User Guide

TRANSIL

AGP Binding Kit

TMP-0211-2096

Version 3, Revision 04

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Part No. TPB-0211-2096M

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 by aspiration/suspension: 20 cycles for small molecules and 120 for large molecules
  OR:
  Incubate the plate on a plate shaker at 1000 rpm for 30 seconds. Then gently shake the plate in your hands for 2 minutes by inverting it top-down and bottom-up.
- 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. Plasma protein binding therefore has a major influence on drug exposure, pharmacokinetics, and pharmacological activity. While human serum albumin is the most abundant plasma protein, α1-acid glycoprotein (AGP) can make a disproportionate contribution to plasma protein binding of many basic and lipophilic drugs.

AGP is present in plasma at much lower concentrations than albumin, but it often binds basic compounds with relatively high affinity. Unlike albumin, AGP is an acute-phase protein, and its plasma concentration can increase several-fold during inflammation, infection, trauma, or cancer. For drugs with strong AGP affinity, this can lead to substantial changes in the unbound fraction and thereby alter drug exposure and pharmacokinetic behavior across physiological and disease conditions.

Because plasma protein binding strongly influences clearance, distribution, half-life, and the interpretation of pharmacological potency, it is routinely assessed during drug discovery and development. Conventional methods such as equilibrium dialysis, ultrafiltration, and ultracentrifugation directly determine the free fraction in plasma, but they can be time-consuming, experimentally demanding, and difficult to apply to highly bound or low-recovery compounds. Membrane adsorption, slow equilibration, compound instability, and limited analytical sensitivity can further complicate accurate measurements.

For AGP in particular, direct fu measurements in plasma provide only a condition-specific result, because the contribution of AGP to overall plasma protein binding depends strongly on its concentration. Determining the dissociation constant (K_D) of the drug–AGP interaction instead provides a mechanistic parameter that can be used to estimate binding at different AGP concentrations and under different physiological conditions. In combination with albumin binding data, AGP binding constants enable a more mechanistic assessment of plasma protein binding in healthy and disease-altered plasma.

This point is not merely theoretical. Several basic drugs with relatively weak albumin contribution but strong AGP binding have shown marked changes in plasma binding as AGP concentrations rise. Lidocaine and imipramine are classic examples of compounds reported to bind predominantly to AGP rather than albumin, and their free fraction tracks AGP levels closely. Similar observations have been reported for disopyramide, methadone, and meperidine, where elevated AGP concentrations were associated with lower unbound

fractions or stronger overall plasma binding. These examples illustrate why AGP affinity can become a major determinant of pharmacokinetic variability: when albumin binding is limited but AGP binding is strong, changes in AGP abundance can translate directly into substantial shifts in free drug exposure.

The TRANSIL AGP Binding Kit applies this principle by determining the binding affinity of test compounds to purified human AGP under well-defined experimental conditions. By measuring the intrinsic drug–AGP interaction in a rapid bead-based assay, the method enables reproducible and mechanistically interpretable characterization of AGP binding without the long equilibration times and matrix-related complications of conventional plasma-based assays. Because the assay isolates binding to a defined protein under controlled conditions, it separates an intrinsic molecular property from the variable plasma environment and supports more reliable translation across patient populations and disease states.

3 Applications of TRANSIL AGP Binding kit

The TRANSIL AGP Binding Kit is designed to characterize the intrinsic affinity of drugs to human α1-acid glycoprotein (AGP) under controlled assay conditions. This is especially relevant for basic and lipophilic compounds for which AGP can make a major contribution to plasma protein binding. Because AGP is an acute-phase protein whose plasma concentration varies substantially with inflammation and disease, AGP binding data help identify compounds for which changes in AGP levels may lead to clinically relevant changes in free fraction. Combined with HSA binding data, the assay supports a more accurate mechanistic assessment of plasma protein binding across different physiological states.

3.1 Simulation of plasma binding in various disease states

In conjunction with the TRANSIL HSA Binding kit you obtain the dissociation constants Kd 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 disease states with elevated AGP levels.

