User Guide

TRANSIL

Equilibrium Shift Assay Kit

TPB-0311-2096 (standard kit without AGP beads)

TPB-0312-2096 (incl. AGP beads)

TPB-0313-2096 (duo kit without AGP beads)

TPB-0314-2096 (duo kit with AGP beads)

Version 2, Revision 01

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List of Abbreviations/ Glossary

a	Plasma dilution factor.
AAG	Synonym for AGP, see AGP.
Analyte	Test item added to the assay kit for measuring plasma binding.
AGP	human α_1-acid glycoprotein, synonymous to AAP and Orosomucoid
APA	Concentration of the free and bound drug in the liquid phase (buffer and plasma). This concentration does not include the drug concentration bound to the immobilized plasma proteins.
Approximated absolute recovery	Recovery derived from the analyte's peak areas in reference wells of the assay. The assay has 5 pairs of references, each containing a different amount of plasma. It is assumed that the highest amount of plasma used in the assay yields the highest absolute recovery. The latter may be close to 100% absolute recovery. A decline in recovery is reflected in decreasing analyte peak areas along with decreasing plasma content of the reference wells. This between plasma concentration comparison allows the approximation of absolute recovery.
Assay volume	"main" tab / assay plate parameters: Total liquid phase of each assay compartment. Refer to the certificate of analysis for each kit to obtain the correct number.
Buffer [µL]	"main" tab / plasma dilution steps: denotes the volume of buffer required to prepare the plasma pre-dilution.
Compound conc.	"main" tab / assay plate parameters: final calculated concentration of the test item in the assay in µM. This is calculated from the assay volume, the sample volume and the compound stock concentration.
Compound name	"main" tab / assay plate parameters: unique identifier of the test item
Compound stock	"main" tab / assay plate parameters: concentration in µM of the test item's stock solution that is added to the assay
c_0	Final test item concentration in the assay.
Confidence interval	"main" tab results: The confidence interval (CI) is an observed interval (i.e. it is calculated from the observations), and is in principle different from sample to

	sample. The CI frequently includes the parameter of interest if the experiment is repeated. The level of confidence of the confidence interval (the spreadsheet uses a level of 95%) indicates the probability that the confidence range captures the true population parameter given a distribution of samples. It does not describe any single sample. A CI does not predict that the true value of the parameter has a particular probability of being in the CI given the data actually obtained.
Date	"main" tab / assay plate parameters: date when the assay was performed.
Dilution	"main" tab / plasma dilution steps: real number of the plasma dilution ratio in the final assay.
DMSO	Dimethyl sulfoxide
ESA	Equilibrium shift assay
f_b	Fraction bound of a drug to plasma proteins
fb/fu	Ratio of the fraction bound over the fraction unbound.
fb/fu*	Predicted ratio of the fraction bound over the fraction unbound.
Final dilution	"main" tab / plasma dilution steps: ratio of the plasma dilution in the final assay.
f_u	Unbound fraction of a drug in plasma
fu' exp	Fraction of drug not bound to TRANSIL beads.
fu(HSA)	"main" tab results: Final estimate of the free fraction of the test item in a 600 µM pure albumin solution. The mean estimate is reported in cell H13 of the "main" tab.
fu(plasma)	"main" tab results: Final estimate of the free fraction of the test item in full (undiluted) plasma. The mean estimate is reported in cell I10 of the "main" tab. A 95% confidence interval around this mean is reported cells H9 and J9 of the "main" tab.
Highest plasma conc.	"main" tab / plasma dilution steps: denotes the plasma dilution ratio in the assay's wells with the highest plasma content. The highest possible ratio 1:3.
HSA	Human Serum Albumin
[HSA]	Concentration human serum albumin immobilized on TRANSIL beads.

HSA content	"main" tab / assay plate parameters: highest albumin concentration immobilized on beads. Refer to the certificate of analysis for each kit to obtain the correct number.
HSA^B	Concentration of HSA covalently immobilized on TRANSIL beads
HSA^S	Concentration of HSA in liquid phase of the assay system (alternative to using plasma)
Internal standard	Reference compound with chemical properties similar to the analyte. We recommend adding an internal standard after performing the assay and before sample preparation.
IS (peak area)	"raw data" tab: refers to the quantification measurement of the internal standard (IS) in each well.
K_D^{HB}	The drugs's dissociation constant to HSA immobilized on TRANSIL beads
K_D^{HS}	The drugs's dissociation constant to HSA in liquid phase
K_D^P	The drugs's dissociation constant to plasma in liquid phase
K_D-value	The dissociation 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.
Lot number	"main" tab / assay plate parameters: unique identifier of the assay kit's production batch shown both on the assay plate's lid and the certificate of analysis.
Matrix	Information on the type of plasma used, e.g. plasma species and coagulant used.
Optional input	Fields with gray background are pre-filled with a default value. Adjustment of the default value is optional.
P	Concentration of plasma based on a somewhat arbitrary assumption of 600 µM. The absolute number is only relevant in relation to the estimate of the dissociation constant KD in relation to plasma.
PBS	Dulbecco's Phosphate buffered saline used in 1x concentration
Peak area	"raw data" tab: refers to the quantification measurement of the analyte in each well.
Plasma volume per preparation	"main" tab / plasma dilution steps: denotes the volume of pure or diluted plasma required for the experiment for each plasma dilution step. Note that the highest plasma

	dilution step requires a higher volume to accommodate for the calibration curve. The exact numbers required for each experiment are shown in the table L20:K26 on the "main" tab.
Plasma volume per well	"main" tab / plasma dilution steps: denotes the volume of plasma to be added to each well.
Pre-dilution	"main" tab / plasma dilution steps: percentage dilution of plasma in the stock solution to be pipetted to the assay kit. If this is 100%, then pure plasma is pipetted to the assay.
Pre-dilution ratio	"main" tab / plasma dilution steps: ratio of the plasma dilution in the stock solution to be pipetted to the assay kit. If this ratio is 1, then pure plasma is pipetted to the assay.
Pure plasma [µL]	"main" tab / plasma dilution steps: denotes the volume of pure plasma required to prepare the plasma pre-dilution.
r^2	Correlation coefficient
Range	"95%CI calc" tab: optional input field for the range of Kd values within the algorithm searches for the 95% confidence interval. If the 95% CI cannot be found within the default range of 4x the final Kd estimate, it is necessary to enter a higher multiple.
Recovery	"main" tab results: lowest approximated absolute recovery in the assay. This number is usually influenced by the approximated absolute recovery estimate in the wells with the lowest plasma concentration.
Required input	Fields with green background that require an input from the user.
Residuals	Difference between the predicted fb/fu ratio and the in the assay observed fb/fu ratio. Note that fb is defined as the fraction of drug bound to the albumin coated beads, while fu is the fraction of drug in the supernatant either free or bound to plasma. The letter equals the concentration APA divided by to the total drug concentration.
Sample	"raw data" tab: refers to the type of the well. Either the well is a reference without beads (=Ref1 or Ref2), or it contains Transil beads coated with albumin. TRANSIL 1 refers to the lowest immobilized albumin concentration, TRANSIL 2 refers to the second lowest immobilized albumin concentration, TRANSIL 3 refers to the third

	lowest albumin concentration, TRANSIL 4 refers to the second highest immobilized albumin concentration, TRANSIL 5 refers to the highest immobilized albumin concentration.
Sample volume	“main” tab / assay plate parameters: volume in µL of the compound stock to be added to each well of the assay.
Scaled peak area	“raw data” tab: refers to the ratio of analyte concentration to internal standard concentration in each well.
SR	Squared residuals
SSR	Sum of squared residuals.
Subsequent dilution steps	“main” tab / plasma dilution steps: denotes the plasma dilution ratio between the series of wells with the highest plasma concentration and the series with the second highest plasma concentration; and the ratio between the second highest plasma concentration and the third highest plasma concentration; and so on to the lowest plasma concentration.
TQI	TRANSIL Quality Index
v/v	volume per volume
Vial ID	“raw data” tab: Logical ID of the assay’s wells referring to the plate layout and colored caps. Use the plate with the vials for the calibration curve (gray caps) oriented on the left side of the plate (Figure 1). Wells for the first plasma dilution (=highest plasma concentration) have brown lids and are in the plate’s position A4 to G4, where A4 and G4 are reference wells and B4 to F4 are wells with increasing concentration of albumin beads. Wells for the second plasma dilution have green caps. Wells for the third plasma dilution have blue caps. Wells for the fourth plasma dilution have pink caps. Wells for the fifth plasma dilution have yellow caps. On the plate with the gray capped calibration wells facing towards the left side the wells with the letter A (A4:brown, A6:green, A8:blue, A10:pink, A12:yellow) face the upper end of the plate, while wells with letter G face the lower end of the plate. NOTE: the wells carry IDs on their bottom, however, this ID will not match the logical ID defined here.

