User Guide

TRANSIL

RSA Binding Kit

TMP-0210-2096

Version 3, Revision 02

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1 Quick Protocol

1. Plate Thawing and preparation

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

2. Drug Candidate Preparation

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

3. Drug Candidate Addition

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

4. Incubation and Supernatant Sampling

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

5. Analysis

- Quantify supernatants by the method of choice.
- For evaluation of the results, please use the supplied MS Excel spreadsheet and refer to the operating instructions for data analysis.

2 Background

Pharmacokinetic characteristics substantially influence safety and efficacy of pharmaceuticals and lack of efficacy and toxicity are considered to be major reasons for drug failures. Therefore, the preclinical pharmacokinetic evaluation facilitates early elimination of weak candidates and directs the entire focus towards fewer potential lead candidates. In vitro profiling comprises assays such as metabolic stability, reaction phenotyping, CYP-450 inhibition and induction, as well as plasma protein binding and provides together with appropriate in vivo data an early indication of whether the compound which worked in animals would work in human as well (Singh, 2006).

It is commonly accepted that only the unbound fraction (f_u) of a drug can penetrate cell membranes and exert its pharmacological effect or become available for elimination. Many drugs, especially lipophilic compounds, bind to circulating plasma proteins, such as albumin, α_1-acid glycoprotein (AGP), globulins, and lipoproteins. Among these proteins, albumin and AGP are the most important for the reduction of the exposure to xenobiotics because of their ability to bind a large array of structurally unrelated drugs in distinct binding sites (Kremer et al., 1988; Peters, 1996).

Albumin (RSA) is also the most abundant protein in rat plasma. It is synthesized in the liver and average concentrations of 42 g/L have been observed (Ohdachi et al., 1999). Its main physiological function is to bind and carry endogenous anions, with long-chain fatty acids. Due to the high sequence homology with human serum albumin, it is assumed that the two high affinity binding sites, which have been described for human serum albumin in subdomains IIA (also known as Sudlow's site I or warfarin site) and IIIA (also known as Site II or benzodiazepine site), can also be found in rat and mouse serum albumin. These comprise highly elongated hydrophobic pockets with charged lysine and arginine residues near the surface that interact with polar ligand parts (Sudlow et al., 1975). In contrast to AGP, HSA blood levels are much more stable.

Given the clinical relevance of fluctuation in the f_u altering drug disposition (Summerfield et al., 2006), active transport (Bow et al., 2006), drug-drug interactions (Christensen et al., 2006) and drug efficacy, especially in the field of chemotherapy of infectious diseases (Boffito et al., 2002; Zhang et al., 1999) there is an increased understanding that binding to plasma proteins should be investigated in the early stages of the drug discovery process. Unfortunately, existing methods for determination of plasma protein binding have low capacity and/or high cost and lack standard evaluation

procedures (for a review, see Boffito et al., 2003). Conventional methods to determine plasma protein binding comprise equilibrium dialysis, ultrafiltration, and ultracentrifugation (Sebille, 1990), ideally using plasma samples of pooled healthy donors, usually with no control over albumin and AGP levels. High throughput methods have been described for plasma equilibrium dialysis combined with liquid chromatography/mass spectrometry (LC-MS/MS) analytics (Wan & Rehngren, 2006). Notably, the resolution of these methods is limited, particularly when examining drugs that are highly bound to plasma proteins. These conventional methods require highly sensitive analytical techniques that exhibit a linear range of more than two orders of magnitude such as LC-MS/MS or the use of radiolabeled compounds to resolve plasma protein binding of compounds with f_u-values smaller than 0.01.

Given the above constraints for the determination of plasma protein binding, the TRANSIL RSA was developed and validated employing a novel method that overcomes these limitations and addresses the problem of varying RSA levels. To overcome the analytical limitations, when examining drugs that are highly bound to plasma proteins, the TRANSIL RSA assay allows K_D-values to be determined by titrating different subphysiological concentrations of RSA against a constant drug concentration.