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 and AGP binding affinity to guide medicinal chemistry efforts. Albumin and AGP 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 Pharmacokinetic (PK) Parameter Prediction

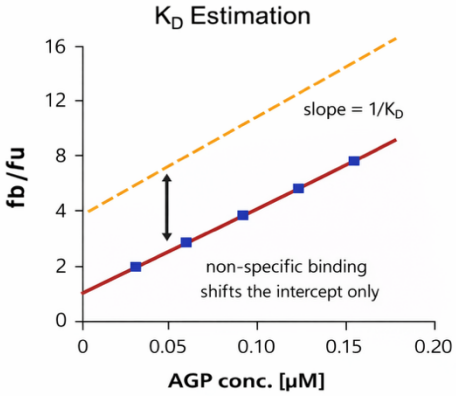
Albumin binding is an important determinant of a drug's pharmacokinetic profile.

- **Volume of Distribution V_d:** HSA and AGP 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.

3.4 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 AGP 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 (KD) 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 AGP Binding Kit works

4.1 Assay Principle overview

The TRANSIL AGP 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.

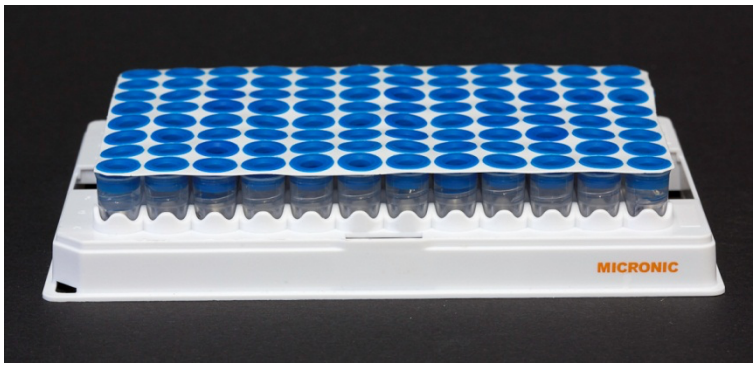
Furthermore, the assay includes integrated internal quality controls, ensuring that recovery and results are validated within the primary run without the need for additional experiments. The measurement remains robust against low recovery, providing reliable data even for challenging compounds. Because 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.

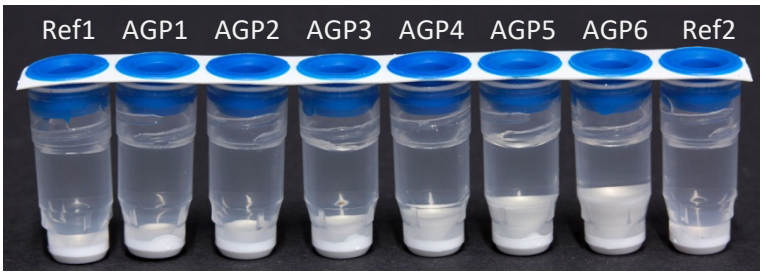
4.2 Technical Assay Principle

The TRANSIL AGP Binding Kit utilizes a bead-based approach to determine the binding affinity of test compounds to AGP. The core of the assay involves incubating a fixed concentration of the test compound with varying concentrations of AGP that has been immobilized on silica beads. This is typically performed using 8 wells for each compound (Figure 1): six wells contain the AGP-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)