1 Quick Protocol

1. Kit preparation

- Equilibrate kit for 1h at room temperature.
- Spin plate quickly for 5 seconds at 750 g to collect all liquid at the bottom.
- Make sure the assay has a working temperature between 20°C and 25°C when starting the experiment.
- Open the lids only immediately before compound addition (step 5).

2. Drug Candidate Preparation

- Prepare 45x stock solutions of each compound in pure solvent (e.g. isopropanol/water).
- 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 a 24 µM stock solution.
- Since each compound is added in an aliquot of 5 µl to 5 x 7 tubes and the calibration tubes, we recommend preparing a volume of at least 200 µl stock solution for each compound.

3. Preparation of plasma and calibration sample pre-dilutions

- Prepare five sequential pre-dilutions (dilution step 1:3) for calibration in pure solvent at 24x concentration of the respective calibration concentration (c.f. section 3).
- Prepare plasma pre-dilutions according to the instructions given in the spreadsheet.

4. Plasma and Drug Candidate Addition

- Open wells with supplied decapper.
- Mix the test compound and calibration curve stock solutions as well as plasma pre-dilutions carefully.
- Add 40 µl pre-diluted plasma to each of the 7 tubes within each plasma dilution series.
- Add 40 µl of pre-dilution with highest plasma concentration to the 5 calibration tubes (or volume of choice for calibration curve that matches the assay's highest plasma concentration).
- Transfer 5 µl of the 45x test compound stock solution to each tube. Change tips after each compound transfer step to avoid carryover of beads.
- Transfer 5 µl of the respective 45x calibration pre-dilution to each calibration tube.
- Close each tube with its respective cap.

5. Incubation and Supernatant Sampling

- Incubate the plates by inverting it on a rotator or by shaking at 800 rpm on a plate shaker.
- Spin the plate in a swing-out centrifuge for 5 minutes at 750 g.
- Quantification by scintillation counting:

  Transfer up to 150 µl supernatant to scintillation vials and proceed with step 8.

  Quantification by LC-MS/MS:

  Transfer 40 µl supernatant to a plate for protein precipitation.

6. Plasma precipitation

- Add 100 µl acetonitrile (or other organic solvent) containing internal standard to each well. Place the plate for 1 minute on at plate shaker at 1000 rpm.
- Incubate 10 minutes at room temperature.
- Centrifuge 30 minutes at 3000 g to precipitate plasma.
- Transfer up to 100 µl of supernatant in a standard 96 well plate for analytical quantification.

7. 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

2.1 Plasma Protein Binding in Drug Discovery

Plasma protein binding is a key determinant of a drug's pharmacokinetic and pharmacodynamic behavior. In plasma, many compounds bind reversibly to abundant proteins such as human serum albumin (HSA) and α1-acid glycoprotein (AGP), leaving only a fraction of the drug unbound. This unbound fraction (fu) is generally considered the pharmacologically active form because only free drug can diffuse across biological membranes, interact with molecular targets, and undergo metabolism and excretion. Accurate determination of plasma protein binding is therefore essential during drug discovery and development, as it influences predictions of tissue distribution, clearance, half-life, drug–drug interactions, and effective dosing. Reliable measurements of fu also support pharmacokinetic modeling and enable rational optimization of compound properties during lead optimization.

2.2 Limitations of Conventional Binding Assays

Many classical plasma protein binding assays, such as equilibrium dialysis or ultrafiltration, rely on analytical models that assume simple linear binding equilibria between drugs and plasma proteins. Under these assumptions, the fraction of drug bound to plasma proteins is expected to scale proportionally with protein concentration across plasma dilution series. However, this assumption is frequently violated for compounds that bind strongly to plasma proteins or interact with a limited number of high-affinity binding sites.

When binding affinity is high, dilution of plasma can change the occupancy of protein binding sites and alter the binding equilibrium in a non-linear manner. Under these conditions, the relationship between plasma concentration and free fraction is no longer proportional, and simple linear extrapolation methods may produce inaccurate estimates of plasma protein binding. The problem becomes particularly pronounced for compounds that exhibit site-specific binding or saturation kinetics, where small changes in protein concentration can lead to large changes in the apparent free fraction.

These effects are increasingly relevant for modern drug modalities, including lipidated peptides, macrocycles, and beyond-Rule-of-5 compounds, which often display strong and complex interactions with plasma proteins such as albumin or α1-acid glycoprotein. In such

systems, conventional plasma protein binding assays may generate results that depend strongly on the chosen dilution conditions or analytical assumptions.

To obtain reliable binding estimates for such compounds, assay approaches are required that explicitly account for non-linear binding equilibria and allow mechanistic interpretation of the observed equilibrium shifts.

2.3 Principle of the Equilibrium Shift Assay

The TRANSIL Equilibrium Shift Assay (ESA) determines plasma protein binding by measuring the distribution of a compound between plasma proteins in solution and albumin immobilized on TRANSIL beads. In contrast to membrane-based equilibrium shift assays, the ESA assay uses protein–protein competition to probe plasma protein binding. Albumin-coated beads act as a defined binding phase that competes with plasma proteins for interaction with the compound, creating a controlled shift in the binding equilibrium.

To generate this equilibrium shift, the assay combines several plasma dilutions with different amounts of albumin-coated beads. Varying the concentration of soluble plasma proteins while simultaneously changing the available albumin binding surface produces a matrix of binding conditions that systematically alters the distribution of the compound between plasma proteins and the immobilized albumin phase. The resulting equilibrium shifts provide the information needed to quantify plasma protein binding.

Importantly, the assay does not require direct measurement of the free drug concentration. Instead, the compound concentration remaining in the liquid phase is quantified after removal of the albumin beads by centrifugation. This liquid phase contains both free compound and compound bound to plasma proteins. Plasma protein binding parameters and the corresponding free fraction are then calculated from the way the equilibrium shifts across the plasma dilution series.

The experimental data are interpreted using a mechanistic kinetic ODE model describing the competitive binding of a compound to the major plasma proteins—albumin and α_1-acid glycoprotein (AGP)—as well as to the immobilized albumin phase on the beads and, optionally, to AGP-coated beads. Each interaction is defined by compound-specific association and dissociation rate constants, allowing the model to capture binding equilibria, displacement, and saturation effects under physiologically relevant protein

concentrations. The model also accounts for non-linear plasma protein binding that can arise when compounds bind weakly to abundant proteins such as albumin (in human around 600 µM) but strongly to lower-abundance proteins like AGP (in human around 20 µM), particularly under conditions of plasma dilution. This model-based approach enables accurate determination of plasma protein binding even when classical linear binding assumptions no longer hold.

Like other TRANSIL assays, the ESA workflow is simple and robust, requiring only standard liquid handling, incubation, and centrifugation steps. The assay can therefore be readily integrated into automated ADME screening workflows while providing mechanistic insight into plasma protein binding behavior.

2.4 Protein-Based Equilibrium Shift in the ESA Assay

In the TRANSIL Equilibrium Shift Assay (ESA), the binding equilibrium is probed using protein–protein competition rather than membrane competition as in the TRANSIL High Sensitivity Binding Kit. Albumin, and optionally also AGP, immobilized on TRANSIL beads serves as a defined binding phase that competes with plasma proteins for interaction with the test compound. By combining different plasma dilutions with varying amounts of albumin-coated beads, the assay systematically shifts the binding equilibrium between soluble plasma proteins and the immobilized albumin phase. These controlled equilibrium shifts change the distribution of the compound between the liquid phase and the bead-bound phase, providing quantitative information about its interaction with plasma proteins. Because the readout measures the compound remaining in the liquid phase after bead removal, the assay avoids the need to directly measure the free drug concentration while still allowing accurate determination of plasma protein binding parameters.

2.5 Mechanistic Model for Binding Analysis

The ESA assay is interpreted using a mechanistic binding model that describes the competitive interaction of a compound with the major plasma proteins and the immobilized protein phase used in the assay. In plasma, drug molecules bind reversibly to proteins such as human serum albumin (HSA) and α1-acid glycoprotein (AGP), which differ strongly in abundance and binding characteristics. The model represents these interactions together with the binding of the compound to the albumin-coated beads used in the assay. By

describing these competing equilibria in a unified framework, the model links the experimentally observed equilibrium shifts to the underlying binding affinities of the compound toward the different protein phases. This approach allows mechanistic interpretation of the assay data and enables reliable estimation of plasma protein binding parameters even when binding behavior becomes non-linear due to plasma dilution or saturation of binding sites.

