Reproducible Characterization of GPCR and Small Molecule Compound Interactions Using Agile™ R100

Savannah Afsahi; Jolie Nokes, PhD; Fariborz Nasertorabi, PhD; Andrii Ishchenko, PhD; Angela Shue; Francie Barron, PhD

- GPCRs are primary drug targets for therapeutic intervention in multiple disease areas, but they are notoriously difficult to characterize due to their inherent instability upon purification. Prior kinetic binding techniques have substantial roadblocks in studying GPCRs, but in this application note, we present Agile R100’s success in quantifying these difficult targets.

- Features of Agile R100 that enable successful detection GPCR interactions include: the ability to detect interactions in complex samples containing detergents and solvents, a non-microfluidic format that allows the sample to be applied directly to the surface of the biosensor chip, temperature versatility, and low target concentration and small sample volume requirements.

- We present 3 example experiments in this application note. The first 2 show that single-concentration kinetic measurements performed in triplicate with Agile R100 is highly consistent with the kinetics found by running a full standard curve. This enables a potential reduction in the required number of measurements, saving both material costs and time.

- The last example experiment displays the temperature versatility of Agile R100. We place the system on a bed of ice to keep the temperature-sensitive GPCR Aβ2 target stable throughout the experiment.

ABSTRACT

G-protein-coupled receptors (GPCRs) are critical therapeutic targets in pharmacological research and drug development, playing active roles in nearly every organ system. However, drug screening efforts are hindered due to impediments intrinsic to GPCRs. GPCRs can require detergents for stabilization, and detergents hinder some detection methodologies. Fluid flow stress, thermal denaturation, and low-yield during purification are also impairments when attempting kinetic characterization of GPCRs. Agile R100 avoids these issues. Employing a new, orthogonal technique, the platform can easily detect interactions in detergents, as well as solvents such as DMSO used to solubilize small molecules. The system also uses a non-microfluidic format, can be deployed in various temperature conditions, and requires very little sample to reproducibly quantify kinetic binding interactions. In this paper, we first run a preliminary single-concentration kinetic measurement of GPCR CR binding with Compound A to inform further in-depth kinetic characterization. The kinetics found with this methodology are $k_\text{on} = 66.8 \pm 16.4 \text{ M}^{-1}\text{s}^{-1}$, $k_\text{off} = 7.38 \pm 3.33 \text{ ks}^{-1}$, and $K_D = 93.9 \pm 24.9 \text{ µM}$. Note that $k_\text{off}$ is reported in ks$^{-1}$ (per kiloseconds) for notation. We then fully characterize the CR and Compound A interaction with the generation of a standard curve. The kinetics found with this methodology are $k_\text{on} = 149 \pm 41.6 \text{ M}^{-1}\text{s}^{-1}$, $k_\text{off} = 10.2 \pm 1.10 \text{ ks}^{-1}$, and $K_D = 68.5 \pm 17.7 \text{ µM}$, in line with the numbers generated by a single-concentration kinetic measurement. In our third experiment, we demonstrate the temperature versatility of Agile R100, preventing the temperature-sensitive GPCR Aβ2 target from degrading by running the measurement on a bed of ice. For this experiment, we perform a single-concentration kinetic measurement in duplicate, and the kinetics found with this methodology are $k_\text{on} = 56.7 \pm 0.20 \text{ M}^{-1}\text{s}^{-1}$, $k_\text{off} = 61.1 \pm 0.46 \text{ ks}^{-1}$, and $K_D = 1.08 \pm 0.089 \text{ µM}$. Agile R100 demonstrates reproducible kinetic characterization of GPCR target and drug compound interactions with little deviation between independent replicate measurements. Advanced kinetic binding analysis is performed in detergents and solvents, bringing the sample directly to the biosensor surface and regulating temperature, with small volumes, making Agile R100 an ideal platform for early-stage GPCR-based drug discovery.