3 Applications of TRANSIL RSA Assay kit

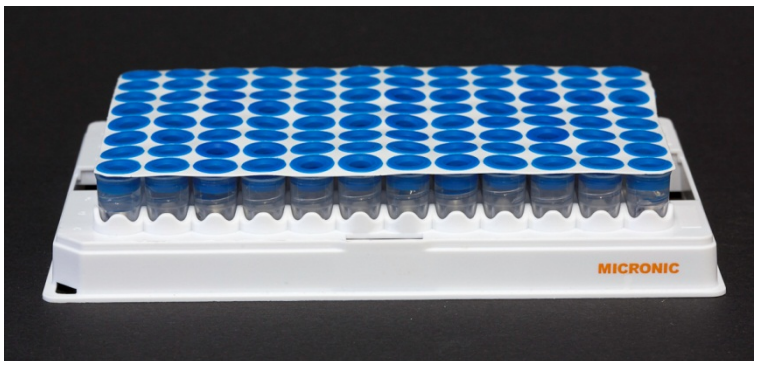
The TRANSIL RSA Binding Kit enables researchers to predict the extent plasma protein binding of molecules. The innovative TRANSIL assay is an *in vitro* kit designed to assess the affinity of drugs to human serum albumin. This enables the researcher to predict the fraction bound to plasma proteins in a highly defined and pH stable assay system.

4 Basic assay principle

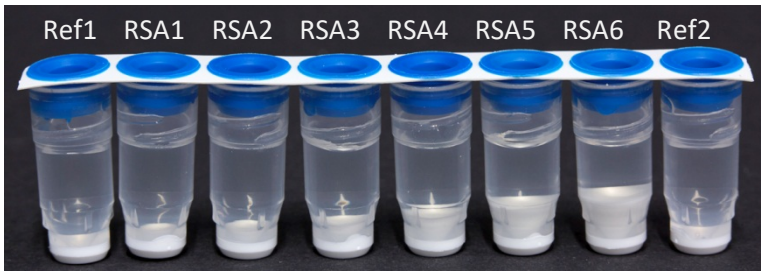
The principle of the TRANSIL RSA Binding Kit is to assess the affinity of test compounds to the human plasma protein albumin (RSA). Albumin binding is determined by incubating a fixed concentration of drug candidate with varying concentrations of albumin immobilized on silica beads. A total of 8 wells of a tube unit are used to determine the plasma protein binding for each compound (Figure 1). Six wells contain RSA covered silica beads, while two serve as references to account for non-specific binding and contain buffer only. Using the spreadsheet and algorithms supplied with the kit, the affinity to albumin is calculated from remaining free compound concentration in the supernatant of each well. Any of the

available detection systems, such as HPLC, LCMS, etc can be used for quantification, as long as it can quantify µM concentrations in volumes of 50 µl or less.

a)



b)



c)