The model describes the reversible binding of the test compound to the principal protein phases present in the assay system. In plasma, the compound can bind to human serum albumin (HSA), the most abundant plasma protein, and to α1-acid glycoprotein (AGP), a lower-abundance protein that often exhibits higher affinity for basic and lipophilic compounds. In addition, the compound can bind to albumin immobilized on the TRANSIL beads, which acts as a defined competing binding phase in the assay. Each of these interactions is treated as a reversible binding process that can form a protein–compound complex. By simultaneously accounting for binding to plasma proteins and to the immobilized albumin phase, the model captures the competitive equilibria that determine the distribution of the compound between the liquid phase and the bead-bound phase during the equilibrium shift experiment.

2.5.1 Kinetic Parameters

Each binding interaction in the model is characterized by compound-specific kinetic parameters that describe the reversible association between the compound and the protein binding sites. For every protein phase represented in the model—such as albumin in plasma, α1-acid glycoprotein (AGP), and the immobilized albumin on the beads—binding is defined by an association rate constant (k_{on}) and a dissociation rate constant (k_{off}). These parameters determine both the speed at which binding equilibria are established and the strength of the interaction. The ratio of the dissociation and association rate constants defines the equilibrium dissociation constant (K_D), which quantifies the affinity of the compound for the respective protein. By estimating these parameters from the observed equilibrium shifts, the model can describe the compound's binding behavior across different plasma dilutions and binding phases and derive the corresponding plasma protein binding characteristics.

In the mechanistic binding model used for data analysis, the association rate constant (k_{on}) is fixed to representative values that reflect the typical diffusion-limited encounter rates of molecules of different size classes. For small molecules, k_{on} is assumed to be 10^6 M^{-1} s^{-1}, which lies near the upper range commonly observed for small-molecule interactions with plasma proteins such as albumin or AGP. For larger ligands such as peptides or protein fragments, a lower value of 10^4 M^{-1} s^{-1} is used to account for their slower diffusion and the greater structural rearrangements often required for productive binding. The dissociation rate constant (k_{off}) is then determined from the fitted equilibrium dissociation constant (K_D) according to $K_D = k_{off}/k_{on}$. This approach is justified because association rates for protein–ligand interactions vary within a relatively narrow range compared with dissociation rates, which can span many orders of magnitude depending on complex stability. As a result, the equilibrium binding affinity—and thus the estimated plasma protein binding—is largely governed by k_{off} rather than k_{on}. Fixing k_{on} to representative diffusion-based values therefore simplifies the model while still providing robust and reliable estimates of K_D and plasma protein binding behavior.

2.5.2 Differential Equation Framework

The competitive binding processes represented in the model are described mathematically using a system of ordinary differential equations (ODEs) that track the time-dependent formation and dissociation of protein–compound complexes. Each equation represents the rate of change of a particular species (free compound, free protein, or protein–compound complex) as a function of the association and dissociation reactions occurring in the system. In this framework, binding of the compound to albumin, α1-acid glycoprotein (AGP), and the immobilized albumin phase on the beads is represented by reversible reactions whose rates are determined by the corresponding kinetic parameters. The ODE system is numerically solved to determine the equilibrium distribution of the compound among the different binding phases under the specific experimental conditions used in the assay. By fitting the model predictions to the experimentally measured compound concentrations in the liquid phase across the equilibrium shift matrix, the binding parameters and resulting plasma protein binding characteristics can be estimated in a mechanistically consistent manner.

Specifically, a system of 9 differential equations describes the mass balance relationships between the concentration of free and bound states in both the liquid and solid phases over time t. The concentration of test compound 1 evolves over time according to this equation:

$$\frac{dS1}{dt} = hm_1HS1[t] - hk_1S1[t]H[t] + am_1AS1[t] - ak_1S1[t]A[t] + bhm_1BHS1[t] - bhk_1S1[t]BH[t] + bam_1BAS1[t] - bak_1S1[t]BA[t]$$

wherein

- $hm_1HS1[t]$ is the off-rate hm_1 times the albumin – test item 1 complex (HS1) concentration;
- $hk_1S1[t]H[t]$is the on-rate hk_1 times the free test item 1 concentration times the free albumin concentration;
- $am_1AS1[t]$ is the off-rate am_1 times the AGP – test item 1 complex (AS_1) concentration,
- $ak_1S1[t]A[t]$is the on-rate ak_1 times the free test item 1 concentration times the free AGP concentration (A);
- $bhm_1BHS1[t]$is the off-rate bhm_1 times the concentration of albumin coated beads with bound test item 1 (BHS_1);
- $bhk_1S1[t]BH[t]$is the on-rate bhk_1 times the free test item 1 concentration times the free concentration of bead-bound albumin;
- $bam_1BAS1[t]$ is the off-rate bam_1 from complex of bead-bound AGP with bound test item 1;
- $bak_1S1[t]BA[t]$ is the on-rate bak_1 times the free test item 1 concentration times the free AGP concentration on beads.

For clarity, only the differential equation describing the evolution of the free compound concentration is shown here. The full mechanistic model consists of a system of nine coupled differential equations that describe the time-dependent mass balance between free and protein-bound species in both the liquid and bead phases. The remaining equations follow the same structure and represent the reversible binding interactions of the compound with albumin, α1-acid glycoprotein (AGP), and the immobilized protein phases. These equations are not shown here for brevity. Numerical solution of the

differential equation system and parameter estimation are performed using Wolfram Mathematica, which enables efficient simulation of the equilibrium states and nonlinear fitting of the model to the experimental data.

2.5.3 Model Fitting and Parameter Estimation

Model parameters are estimated by fitting the mechanistic binding model to the experimentally measured compound concentrations in the liquid phase obtained across the matrix of plasma dilutions and bead surface areas. For each experimental condition, the differential equation model predicts the equilibrium distribution of the compound between plasma proteins, the immobilized albumin phase, and the free solution phase. Parameter estimation is performed using nonlinear regression, where the model parameters are iteratively adjusted to minimize the difference between predicted and measured concentrations. The fitting procedure yields estimates of the equilibrium dissociation constants for the relevant protein interactions and allows calculation of the corresponding plasma protein binding parameters, including the fraction unbound (fu). Because the fitting is based on a mechanistic representation of the competing binding equilibria, the resulting parameter estimates remain robust even when binding behavior deviates from simple linear assumptions due to strong binding, plasma dilution, or saturation effects.

Numerical solutions of the model yield the dissociation rate constants (k_{off}) for binding to albumin and AGP. The corresponding equilibrium dissociation constants are then calculated according to

$$K_D = \frac{k_{off}}{k_{on}}$$

The fraction unbound (f_u) is subsequently calculated from the resulting dissociation constants using

$$f_u = \frac{1}{1 + \frac{[HSA]}{K_D^{HSA}} + \frac{[AGP]}{K_D^{AGP}}}$$

It should be noted that the dissociation constants determined by the model represent effective or pseudo-KD values, because the model describes the interaction of the

compound with plasma proteins as an ensemble rather than resolving binding to individual protein species in detail. Consequently, the pseudo-KD depends on the assumed total plasma protein concentration used in the calculation. Since binding typically occurs only to a subset of plasma proteins, the exact molar concentration of relevant binding sites cannot be defined precisely unless there are experimentally quantified.

2.5.4 Non-Linear Binding Behavior

Plasma protein binding can become non-linear when a compound interacts with multiple plasma proteins that differ strongly in both abundance and binding affinity. In human plasma, albumin is present at high concentration but often binds compounds with moderate affinity, whereas α1-acid glycoprotein (AGP) is present at much lower concentration but can provide high-affinity binding sites. When a compound binds preferentially to AGP, these high-affinity sites may become partially saturated within the concentration range studied. As a result, the distribution of the compound between AGP, albumin, and the free solution phase changes as plasma is diluted or compound concentration varies. This shift in binding site occupancy alters the relative contribution of the different protein phases to overall binding and leads to non-linear plasma protein binding behavior. Mechanistic modeling is therefore required to correctly interpret the resulting equilibrium shifts and to separate true binding saturation effects from changes caused by experimental conditions.

2.5.5 Model Assumptions and Limitations

The mechanistic binding model used to interpret ESA data is based on several simplifying assumptions that allow robust estimation of plasma protein binding parameters while maintaining a tractable mathematical framework. The model assumes that binding of the compound to albumin, α1-acid glycoprotein (AGP), and the immobilized albumin phase on the beads is reversible and independent, with each protein represented by a single effective binding site (or multiple independent but identical binding sites). It further assumes that the incubation time is sufficient for the system to reach equilibrium and that the measured compound concentration in the liquid phase accurately reflects the distribution between free compound and protein-bound species in solution. As with any simplified representation of complex biological systems, certain compound classes may violate these

assumptions. For example, molecules capable of binding simultaneously to multiple albumin molecules or interacting with additional plasma components may introduce binding behaviors not fully captured by the current model. Nevertheless, within these assumptions the framework provides a robust and mechanistically consistent method for estimating plasma protein binding parameters across a wide range of compounds.