INTRODUCTION

In both academic and industry settings, pharmacology researchers strive to identify novel therapeutics to treat modern ailments. A crucial step in the drug development process is identifying key interactions in biological systems as opportunities for therapeutic intervention. Only ~22% of all proteins encoded by the human genome are present at the cell surface, yet these proteins make up 60% of current drug targets. Currently, the leading protein of interest is a family of proteins called G-protein-coupled receptors (GPCRs). GPCRs are seven transmembrane domain receptors that transduce exterior stimuli into intracellular signals. GPCRs function as critical regulators in a variety of biological processes and are essential to neurological, cardiovascular, endocrine, and respiratory systems, making them attractive targets in pharmaceutical research. Diseases such as cancer, obesity, heart disease, neuropathy, and inflammation are currently being investigated using GPCR targets. The majority of best-selling drugs and roughly 40% of all prescription
pharmaceuticals on the market are GPCR agonists or antagonists.\textsuperscript{4–8} For example, a popular GPCR-specific drug is Zantac, a reversible GPCR inhibitor commonly prescribed to treat heartburn.\textsuperscript{8}

Despite numerous advances in biochemical techniques, studying GPCR structure and function remains challenging, and drug discovery efforts are limited by the innate instability of GPCRs during purification and functional analysis. Due to the hydrophobic nature of the transmembrane domains, detergents are often required to solubilize GPCRs and stabilize the membrane-spanning regions during purification. Since GPCRs are membrane-bound, fluid-flow stress can disrupt the structure of purified proteins during \textit{in vitro} kinetic binding assays. Further, most GPCRs exhibit significant thermal instability and consequently require stringent temperature controls to maintain stability. The complex structure and relative fragility of these molecules often limit the success of protein purification, resulting in low yields of functional GPCR. These strict requirements of GPCRs complicate hit identification and optimization efforts in drug discovery.

Common methods used for GPCR-focused hit identification and lead optimization include a combination of radioligand labeling and computer simulations. However, radioligand labeling involves the use of dangerous radioactive materials, handling of which requires dedicated facilities and trained personnel. The risks and cost of radioligand labeling make it a prohibitive method for drug screening, especially for facilities without access to the necessary equipment and resources.\textsuperscript{9} Further, binding parameters may be affected by specific radioactivity, ionic strength of buffers, divalent ions, and temperature. As a result, radioligand binding is not a comprehensive tool to characterize binding interactions.\textsuperscript{10}

While \textit{in silico} computer simulations provide a safer method to reduce compounds in a library, they require a large amount of structural and biological information from both target and compound.\textsuperscript{11} This limits the ability to fully represent the complexity of \textit{in vivo} interactions and may decrease the success of hits. With limited structural information, most drug discovery breakthroughs result from trial-and-error.\textsuperscript{8} GPCRs are notoriously difficult to crystallize, thus, computer simulations are modeled on homology to available GPCR structures. The need for experimental information is critical in creating better GPCR models as \textit{in silico} computer simulations can only go so far. With 6 classes of GPCRs and dozens of subtypes, computer simulations cannot accurately accommodate the variety in GPCR structure and function. While these \textit{in silico} methods can inform drug discovery efforts, real-time, label-free kinetic quantification is needed to fully characterize GPCR binding interactions.

To monitor real-time, label-free kinetic binding interactions between a GPCR target and low molecular weight compound, costly tools such as surface plasmon resonance (SPR) and Bio-Layer Interferometry (BLI) are utilized. However, these kinetic binding assays are hampered due to intrinsic incompatibilities with the stability requirements of GPCRs. A key requirement to keep purified GPCRs stable is a high concentration of detergents to solubilize membrane-spanning regions. SPR and BLI have difficulties with detergents because they are optical tools, and optically-dense detergents cause significant amounts of background noise, requiring time-consuming and error-prone solvent correction processes.\textsuperscript{12} These tools have similar difficulties with solvents such as dimethyl sulfoxide (DMSO), which is an industry-standard solvent for dissolving compounds. SPR has 3 added complications due to its microfluidic format. First, detergents and solvents can clog microfluidic tubing. Second, the natural state of GPCRs is membrane-bound, requiring purified proteins to be within a structured environment, and flow can disrupt purified GPCRs. Last, the use of a flow-cell necessitates larger volumes and higher concentrations of target, which is particularly difficult in GPCR research where purification of concentrated target is challenged by stability constraints and low yields during purification. Both SPR and BLI are limited in their ability to regulate temperature, a critical component of GPCR stability. Many GPCRs are highly unstable at room temperature, making full kinetic characterization difficult, if not impossible, for some targets. The current challenges in GPCR-focused drug discovery remain unsolved by prior kinetic binding tools, necessitating innovative, next-generation solutions.

