4	D-4	Well 3	0.449		
cmp4	E-4	Well 4	0.346		
cmp4	F-4	Well 5	0.250		
cmp4	G-4	Well 6	0.151		
cmp4	H-4	Ref 2	0.639		
cmp5	A-5	Ref 1	0.601		
cmp5	B-5	Well 1	0.484		
cmp5	C-5	Well 2	0.367		
cmp5	D-5	Well 3	0.298		
cmp5	E-5	Well 4	0.195		
cmp5	F-5	Well 5	0.142		
cmp5	G-5	Well 6	0.074		
cmp5	H-5	Ref 2	0.563		
cmp6	A-6	Ref 1	0.601		
cmp6	B-6	Well 1	0.360		
cmp6	C-6	Well 2	0.279		
cmp6	D-6	Well 3	0.199		
cmp6	E-6	Well 4	0.129		
cmp6	F-6	Well 5	0.055		
cmp6	G-6	Well 6	0.038		
cmp6	H-6	Ref 2	0.633		
cmp7	A-7	Ref 1	0.601		
cmp7	B-7	Well 1	0.370		
cmp7	C-7	Well 2	0.253		

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 MSA Binding Kit. The “rawdata” tab is used to enter peak area or concentration data from the supernatants of the assay plate after incubation and centrifugation.

14.4 Results

The spreadsheet calculates protein binding 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 MSA protein binding

Plasma protein binding is measured in terms of the dissociation constant K_D:

$$K_D = \frac{[A]\cdot[P]}{[AP]} \quad (1)$$

where [AP] is the concentration of drug A bound to the protein P and where [A] denotes the free concentration of drug and [P] denotes the free concentration of protein. The free concentration of drug can also be expressed as

$$[A] = f_u \cdot ([A]+[AP]) \quad (2)$$

When entering (2) into (1) and rearranging, we obtain a linear model that can be fitted to the data from the TRANSIL MSA Binding Kit

$$\frac{f_b}{f_u} = \frac{1}{K_D}\cdot P \quad (3)$$

with the slope $1/K_D$ and an intercept of 0. Note that this equation requires that the concentration of the protein-drug complex [AP] should be much smaller than the total protein concentration in each well.

The fraction bound can be predicted from the K_D to MSA as well as the physiological concentration of this plasma protein in mouse blood:

$$\mathrm{f_b} = 1 - \frac{1}{1+\frac{[\mathrm{MSA}]}{\mathrm{K_D^{MSA}}}}. \quad (4)$$

This represents the fraction bound to mouse serum albumin. Contributions from AGP and other plasma proteins are not included and may need to be considered separately.

Differences between TRANSIL measurements and plasma-based methods may arise due to differences in protein composition, binding site accessibility, and experimental conditions.

The results for the fraction bound to plasma is reported in column F17 to F28 of the “main” tab of the spreadsheet, while the respective measurement errors are reported in column G17 to G28.

14.5 TRANSIL Quality Index

14.5.1 TRANSIL Quality Index for Protein Binding

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.

14.5.1.1 Model fit

Plasma protein binding is calculated according to equation (3). Fitting optimal data to equation (3) will yield a slope that exactly represents the true affinity constant, K_D, and an intercept of zero (Figure 5). In fact, a biased estimation of the slope will typically result in a biased estimation of the intercept as well. Since the intercept equals zero, the estimated intercept is used as a quality control parameter. If the estimated intercept is within the interval [-0.5,0.5] a partial quality score of 10 is attributed. If the estimated intercept is within the interval [-1.5,1.5] a partial quality score of 5 is attributed. If the estimated intercept is outside the latter range, a sore of 0 is attributed.

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

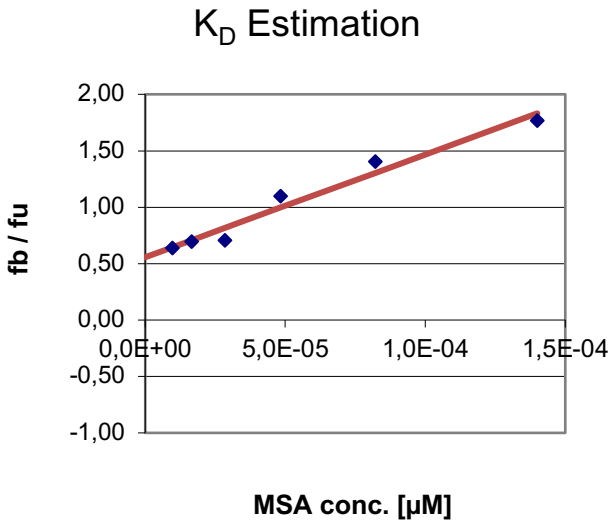


Figure 5: Illustration of fitting experimental data to equation (3) to determine the affinity to MSA. The intercept was estimated to 0.56, hence a quality score of 5 was attributed to the model fit.