2.6 Non-Linear Plasma Protein Binding

Non-linear plasma protein binding can have important pharmacokinetic consequences because the fraction of unbound drug is no longer constant across the concentration range encountered in vivo. When high-affinity binding sites become partially saturated, the unbound fraction may increase with rising drug concentrations, altering the effective exposure of free drug in plasma and tissues. Because pharmacokinetic processes such as distribution, clearance, and target engagement are generally governed by the unbound drug concentration, such concentration-dependent binding can lead to non-linear relationships between dose, plasma concentration, and pharmacological effect. Accurate characterization of these effects is therefore essential for reliable pharmacokinetic modeling, dose prediction, and interpretation of exposure–response relationships during drug development.

2.7 Scope and Applicability of the ESA Assay

The TRANSIL Equilibrium Shift Assay (ESA) is designed to determine plasma protein binding for compounds whose binding behavior cannot be reliably characterized using conventional methods such as equilibrium dialysis or ultrafiltration. The assay is particularly well suited for compounds that exhibit strong plasma protein binding, complex or non-linear binding behavior, or concentration-dependent interactions with multiple plasma proteins. Because the method relies on protein-based competition rather than membrane partitioning, it is also applicable to compounds with low membrane affinity, including many hydrophilic molecules and peptide-based therapeutics. The ESA assay is therefore suitable for a broad range of drug modalities, including small molecules, peptides, and other complex structures, and can provide reliable plasma protein binding estimates even under conditions where traditional approaches are limited by very low unbound fractions, slow equilibration, or experimental artifacts.

2.8 Relationship to Other TRANSIL Assays

The TRANSIL Equilibrium Shift Assay (ESA) is closely related to the TRANSIL High Sensitivity Binding (HSB) Kit, but the two assays use different competitive binding principles and therefore complement each other in their applicability. The HSB assay employs membrane-coated beads to generate an equilibrium shift through competition between plasma proteins and a phospholipid membrane phase. This approach is particularly effective for compounds that exhibit significant reversible membrane affinity, such as highly lipophilic small molecules with very low unbound fractions. In contrast, the ESA assay uses albumin-coated beads to generate the equilibrium shift through protein-based competition, making it more suitable for compounds with low membrane affinity or complex protein binding behavior, including hydrophilic molecules and many peptide-based therapeutics. The two assays therefore address different physicochemical and mechanistic regimes of plasma protein binding. As summarized in Table X, HSB is often preferred for strongly lipophilic compounds or compounds with extremely low fu, whereas ESA is advantageous when binding behavior becomes non-linear, membrane affinity is weak, or protein-based interactions dominate the binding equilibrium.

The table below summarizes typical use cases for the HSB and ESA assays based on compound properties and expected binding behavior.

	HSB	ESA
Lipidated peptides (GLP-1 analogs)	0	+
complex or non-linear plasma protein binding	+	++
Very low fu	++	+
Strong non-specific binding to assay surfaces	+	+
Very low or poorly reversible membrane affinity	-	++
Hydrophilic compound	-	+
PROTAC with 2 albumin binding domains	++	-
PROTAC with one or no albumin binding domain	+	+

++ preferred assay, **+** suitable, **0** possible but not ideal, **–** not recommended

PROTACs containing two albumin-binding domains may bind simultaneously to immobilized albumin on the beads and to plasma albumin. This dual binding depletes compound from the plasma phase and introduces non-linear effects that are not captured by the current ESA model.

3 Kit components

The table below lists the contents of the kit and Figure 1 shows the supplied prefilled tubes.

No.	Qty.	Item
2	5	Prefilled tubes for calibration curve in plasma dilution 1 (cal-1 to cal-5, color code: ●).
1	7	Prefilled tubes for plasma dilution 1 (color code: ●).
3	7	Prefilled tubes for plasma dilution 2 (color code: ●).
4	7	Prefilled tubes for plasma dilution 3 (color code: ●).
5	7	Prefilled tubes for plasma dilution 4 (color code: ●).
6	7	Prefilled tubes for plasma dilution 5 (color code: ●).
7	1	Instruction manual
8	1	CD with spreadsheet calculation

Figure 1: Prefilled tubes supplied with the TRANSIL Equilibrium Shift Assay kit. For each plasma dilution the kit supplies 5 tubes with varying TRANSIL content and 2 reference tubes. For the lowest and highest plasma dilution (i.e. highest plasma concentration), the kit supplies 5 tubes for preparing a 5-point calibration curve.

4 Reagents

No.	Reagent	Specification
1	Full plasma	A total of 4.5 ml plasma is required per compound
2	Dulbecco's PBS (1x)	For dilution of plasma
3	45x sample stock solution	
4	Sample dilution for calibration	Prepare appropriate sample dilutions for a 5 point calibration curve

5 Equipment

No.	Instrument	Specification
1	Vortexer	
2	Test tube rotator or roller	
3	Pipette	
4	Centrifuge	Including rotor for SBS plates

6 Methodological description

6.1.1 Assay procedure

The TRANSIL ESA kit single-step-design consists of the following steps: (i) addition of 40 µl of pre-diluted plasma to each of the 7 vials within one of the 5 plasma dilution series and to each of 5 vials for calibration (ii) addition of 5 µL compound (24x) to each of the 7 sample vials per plasma dilution and to the 5 calibration curve vials, (iii) mixing by vortexing and 30 minutes incubation while rotating/shaking vials, (iv) separation of beads and buffer by centrifugation of the assay plate for 10 minutes at 1200 g, and (v) transfer of 50 µl supernatant to analytical quantification by scintillation counting. The assay principle is illustrated by Figure 2.

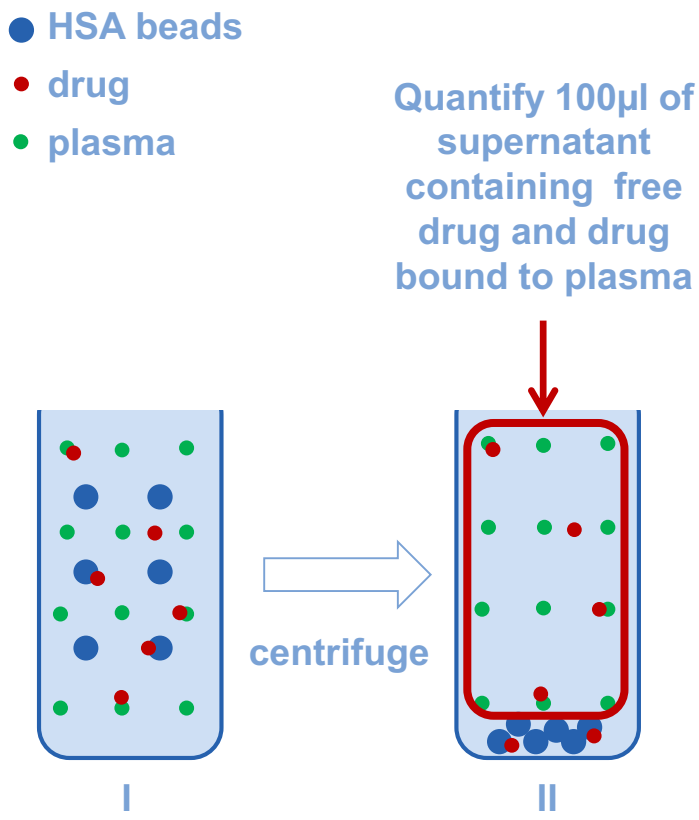


Figure 2: Schematic illustration of the TRANSIL ESA to assess plasma protein binding of the test drug. The assay principle consists of two competitive binding events of the drug candidate to immobilized HSA (HSA^B) and to the plasma proteins. The affinity of drugs to plasma is determined by measuring the change of drug bound to HSA^B in response to changing the plasma concentration in the assay system. The higher the amount of plasma that is added to this system, the more drug binds to plasma. This reduces the amount of free drug which can equilibrate with the HSA^B. Hence, less drug will be bound to HSA^B. After equilibration of the reaction setup (I), TRANSIL HSA beads are captured by centrifugation (II) and the supernatant, containing both free drug and drug bound to plasma proteins, is transferred to a new reaction vial for analytical

quantification by scintillation counting. The concentration of the drug in the supernatant (framed in red) is referred to as APA in the data analysis.

6.1.1.1 Drug candidate preparation

Prepare a 24x stock solution for the drug candidate. The stock solution may be set up in the appropriate solvent analogously to the original test compound solution (Isopropanol/Water).

It is necessary to have at least 200 µl of the stock solution for the test candidate since 5 µl of compound is added to each out of 5 x 7 vials. To accommodate for preparation of the calibration curves as well, prepare a volume of at least 400µl.