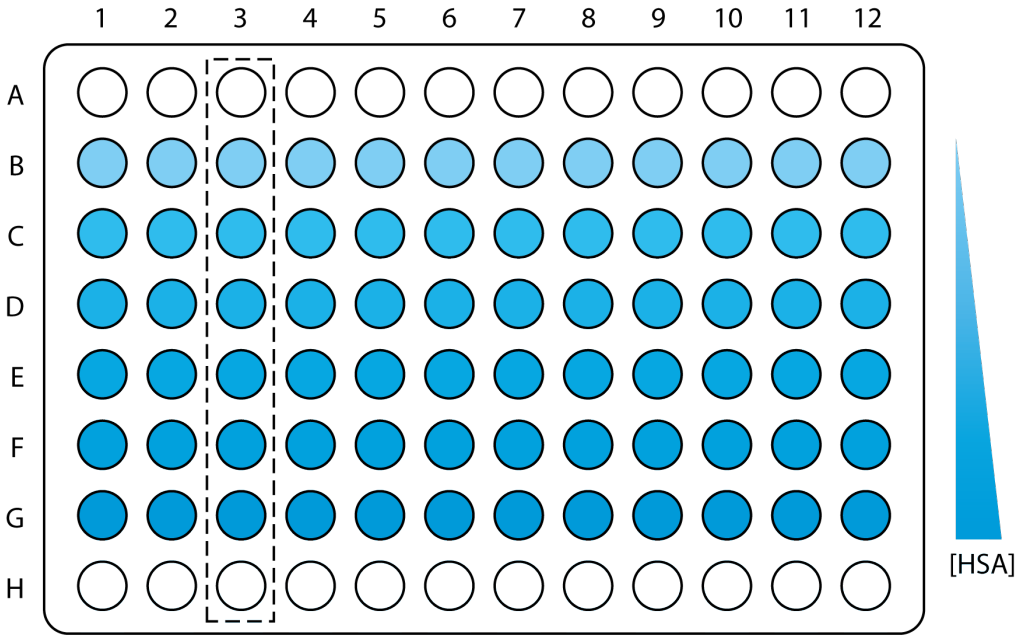


Figure 1: The TRANSIL RSA Binding Kit uses a column of 8 wells to determine the affinity to human serum albumin (RSA). a) Photography of the assay plate and b) the annotated tube units supplied. c) Illustration of the assay plate showing the reference rows A and H (white wells) as well as the increasing RSA 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 RSA 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 (RSA) 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	human α_1 acid glycoprotein, synonymous to AAG
cmp	Compound
conc	Concentration
DMSO	Dimethyl sulfoxide
K_D	Dissociation constant
RSA	Human 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

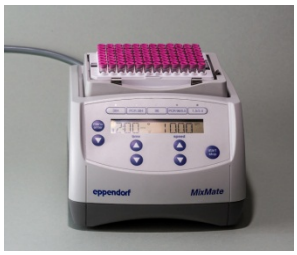
7 Reagents

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

No.	Instrument	Specification
1	Plate shaker	For highspeed 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 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 RSA 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 RSA Binding assay is designed such that one compound utilizes 8 wells – two references and 6 wells with increasing RSA 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 RSA Binding assay is illustrated in Figure 2.

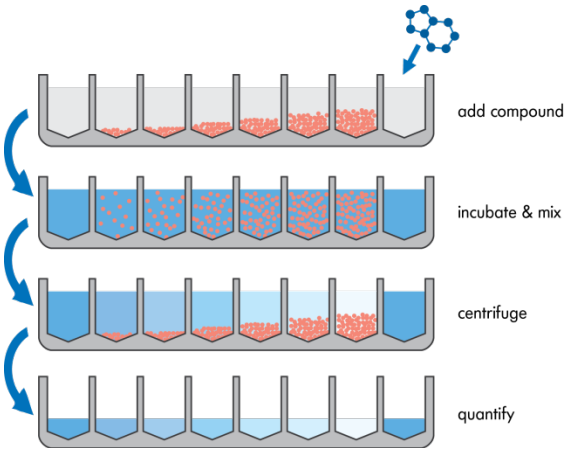


Figure 2: TRANSIL RSA Binding Assay workflow: The same amount of drug is added to all wells followed by a mixing step. After 12 minutes incubation, beads are separated from the buffer by centrifugation and the remaining supernatant is sampled for quantification.

Follow the following 5 steps for the assay procedure:

12.1 Compound addition

Mix the compound stock solution carefully by vortexing. When the TRANSIL RSA 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 plates for twelve minutes on a plate shaker at 1000 rpm at RT.

NB: The first time a plate shaker is used for TRANSIL assays it is essential to determine that all the beads are resuspended in solution. To ensure beads are resuspended, visually

inspect the plate after 1 min. If necessary increase the mixing speed until all beads are resuspended. Alternatively, manually invert plate to ensure all beads are resuspended.