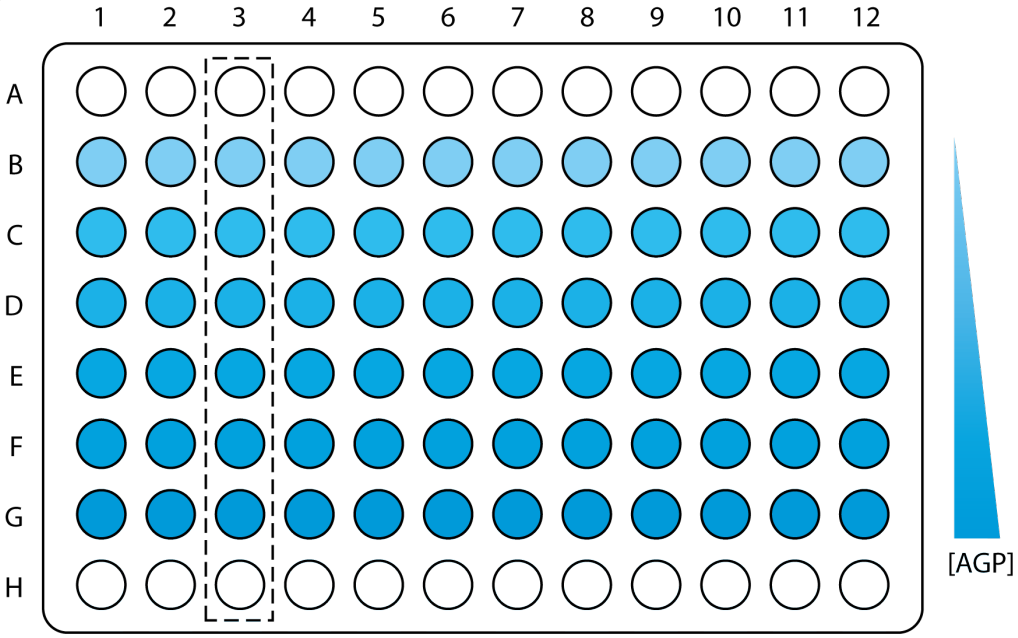


Figure 1: The TRANSIL AGP Binding Kit uses a column of 8 wells to determine the affinity to α_1-acid glycoprotein (AGP). a) Photography of the assay plate and b) the annotated tube units supplied. c) Illustration of the assay plate showing the reference rows A and H (white wells) as well as the increasing AGP 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 AGP 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 (AGP) 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
HSA	Human Serum Albumin
$\log K_{b/f}$	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.
PBS	Dulbecco's Phosphate buffered saline used in 1x concentration
PPB	Plasma protein binding

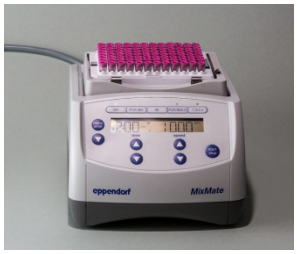
7 Reagents

The following reagents are required to run the TRANSIL AGP 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 AGP Binding 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 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 AGP 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 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 AGP Binding assay is designed such that one compound utilizes 8 wells – two references and 6 wells with increasing AGP 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 AGP Binding assay is illustrated in Figure 2.

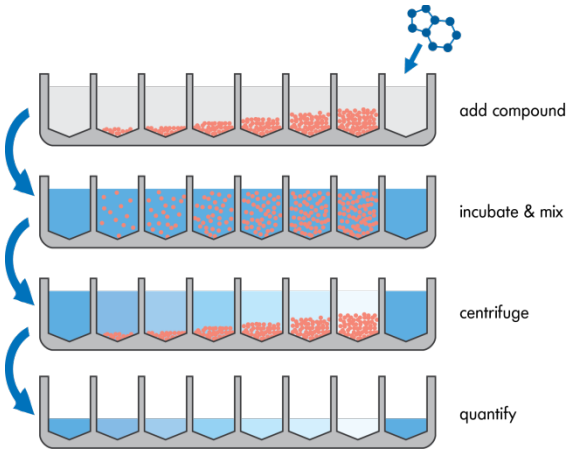


Figure 2: TRANSIL AGP Binding Assay workflow: The same amount of drug is added to all wells followed by a mixing step. After 12 minutes incubation, beads are separated from the buffer by centrifugation and the remaining supernatant is sampled for quantification.

Follow the following 5 steps for the assay procedure:

12.1 Compound addition

Mix the compound stock solution carefully by vortexing. When the TRANSIL AGP 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 with repeated aspiration and suspension to ensure proper mixing:

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

NB: Alternatively, a plate shaker may be used @ 800 RPM for 12 minutes. 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. But note that gentle mixing via aspiration and suspension is best AGP beads.