6.1.1.2 Replicates

The assay kit is designed such that the HSA binding of the test compound is determined in one plasma dilution in 7 vials – two references and 5 vials with increasing HSA^B concentrations.

6.1.1.3 Plasma Dilutions

The assay utilizes a series of 5 HSA^B concentrations and 5 plasma dilutions to determine the affinity of drugs to plasma via measuring how much drug binds to HSA^B under the resulting 25 different experimental conditions. The gradient of HSA^B is supplied with the kit, however, the gradient of plasma dilutions has to be set up individually. Compound properties, the HSA^B gradient and the plasma dilution series influence the accuracy of plasma binding results. Therefore, care must be taken to choose an appropriate plasma dilution series. We suggest a dilution series based on four equal dilution steps starting with a 1:3 dilution for the highest plasma concentration (corresponds to pure plasma without any pre-dilution) and four equal dilution steps with a dilution factor of 2. Based on this dilution series definition the spreadsheet automatically calculates a pipetting scheme for setting up the plasma pre-dilution series in PBS buffer (Figure 3).

Prepare five different plasma dilutions according to the dilution scheme provided by Figure 3. Add a volume of 75 µl of pre-diluted plasma to each of the 7 vials within one of the 5 plasma dilution series. Add an appropriate volume of pre-diluted plasma (first pre-dilution

– in most cases pure plasma) to the 5 vials provided for calibration as well to obtain the same plasma dilution as in the assay's first plasma dilution.

Plasma Dilution Steps

highest plasma conc: 1:	3.0
subsequent dilution steps: 1:	2.0
plasma volume per well:	75 µL
plasma volume per preparation:	500 µL

	final dilution	Assay	dilution	Pre-dilution ratio	Pre-dilution	buffer [µL]	pure plasma [µL]
No 1	1: 3		0.333333	1	100%	0.0	1000.0
No 2	1: 6		0.166667	1: 2	50%	250.0	250.0
No 3	1: 12		0.083333	1: 4	25%	375.0	125.0
No 4	1: 24		0.041667	1: 8	13%	437.5	62.5
No 5	1: 48		0.020833	1: 16	6%	468.75	31.3
						sum:	1468.8

Figure 3: Screen shot of the table "Plasma dilution steps" from the "main" tab of the spreadsheet for analyzing data from the TRANSIL ESA kit. The table shows calculated volumes (blue shaded area) for setting up the plasma pre-dilution series based on the choice of dilution series specified in fields D21 and D22. The final dilution is obtained when 75 µl of each plasma pre-dilution are added to 150 µl assay volume making a total assay volume of 225 µl. The total amount of pure plasma required is shown in cell L26.

6.1.1.4 Calibration

The calibration curve in the TRANSIL ESA is used only to assess apparent recovery. The assay supplies 5 vials for the calibration curve at the highest plasma concentration in the assay. The calibration curve with the highest plasma concentration is used to assess the maximal apparent recovery in the assay. We recommend to prepare the calibration curve with 1:3 dilution steps between the different calibration samples. An automatic calculation of the pre-dilutions, being concentrated 24x higher than the final concentrations in the calibration sample vials, is integrated in the "main" tab of the spreadsheet delivered with the Kit (Figure 4).

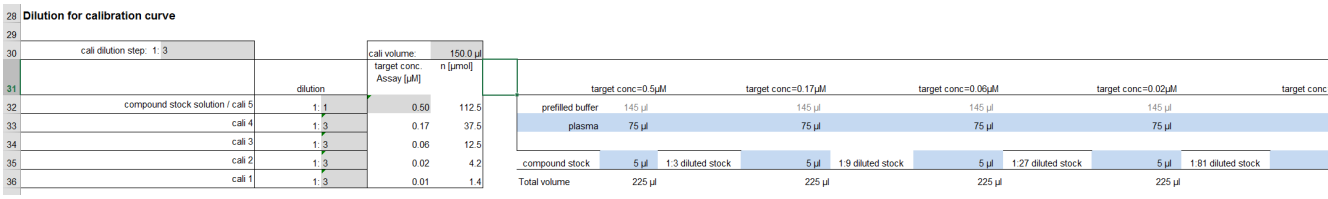
The spreadsheet also provides an approximation of the absolute recovery based on calculating apparent recovery at the highest plasma concentration (assuming this is close to 100% absolute recovery) and by evaluating the decrease of scintillation counts in reference vials from the highest to the lowest plasma concentration.

6.1.1.5 Compound addition

After the addition of plasma to the 35 vials of the experiment and to the 5 vials of the calibration, add 5 µL sample to each vial (assay and calibration).

6.1.1.6 Incubation

Incubate samples for 30 minutes at room temperature while shaking vials or by inverting the plate on a rotator.



28	Dilution for calibration curve																			
29																				
30	cali dilution step: 1: 3			cali volume:	150.0 µl															
31		dilution		target conc. Assay [µM]	n [µmol]		target conc=0.5µM		target conc=0.17µM		target conc=0.06µM		target conc=0.02µM		target conc					
32	compound stock solution / cali 5	1: 1		0.50	112.5	prefilled buffer	145 µl		145 µl		145 µl		145 µl							
33	cali 4	1: 3		0.17	37.5	plasma	75 µl		75 µl		75 µl		75 µl							
34	cali 3	1: 3		0.06	12.5															
35	cali 2	1: 3		0.02	4.2	compound stock	5 µl	1:3 diluted stock	5 µl	1:9 diluted stock	5 µl	1:27 diluted stock	5 µl	1:81 diluted stock						
36	cali 1	1: 3		0.01	1.4	Total volume	225 µl		225 µl		225 µl		225 µl							

Figure 4: Screen shot of the table “Dilution for calibration curve” from the “main” tab of the spreadsheet for analyzing data from the TRANSIL ESA kit. The table shows calculated volumes (highlighted in blue) for setting up the calibration dilution series of the test compound. All calculations are run automatically after the test compound concentration has been specified in the “main” tab of the spreadsheet. It is recommended to prepare a dilution series with 1:3 dilution steps starting with the 45x compound stock solution.

6.1.1.7 Separation of beads and sample quantification

Centrifuge plate at 800 g for 5 minutes to remove the TRANSIL beads. Remove an appropriate aliquot (up to 150 µl) for quantification.

6.1.2 Data analysis

Please enter the LC–MS/MS results into the **“Raw Data”** tab of the supplied spreadsheet template and submit the completed file to Sovicell for analysis. The data will then be processed using the full non-linear mechanistic model described above. Until dedicated data analysis software becomes available, this approach ensures accurate and consistent evaluation of the equilibrium shift experiments.

6.2 Assay parameters

After opening the spreadsheet navigate to the “main” tab and enter the lot specific parameters from the certificate of analysis that came with the assay kit. Also, enter the lot number and the assay date (Figure 5).