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 RSA 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} HSA Binding Kit

required input
optional input

Assay Plate Parameters

Lot Number:	ABCD
Date:	dd.mm.yyyy
Assay volume HSA:	240.0 µl
HSA 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]	K_d HSA	fb		r^2	TQI[1]
		15.0 µl	80.0 µM					
No 1	cmp1	15.0 µl	80.0 µM	2.52E-05	95.9%	± 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	95.9%	± 0.01	0.9966	9.5
No 5	cmp5	15.0 µl	80.0 µM	1.65E-03	26.2%	± 0.07	0.9609	9.4
No 6	cmp6	15.0 µl	80.0 µM	2.45E-05	96.0%	± 0.01	0.9844	9.4
No 7	cmp7	15.0 µl	80.0 µM	2.08E-03	22.0%	± 0.05	0.8452	9.3
No 8	cmp8	15.0 µl	80.0 µM	9.26E-05	86.4%	± 0.03	0.7795	9.1
No 9	cmp9	15.0 µl	80.0 µM	2.29E-03	20.4%	± 0.06	0.8847	9.2
No 10	cmp10	15.0 µl	80.0 µM	9.26E-05	86.4%	± 0.03	0.7795	9.1
No 11	cmp11	15.0 µl	80.0 µM	2.18E-04	72.9%	± 0.03	0.9840	9.3
No 12	cmp12	15.0 µl	80.0 µM	1.21E-04	83.0%	± 0.02	0.9955	9.1

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

ID	Compound name	K_d AGP	fu*
No 1	cmp1	1.00E-04	4.1%
No 2	cmp2	1.00E-06	0.2%
No 3	cmp3	1.00E-07	0.2%
No 4	cmp4	1.00E-04	4.1%

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 RSA 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 RSA 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 RSA and AGP 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 RSA 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 total fraction bound can be predicted from the K_D's to AGP and RSA as well as the physiological concentration of these plasma proteins in human blood:

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

This is an estimation of the fraction bound to plasma proteins based on human serum albumin and human α_1 acid glycoprotein. Binding to other low abundance plasma proteins like lipoproteins, transcortin, and sex hormone binding protein is not considered.

The estimation errors tend to be small and may be of similar order of magnitude as dialysis estimation errors due to variations in plasma composition and pH variation.

When comparing TRANSIL plasma protein binding with equilibrium dialysis using human plasma, different results can be expected due to following differences between the two approaches:

- TRANSIL technology is based on purified proteins; therefore, all binding sites are expected to be available. Whereas, in plasma the binding sites may be masked; this may result in higher binding estimates by TRANSIL protein assay
- TRANSIL protein assay measures the K_Ds to RSA or AGP or both (the combined assay) and calculates the binding based on assumptions of the absolute abundance of these proteins. The user can change these assumptions and adjust them to his/her own needs; however, they may not reflect the plasma composition chosen in the dialysis experiment. Thus, deviations of binding can go both ways depending upon the physiological and disease state of the plasma donor.
- TRANSIL measures only RSA and AGP binding, which could cause underestimation of binding, since compounds could also bind to lipoproteins or other plasma proteins with low abundance.

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. Column D32 to D43 lists the predicted free fraction in plasma given a K_D value for binding to AGP is entered for each drug in column C32 to C43.

14.5 TRANSIL Quality Index

14.5.1 TRANSIL Quality Index for Protein Binding

The TRANSIL Quality Index (TQI) is based 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.