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

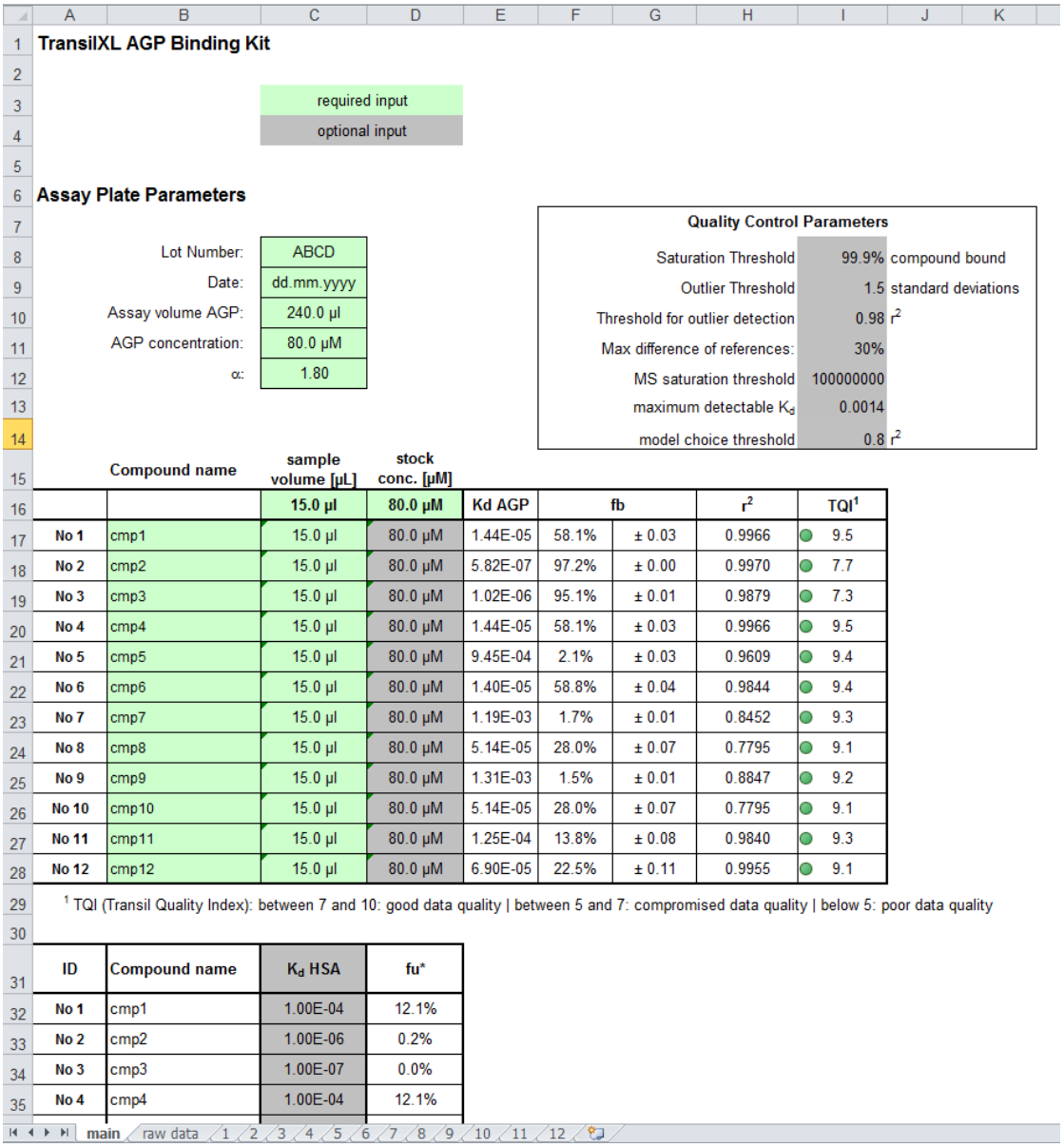
Use your analytical technique of choice for quantifying the compound concentration in the supernatant obtained in the last assay step.