14.5.1.2 Match of measured versus predicted reference signal

When determining the affinity constants via 5 or 6 different protein concentrations 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 protein binding is assumed to be a non-cooperative process (Figure 6). This score has a weight of 3 in the TQI.

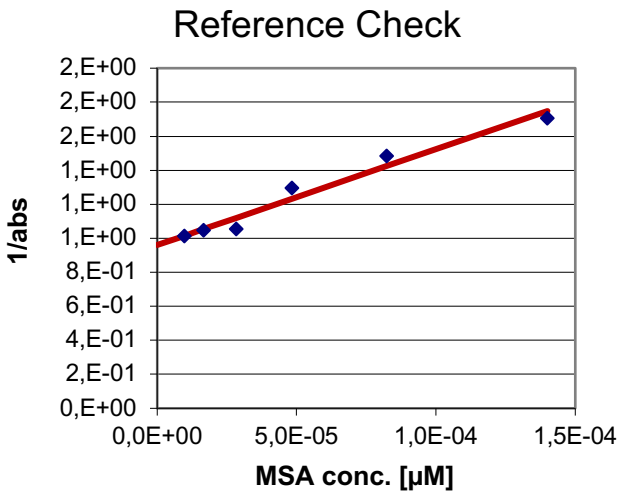


Figure 6: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the protein content. The inverse of the intercept represents the expected peak area of the references.

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 proteins on 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

14.5.1.3 Correlation coefficient

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

14.5.1.4 Number of outliers or missing data

The number of data points used to calculate the affinity constant 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

14.5.1.5 Data consistency

With increasing protein concentration the binding the test items to the proteins should increase proportionally. At least the binding should increase with increasing protein concentration, if binding is non-linear. If the measured peak area suggests decreased binding compared to binding in the TRANSIL well with the next lower protein concentration, then this data point is considered to be inconsistent with the fundamental assumption about protein binding. If this happens for more than one consecutive TRANSIL well, the data point will be excluded from the calculation. Irrespective of inclusion or exclusion, a partial quality score will be attributed to the data set based on consistency according to table 4. This score has a weight of only 1 in the TQI as it may affect also the number or data points.

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

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

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. This requires the addition of a higher volume of test compound increasing the total assay volume. Please contact our technical support team for details on how to adapt the spreadsheet accordingly.

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 “KD Estimation plot” in the individual data analysis tabs of the spreadsheet; see Figure 7) may indicate non-linearity response issues.