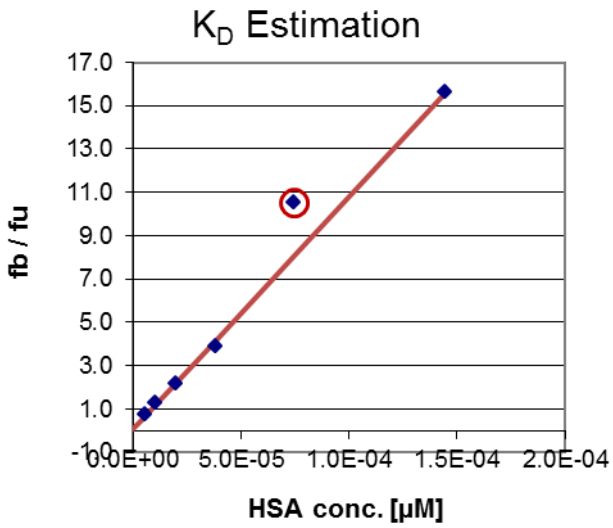


Figure 5: Illustration of fitting experimental data to equation (3) to determine the affinity to RSA. The intercept was estimated to -0.64, hence a quality score of 5 was attributed to the model fit.

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.

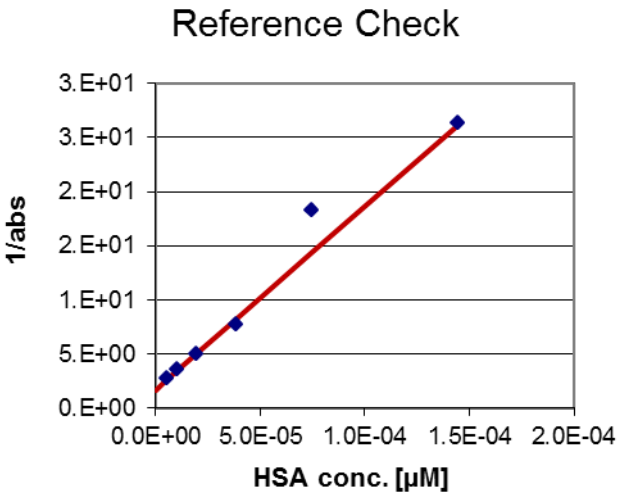


Figure 6: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the protein content. The inverse of the intercept represents the expected peak area of the references.

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.