14 Data analysis

Open the supplied spreadsheet for data analysis and follow the steps below to obtain the results for the TRANSIL AGP 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.



TransilXL AGP Binding Kit

required input
optional input

Assay Plate Parameters

Lot Number:	ABCD
Date:	dd.mm.yyyy
Assay volume AGP:	240.0 µl
AGP concentration:	80.0 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]					
		15.0 µl	80.0 µM	Kd AGP	fb		r^2	TQI[1]
No 1	cmp1	15.0 µl	80.0 µM	1.44E-05	58.1%	± 0.03	0.9966	9.5
No 2	cmp2	15.0 µl	80.0 µM	5.82E-07	97.2%	± 0.00	0.9970	7.7
No 3	cmp3	15.0 µl	80.0 µM	1.02E-06	95.1%	± 0.01	0.9879	7.3
No 4	cmp4	15.0 µl	80.0 µM	1.44E-05	58.1%	± 0.03	0.9966	9.5
No 5	cmp5	15.0 µl	80.0 µM	9.45E-04	2.1%	± 0.03	0.9609	9.4
No 6	cmp6	15.0 µl	80.0 µM	1.40E-05	58.8%	± 0.04	0.9844	9.4
No 7	cmp7	15.0 µl	80.0 µM	1.19E-03	1.7%	± 0.01	0.8452	9.3
No 8	cmp8	15.0 µl	80.0 µM	5.14E-05	28.0%	± 0.07	0.7795	9.1
No 9	cmp9	15.0 µl	80.0 µM	1.31E-03	1.5%	± 0.01	0.8847	9.2
No 10	cmp10	15.0 µl	80.0 µM	5.14E-05	28.0%	± 0.07	0.7795	9.1
No 11	cmp11	15.0 µl	80.0 µM	1.25E-04	13.8%	± 0.08	0.9840	9.3
No 12	cmp12	15.0 µl	80.0 µM	6.90E-05	22.5%	± 0.11	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 HSA	fu*
No 1	cmp1	1.00E-04	12.1%
No 2	cmp2	1.00E-06	0.2%
No 3	cmp3	1.00E-07	0.0%
No 4	cmp4	1.00E-04	12.1%

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 AGP 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	2.478		
cmp1	B-1	Well 1	2.156		
cmp1	C-1	Well 2	1.873		
cmp1	D-1	Well 3	1.401		
cmp1	E-1	Well 4	0.875		
cmp1	F-1	Well 5	0.522		
cmp1	G-1	Well 6	0.385		
cmp1	H-1	Ref 2	2.430		
cmp2	A-2	Ref 1	3.870		
cmp2	B-2	Well 1	1.216		
cmp2	C-2	Well 2	0.399		
cmp2	D-2	Well 3	0.157		
cmp2	E-2	Well 4	0.085		
cmp2	F-2	Well 5	0.053		
cmp2	G-2	Well 6	0.028		
cmp2	H-2	Ref 2	3.720		
cmp3	A-3	Ref 1	6.980		
cmp3	B-3	Well 1	2.918		
cmp3	C-3	Well 2	1.643		
cmp3	D-3	Well 3	0.553		
cmp3	E-3	Well 4	0.222		
cmp3	F-3	Well 5	0.122		
cmp3	G-3	Well 6	0.085		
cmp3	H-3	Ref 2	5.910		
cmp4	A-4	Ref 1	5.452		
cmp4	B-4	Well 1	4.744		
cmp4	C-4	Well 2	4.120		
cmp4	D-4	Well 3	3.081		
cmp4	E-4	Well 4	1.925		
cmp4	F-4	Well 5	1.147		
cmp4	G-4	Well 6	0.847		
cmp4	H-4	Ref 2	5.346		
cmp5	A-5	Ref 1	5.930		
cmp5	B-5	Well 1	5.723		
cmp5	C-5	Well 2	5.510		
cmp5	D-5	Well 3	5.414		
cmp5	E-5	Well 4	5.386		
cmp5	F-5	Well 5	5.254		
cmp5	G-5	Well 6	5.136		
cmp5	H-5	Ref 2	6.250		
cmp6	A-6	Ref 1	4.634		
cmp6	B-6	Well 1	4.054		
cmp6	C-6	Well 2	3.540		
cmp6	D-6	Well 3	2.675		
cmp6	E-6	Well 4	1.715		
cmp6	F-6	Well 5	1.006		
cmp6	G-6	Well 6	0.720		
cmp6	H-6	Ref 2	4.471		
cmp7	A-7	Ref 1	1.620		
cmp7	B-7	Well 1	1.592		