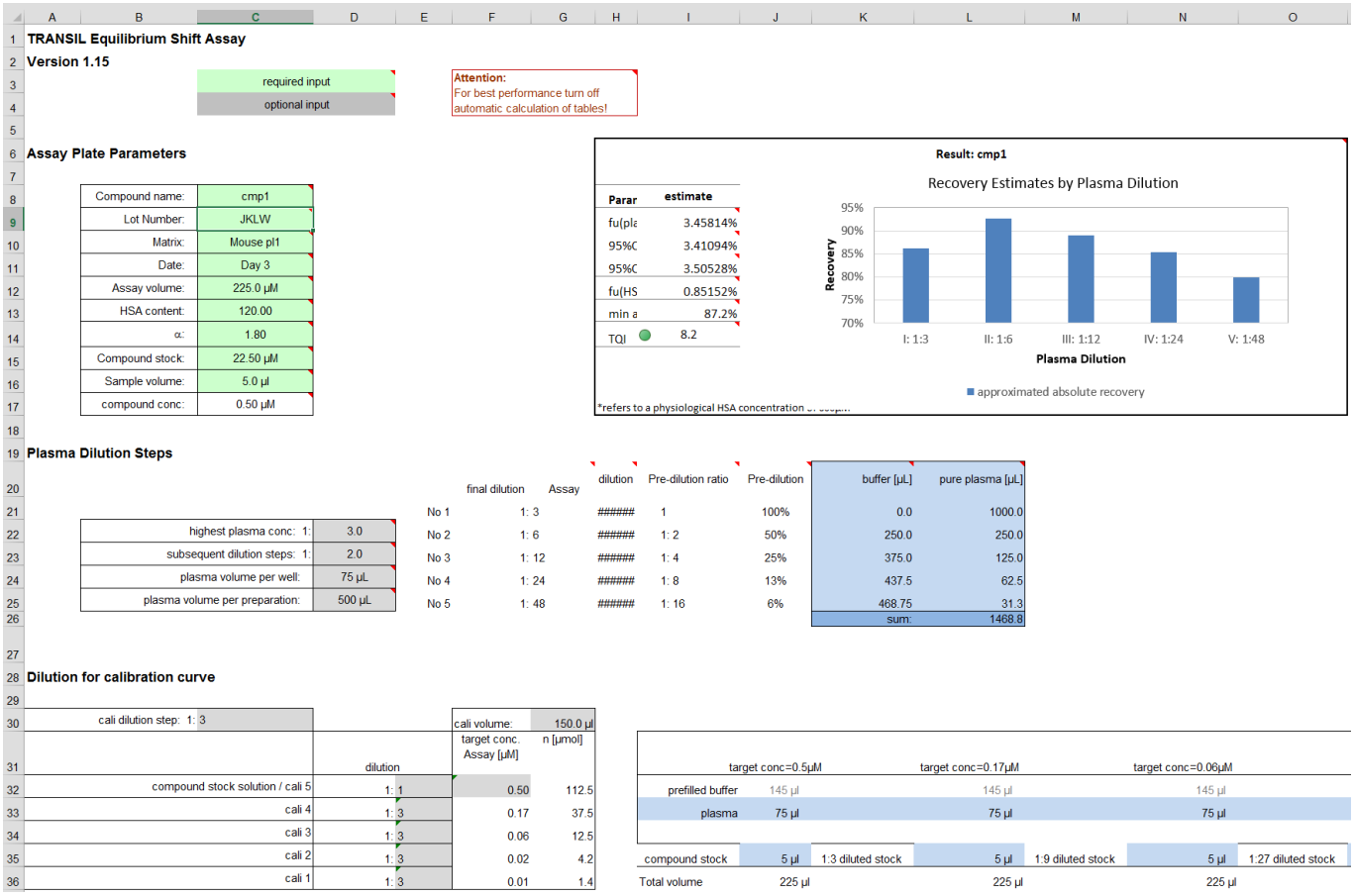


Figure 5: Screen shot of the spreadsheet's main tab where data from the certificate of analysis needs to be entered and the plasma dilution has to be specified. The tab then provides pipetting schemes for the plasma dilution and the calibration curve. The main tab also provides the final results. Note however, that it is necessary to initiate the calculations on the "fu_calc" and "95%Ci_calc" tab.

6.3 Compound information and plasma dilutions

Please enter the name of the compound (cell C8), the concentration of the compound stock (cell C15), and the sample volume added (cell C16) on the "main" tab.

6.4 Raw data from sample quantification

Please enter the raw data from the sample quantification in the "raw data" tab of spreadsheet. The spreadsheet provides sections for each plasma dilution step and fields for entering the peak areas form quantifying the drug as well as from quantifying the internal standard. If no internal standard is used, please enter "1" in all fields requiring internal standard input data. In case of missing values enter "NaN" in the respective field.

6.5 Analysis of Data Quality

6.5.1 Analysis of raw data

After entering the data in the “raw data” tab for plots are shown to inspect elementary data quality. The data from the calibration curve are plotted as shown in Figure 6. This plot indicates potential non-linearity in quantification process.

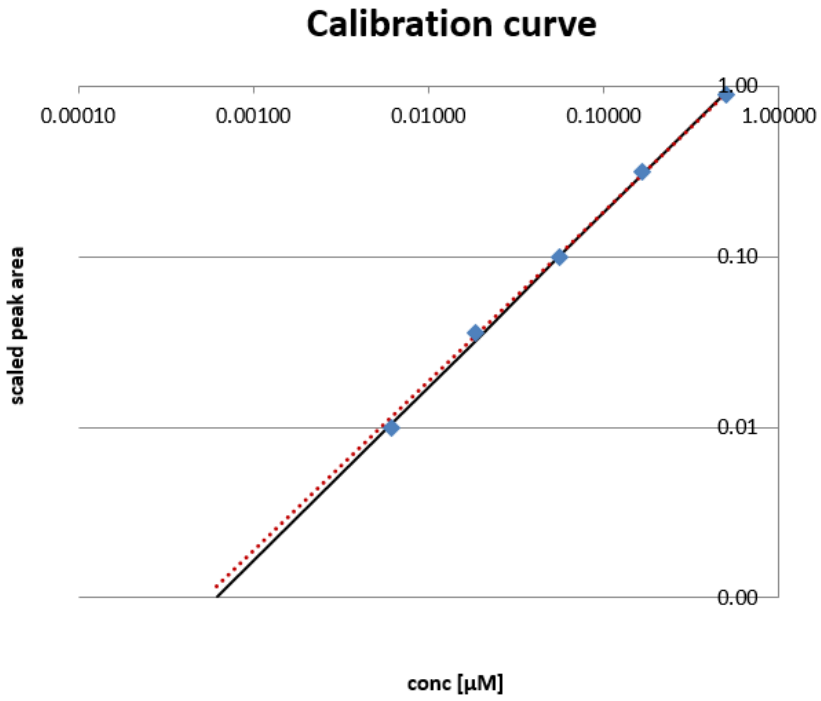


Figure 6: Calibration curve as shown on the “raw data” tab. The blue dots denote the actual measurements and the black line represents the regression line using all measured data. The red dotted line represents the expected decline in signal intensity based on the assumption that the instrument’s response is linear and thus that the relative signal change observed between the highest and second highest concentration is proportional to the changes between all other concentrations measured. A deviation of the red dotted line from the black line indicates non-linearity of the quantification process.

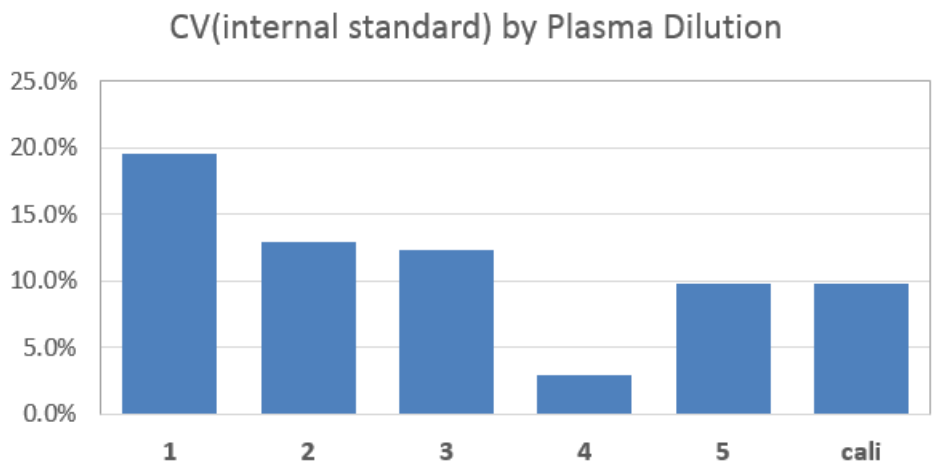


Figure 7: Plot of the internal standards coefficient of variation in the five plasma dilution series and the calibration curve. Data with a CV exceeding 15% can result in inaccurate estimates of the fraction bound in plasma.

Another plot analyzes the coefficients of variation (CV) of the internal standard measurements within each plasma dilution and the calibration curve Figure 7. Ideally, all CVs should be below 15%, or better below 10%.

When quantifying the test items with LC-MS/MS instrument drift can be observed occasionally. Such drift becomes most visible in the internal standard measurements. This is uncritical as long as the internal standard and the analyte exhibit similar drift. To assess this issue without knowledge of the order of sample processing, we designed a graph showing the relationship between the scaled peak areas, which are the ratios of analyte peak areas over the internal standard peak areas, plotted versus the peak area of either the analyte or the internal standard Figure 8. As long as the data points for the analyte and the internal standard from coherent clouds with similar shapes, it can be assumed that the drift – if any – did not affect the data quality. However, if individual data points are distinctly separated from the majority of data points, or when a clear trend within the analyte data is opposed to a trend in the internal standard data, data quality can be compromised and may have to be separately analyzed investigating the time series of the measurements.