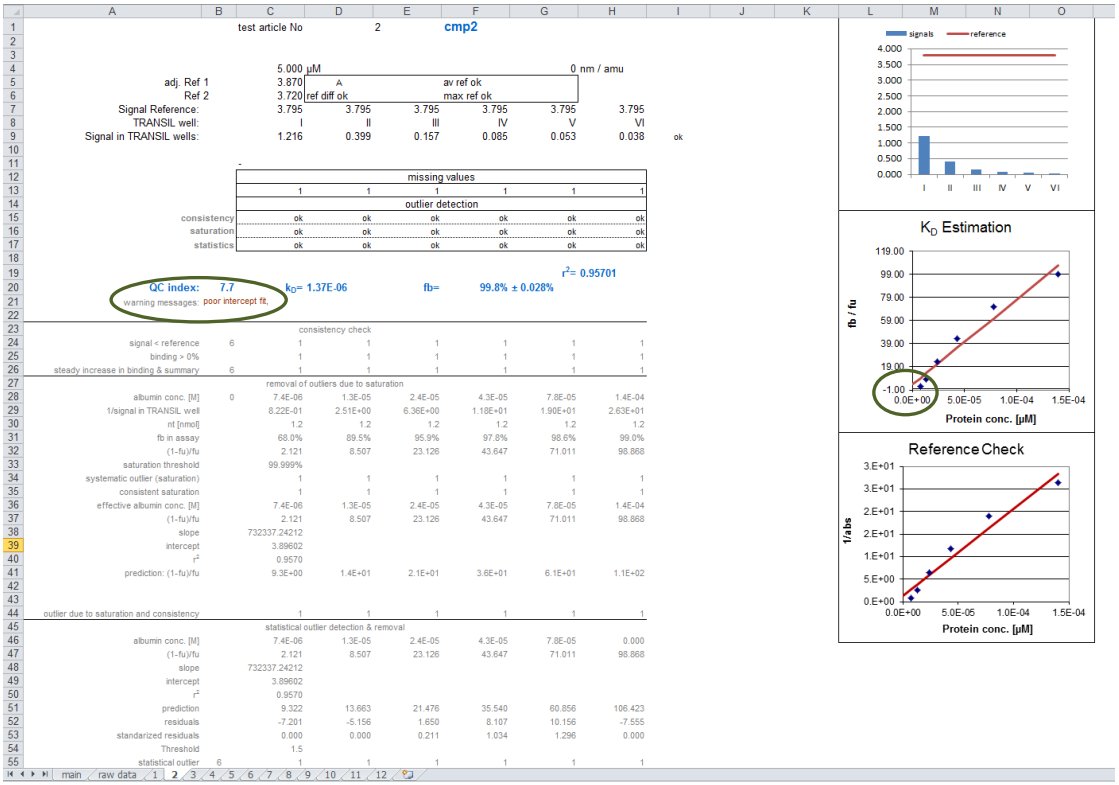


Figure 7: Illustration of the non-linear response issue which can be recognized by a poor intercept fit (green circles) or non-linear shape of the regression plotted in the “KD Estimation” graph.

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. If test compound concentration is limited by poor compound solubility, the TRANSIL High Sensitivity Binding Kit can be used to measure MSA or full plasma protein binding.

iii. Alternatively, a detailed calibration curve covering the non-linear response can be recorded and used to calculate test compound concentrations. These concentrations can then directly be applied to the spreadsheet for MSA binding calculation. 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.

16.3 Strong Binders

16.3.1 Challenges and problem identification

Compounds exhibiting very strong binding (fu < 0.1%) are not accurately resolved in the standard assay design. Moreover, the non-linear instrument response issue is frequently observed for compounds with such protein binding characteristics.

16.3.2 Problem-solving approaches

For accurate prediction of very low free fractions we recommend the TRANSIL High Sensitivity Binding assay.

16.4 Low Binders

16.4.1 Challenges and problem identification

Compounds with very low affinity to plasma proteins and hence high free fractions (fu > 30%) are not accurately predicted. Low affinity compounds yield supernatant concentrations in the assay that deviate only marginally from the reference signals (Figure 8).

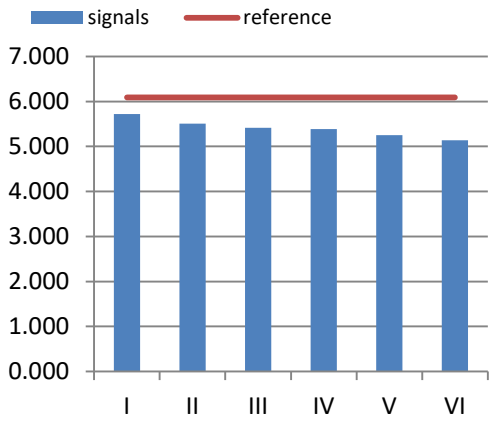


Figure 8: Illustration of a bar plot of a compound exhibiting weak protein binding (c.f. individual data analysis tabs of the spreadsheet). The blue bars show the detected signals in the supernatants of TRANSIL wells I to VI. As the compound binds only weekly to the proteins, supernatant concentrations differ only marginally from the reference signals (red line).

16.4.2 Problem-solving approaches

Please contact Sovicell support to receive a kit with adjusted TRANSIL content (higher than the standard kit) if exact predictions are required.

Technical Support

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Email: contact@sovicell.com

17 Glossary

AAG	human α_1 acid glycoprotein, synonymous to AGP
AGP	human α_1 acid glycoprotein, synonymous to AAG and orosomucoid
MSA	Mouse Serum Albumin
K_D-values	The affinity constant K_D is directly related to the ratio of the forward rate constant over the reverse rate constant of the binding interaction A + B = AB.
Orosomucoid	human α_1 acid glycoprotein, synonymous to AAG and AGP
Sudlow's site I	Structurally defined region of albumin binding warfarin and other coumarin anticoagulants (c.f. Sudlow et al. 1975).
Sudlow's site II	Structurally defined region of albumin binding diazepam and other drug molecules (c.f. Sudlow et al. 1975).

18 References

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