main | raw data | 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 AGP 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 AGP and HSA 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 AGP 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 HSA 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 AGP or HSA 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 AGP and HSA 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 HSA 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.

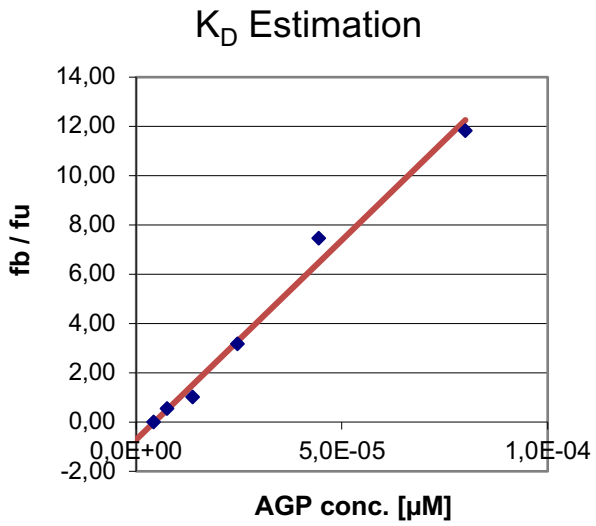


Figure 5: Illustration of fitting experimental data to equation (3) to determine the affinity to AGP. The intercept was estimated to -0.7, hence a quality score of 5 was attributed to the model fit.

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.

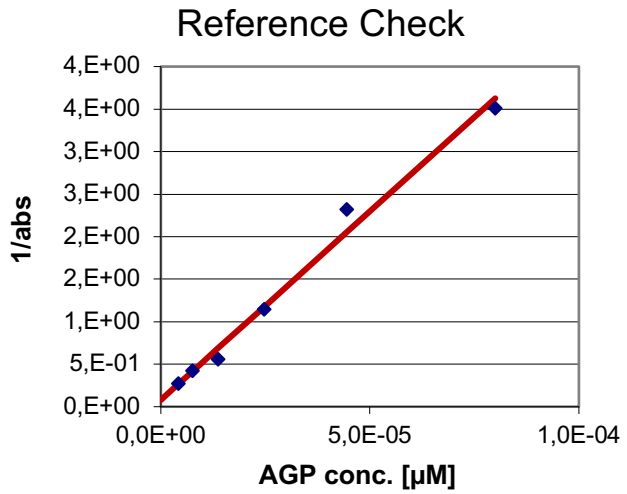


Figure 6: Illustration of estimating the peak area in the reference vials by plotting the inverse of the peak areas of the compound concentration of supernatants in TRANSIL vials against the protein content. The inverse of the intercept represents the expected peak area of the references.

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.