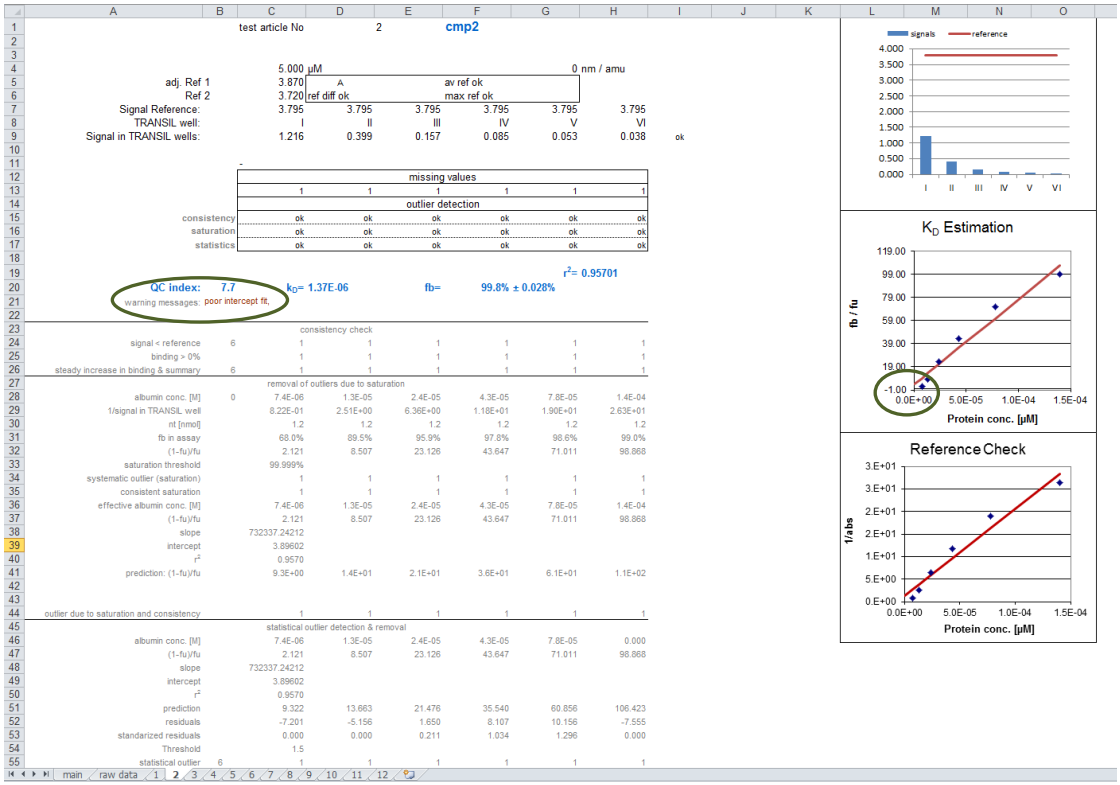


Figure 7: Illustration of the non-linear response issue which can be recognized by a poor intercept fit (green circles) or non-linear shape of the regression plotted in the “KD Estimation” graph.

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 RSA 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 RSA 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).

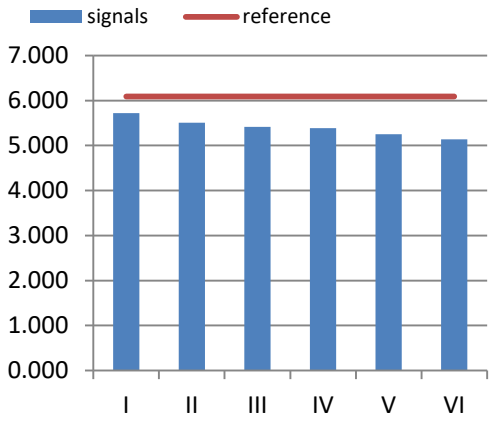


Figure 8: Illustration of a bar plot of a compound exhibiting weak protein binding (c.f. individual data analysis tabs of the spreadsheet). The blue bars show the detected signals in the supernatants of TRANSIL wells I to VI. As the compound binds only weekly to the proteins, supernatant concentrations 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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17 Glossary

AAG	human α_1 acid glycoprotein, synonymous to AGP
AGP	human α_1 acid glycoprotein, synonymous to AAG and orosomucoid
RSA	Human Serum Albumin
Hyperalbuminemia	Typically this condition is a sign of severe or chronic dehydration. Chronic dehydration needs to be treated with zinc as well as with water. Zinc reduces cell swelling caused by increased intake of water (hypotonicity) and also increases retention of salt. In the dehydrated state the body has too high of an osmolarity and apparently discards zinc to prevent this. Hyperalbuminemia is also associated with high protein diets.
Hypoalbuminemia	Low blood albumin levels (hypoalbuminemia) can be caused by: Liver disease; cirrhosis of the liver is most common;Excess excretion by the kidneys (as in nephrotic syndrome); Excess loss in bowel (protein losing enteropathy e.g. Menetrier's); Burns (plasma loss in the absence of skin barrier); Redistribution (hemodilution [as in pregnancy], increased vascular permeability or decreased lymphatic clearance); Acute disease states (referred to as a negative acute phase protein); Mutation causing analbuminemia (very rare).

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 I	Structurally defined region of albumin binding binding diazepam and other drug molecules (c.f. Sudlow et al. 1975).
Xenobiotics	A xenobiotic is a chemical which is found in an organism but which is not normally produced or expected to be present in it. It can also cover substances which are present in much higher concentrations than are usual. Specifically, drugs such as antibiotics are xenobiotics in humans because the human body does not produce them itself, nor are they part of a normal diet.

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