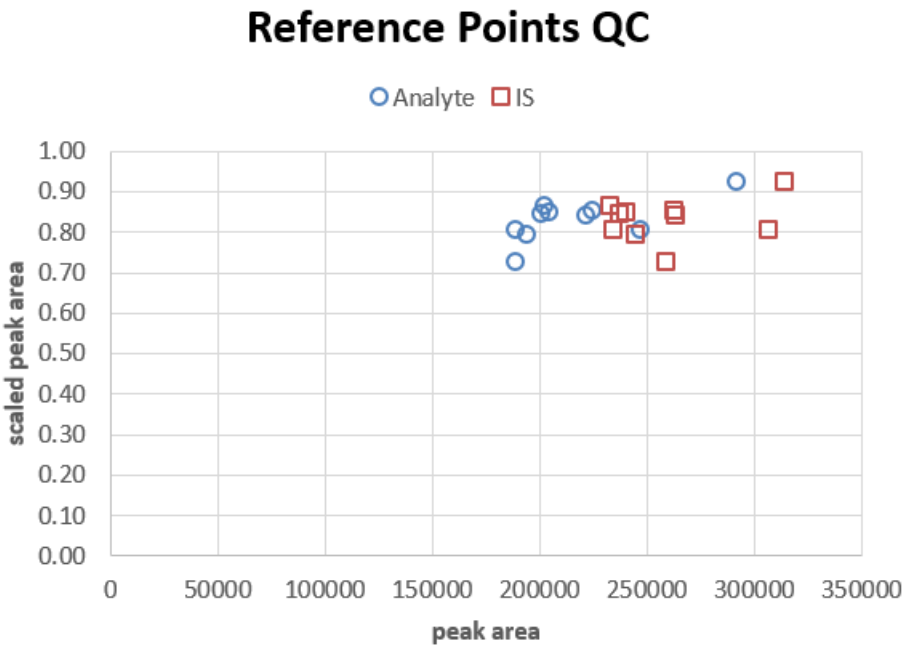


Figure 8: Plot of the scaled peak area versus the absolute peak area of the analyte (blue circles) and the internal standard (red squares).

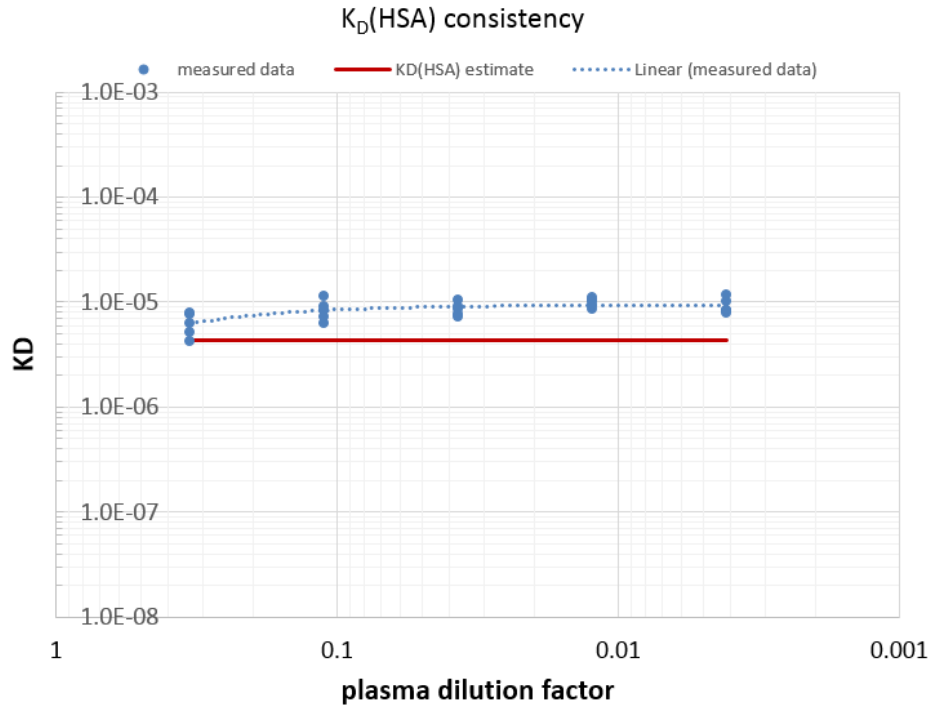


Figure 9: QC plot of the KD from the HSA beads versus the plasma dilution factor. It is assumed that the final estimate for the drug's plasma binding is the true value. Under this assumption, the KD from HSA on the beads is calculated for each individual incubation. These KD values are shown as blue dots. The dotted line is a spline curve connecting the dots across all plasma dilutions. The red line represents the final KD(HSA) estimate. Strong slopes of the blue dotted line indicate APA measurements that are inconsistent with the protein binding model. Heteroscedasticity (i.e. a trend of increasing variability towards left or right of the curve) indicates systematic measurement errors that potentially cause a poor estimate of the final result.

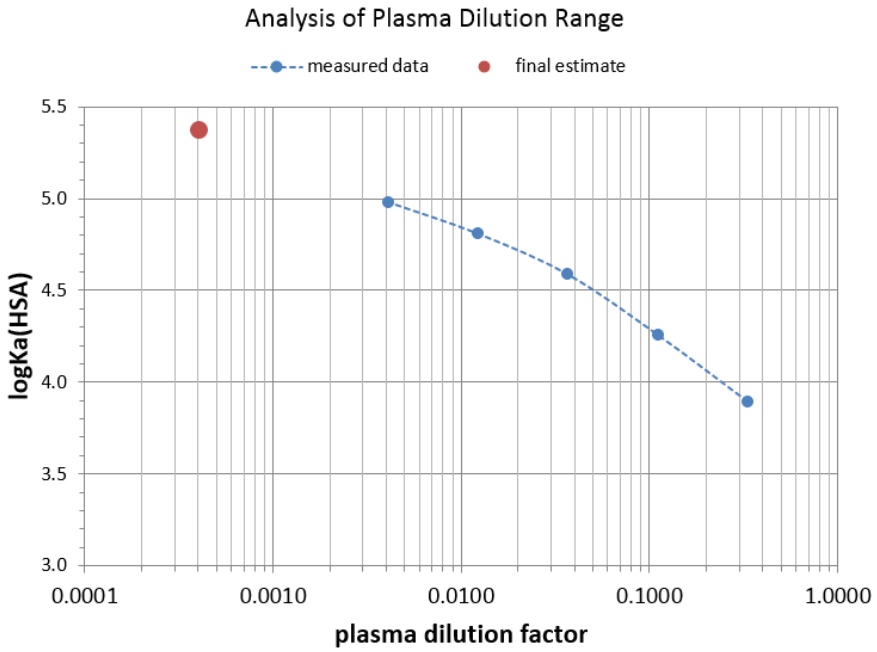


Figure 10: QC plot of the log of the affinity constant to the albumin on beads (inverse of the KD) versus the plasma dilution factor. The plot can be used to evaluate the appropriateness of the chose plasma dilution range.

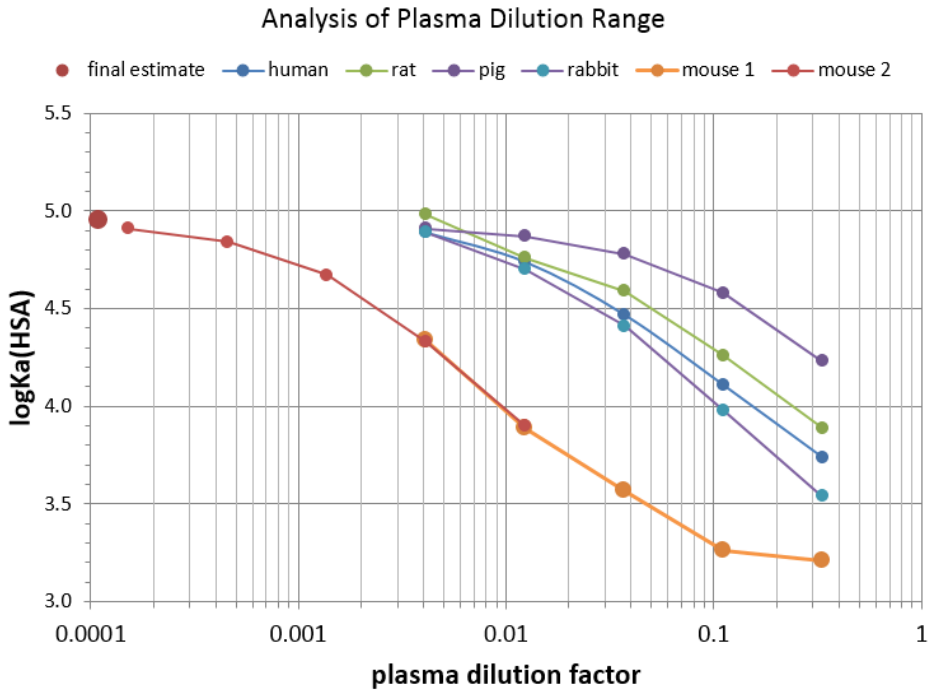


Figure 11: Sample QC plot of the log of the affinity constant to the albumin on beads (inverse of the KD) versus the plasma dilution factor showing data from multiple experiments. The plot is used to evaluate the appropriateness of the chose plasma dilution range. Here data are shown for one compound incubated with human, rat, pig, rabbit and mouse plasma. Looking at the graph from right to left, the log K_a values increase the stronger the plasma is diluted. Data from human, rat, pig, and rabbit all converge to the same Ka value with in a plasma dilution range between 1:3 and 1:243. However, the data from mouse plasma are not converging within this plasma dilution range. In fact, they require a 1:2187 plasma dilution to yield an accurate Ka (inverse of K_D) estimate. Hence, the shape of the curve can be used as an indicator for an appropriate plasma dilution range. When the slope of this plot decreases from right to left, the plasma

dilution range will be most likely ok; while a slope that is increasing from right to left indicates too high plasma dilutions.