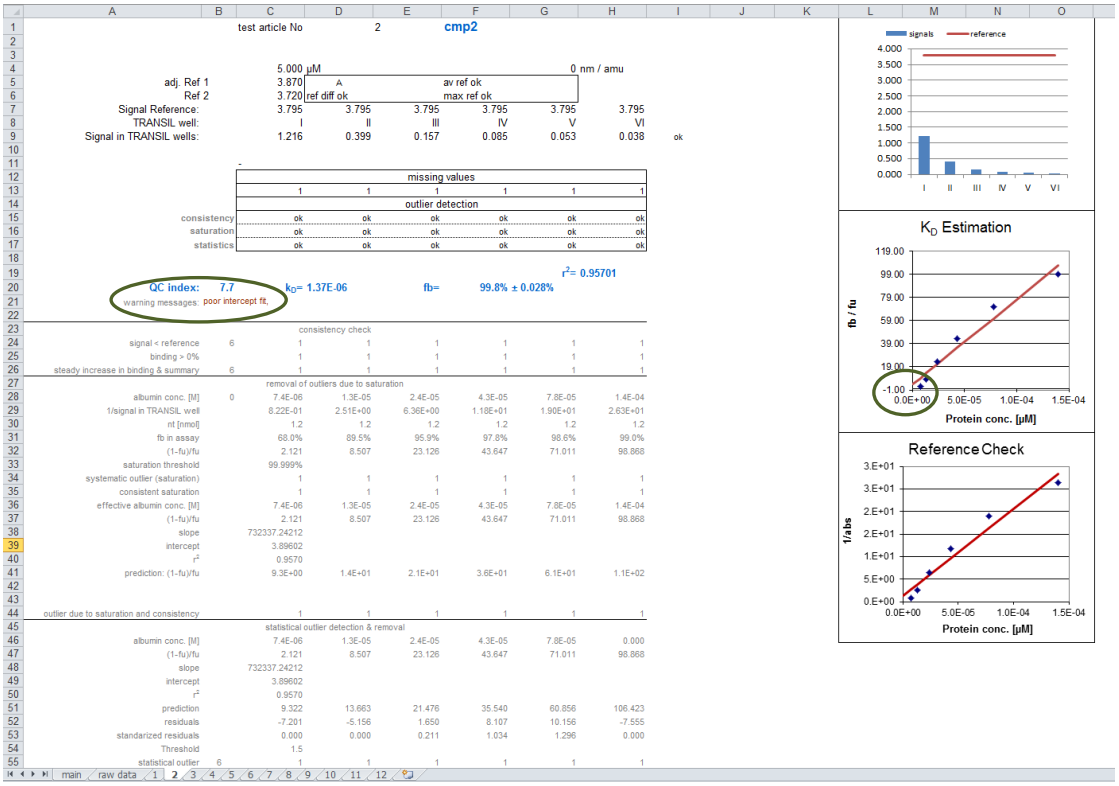


Figure 7: Illustration of the non-linear response issue which can be recognized by a poor intercept fit (green circles) or non-linear shape of the regression plotted in the “KD Estimation” graph.

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 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 protein 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

The TRANSIL AGP binding kit is optimized for strong binding compounds. However, there are still limits to measuring very low dissociation constants. Please contact our technical support if it is required to estimate dissociation constants of 10 nM or less. However, if it suffices to accurately measure plasma protein binding of tightly bound drugs we recommend using 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). Due to the low abundance of AGP in the physiological composition of plasma it has generally no relevance to measure weak AGP binding more accurately as the contribution to total plasma binding will be insignificant.

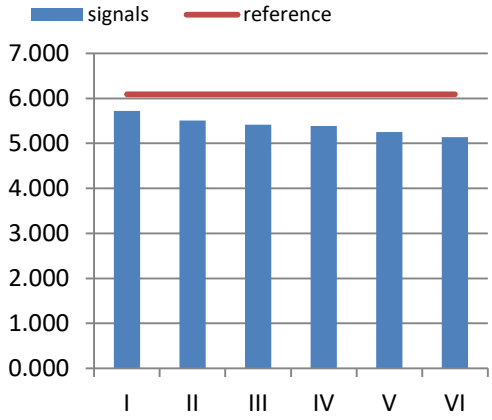


Figure 8: Illustration of a bar plot of a compound exhibiting weak protein binding (c.f. individual data analysis tabs of the spreadsheet). The blue bars show the detected signals in the supernatants of TRANSIL wells I to VI. As the compound binds only weekly to the proteins, supernatant concentrations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

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
HSA	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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