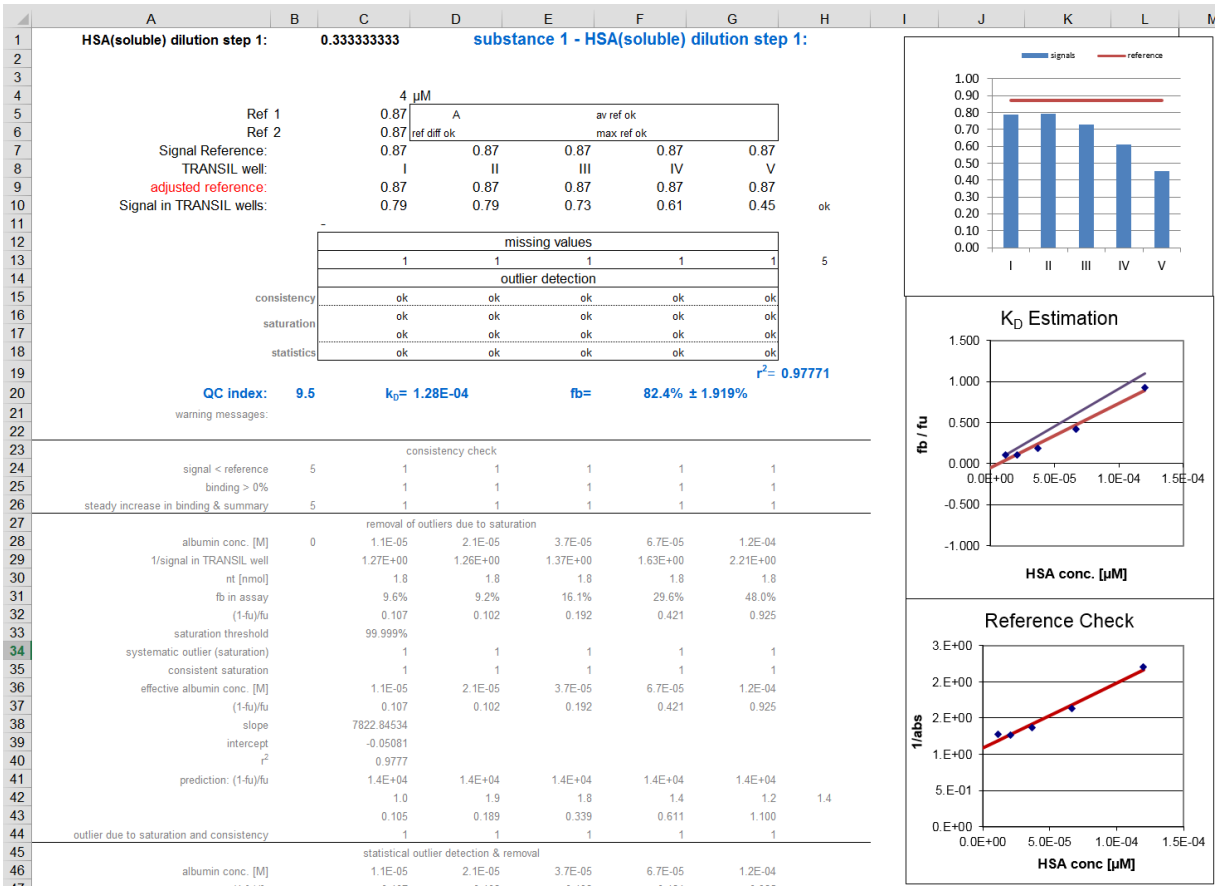
6.5.2 Overall model fit

The quality of the final K_D(HAS) and fu(plasma) estimate can be inspected on the "QC plots 2" tab. Figure 9 illustrates how consistent the KD of albumin on the TRANSIL beads is estimated in the experiment across all plasma dilutions. For this purpose, it is assumed that final estimate of the plasma binding is correct and the KD to the beads is estimated in each incubation independently (blue dots). The final (overall) estimate of KD to the beads is shown as red line. Systematic change in the scatter of the individual KD estimates indicates heteroscedasticity and hence potential inaccuracy of the final estimate.

The apparent affinity to the beads changes with the plasma dilution and the higher diluted the plasma becomes, the closer becomes the estimate of the apparent affinity to the true affinity. For this purpose, the apparent affinity in each incubation is estimated ignoring the test item's binding to plasma. Figure 10 shows a plot of the log of the estimated apparent affinity constant to the albumin beads versus the plasma dilution factor. The overall estimate of the affinity constant is show as a red dot. The measurements in the 5 plasma dilutions should converge towards the final estimate (red dot). As shown in a sample plot that combines several experiments with measurements from various plasma species and different plasma dilution series (Figure 11) the graph can be used to evaluate the suitability of the chosen plasma dilution series.

6.5.3 Detailed QC within each plasma dilution

Calculations of the apparent KD to the beads for each plasma dilution are shown in the tabs "KD d1" to "KD d5". The data analysis and plots shown serve only quality control purposes, as the calculation of the full model parameters is done independently on the "fu calc" tab. However, linearity and consistency of the individual data points can be visualized more easily for each plasma dilution independently. An example of this analysis is shown in Figure 12. A compilation of all individual diagnostic plots is shown on the "QC plots 1" tab. This tab allows easy comparison of the data across all plasma dilutions.



1	HSA(soluble) dilution step 1:		0.333333333		substance 1 - HSA(soluble) dilution step 1:			
2								
3								
4			4 µM					
5	Ref 1		0.87	A		av ref ok		
6	Ref 2		0.87	ref diff ok		max ref ok		
7	Signal Reference:		0.87	0.87	0.87	0.87	0.87	
8	TRANSIL well:		I	II	III	IV	V	
9	adjusted reference:		0.87	0.87	0.87	0.87	0.87	
10	Signal in TRANSIL wells:		0.79	0.79	0.73	0.61	0.45	ok
11								
12			missing values					
13			1	1	1	1	1	5
14			outlier detection					
15	consistency		ok	ok	ok	ok	ok	
16	saturation		ok	ok	ok	ok	ok	
17			ok	ok	ok	ok	ok	
18	statistics		ok	ok	ok	ok	ok	
19							r^2= 0.97771	
20	QC index:	9.5	k_D= 1.28E-04		fb=	82.4% ± 1.919%		
21	warning messages:							
22								
23			consistency check					
24	signal < reference	5	1	1	1	1	1	
25	binding > 0%		1	1	1	1	1	
26	steady increase in binding & summary	5	1	1	1	1	1	
27			removal of outliers due to saturation					
28	albumin conc. [M]	0	1.1E-05	2.1E-05	3.7E-05	6.7E-05	1.2E-04	
29	1/signal in TRANSIL well		1.27E+00	1.26E+00	1.37E+00	1.63E+00	2.21E+00	
30	nt [nmol]		1.8	1.8	1.8	1.8	1.8	
31	fb in assay		9.6%	9.2%	16.1%	29.6%	48.0%	
32	(1-fu)/fu		0.107	0.102	0.192	0.421	0.925	
33	saturation threshold		99.999%					
34	systematic outlier (saturation)		1	1	1	1	1	
35	consistent saturation		1	1	1	1	1	
36	effective albumin conc. [M]		1.1E-05	2.1E-05	3.7E-05	6.7E-05	1.2E-04	
37	(1-fu)/fu		0.107	0.102	0.192	0.421	0.925	
38	slope		7822.84534					
39	intercept		-0.05081					
40	r^2		0.9777					
41	prediction: (1-fu)/fu		1.4E+04	1.4E+04	1.4E+04	1.4E+04	1.4E+04	
42			1.0	1.9	1.8	1.4	1.2	1.4
43			0.105	0.189	0.339	0.611	1.100	
44	outlier due to saturation and consistency		1	1	1	1	1	
45			statistical outlier detection & removal					
46	albumin conc. [M]		1.1E-05	2.1E-05	3.7E-05	6.7E-05	1.2E-04	

Figure 12: Screen shot of QC analysis for individual plasma dilutions. Shown here is the first plasma dilution step.

7 Glossary

AAG	human α_1 acid glycoprotein, synonymous to AGP
AGP	human α_1 acid glycoprotein, synonymous to AAG
HSA	Human 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.
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 binding diazepam and other drug molecules (c.f. Sudlow et al. 1975).

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