Here, we introduce Agile R100, a breakthrough, orthogonal kinetic binding platform with the potential to revolutionize lead optimization for GPCRs. Agile R100 is a real-time, label-free kinetic characterization system that offers many advantages over prior techniques and mitigates a good number of GPCR research constraints. Agile R100 utilizes Field Effect Biosensing (FEB) technology which demonstrates advantages in sensitivity, selectivity, response time, and ease of use over alternative kinetic binding techniques.\textsuperscript{13,14} FEB is an electrical sensing methodology as opposed to an optical one, enabling Agile R100 to detect target and drug compound interactions in optically impure samples containing detergents and solvents. Agile R100 applies the sample directly to the sensor surface, creating ideal, static surface conditions for GPCRs. Its non-microfluidic format conserves sample by reducing sample volume requirements. Additionally, Agile R100’s small, robust design enables temperature control for GPCR stability. Agile R100 provides reproducible measurements in a wide range of conditions for challenging targets, making it an ideal platform for GPCR-focused drug discovery research.
**MATERIALS AND METHODS**

**GPCRs and Small Molecules**

**CR and Compound A**

CR (47.7 kDa) was engineered with a N-terminal FLAG tag and a C-terminal 10x His-tag to facilitate immobilization to the NTA biosensor chip in an orientation that facilitates solvent exposure. CR was purified to homogeneity by a 2-step (immobilized metal affinity chromatography [IMAC] and M2 anti-FLAG agarose) affinity chromatography process. Briefly, HEK293T cells overexpressing CR were lysed by sonication in hypotonic buffer, and crude membranes were prepared by standard methods. Membranes were washed with high salt buffer and subsequently solubilized using a 1% n-Dodecyl-β-D-maltoside (DDM) and 0.2% cholesteryl hemisuccinate (CHS) detergent mixture. Solubilized CR was captured on Clontech Talon resin. The resin was washed extensively, and protein eluted with imidazole. Sample was diluted 2-fold with buffer without imidazole, and M2 anti-FLAG affinity resin was added and incubated in the cold with mixing for 3 hrs. The resin was washed, and the protein eluted with 4 column volumes of buffer (1X phosphate buffered saline [PBS], 0.05% DDM, 0.01% CHS) containing 300 μg/mL FLAG peptide. The functional protein was further concentrated to 2 mg/ml using a spin filter with 100 kDa molecular weight cutoff (MWCO). Compound A (434.89 g/mol) was purchased from Tocris and dissolved in 100% DMSO at a stock concentration of 50 mM.

**A2A and TH**

The human A2A (50 kDa) receptor construct was previously optimized to improve its stability for crystallization studies\(^1\) by N-terminal and C-terminal truncations and fusion of b562RIL cytochrome (BRL) into the third intracellular loop. The GPCR A2A was expressed in Sf9 cells and purified using IMAC without ligand present throughout the purification process. The compound was the low affinity antagonist TH (180.16 g/mol) from Tocris. The functional GPCR A2A also contains a C-terminal His-tag to specify immobilization orientation to the NTA biosensor chip. No engineered modifications were introduced after purification. The GPCR A2A is highly sensitive to thermal degradation and thus requires environmental controls to maintain a constant temperature below 10°C.

**Target Immobilization**

The volume used for all measurements was 30 μL. To immobilize His-tagged GPCR targets to the NTA biosensor chip, the NTA biosensor chip was activated by incubating the chips in 11.3 mM NiCl\(_2\) for 15 minutes and washing several times with DI water and immobilization buffer before proceeding to the target immobilization.

**GPCR CR**

After recalibration in immobilization buffer containing 1X PBS, 0.05% DDM, 0.01% CHS, 14.3 μg/mL of target GPCR CR in immobilization buffer was incubated on the biosensor chip for 60 minutes at room temperature.

**GPCR A2A**

To maintain its structural integrity, GPCR A2A needs to be kept below 10°C. Agile R100 was placed on a bed of ice during the immobilization protocol to preserve the stability of the GPCR A2A target. Standard Agile R100 biosensor chips contain built-in thermometers that monitored the temperature in real-time to ensure it remained below 10°C. After recalibration in immobilization buffer containing 50 mM 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES) pH 7.5, 0.025% DDM, 0.005% CHS, 15.0 μg/mL GPCR A2A target in immobilization buffer was incubated on the biosensor chip for 60 minutes.

**GPCR and Compound Measurement**

**CR and Compound A Single-Concentration Kinetic Measurement**

After equilibration in assay buffer (1X PBS pH 7.5, 0.05% DDM, 0.01% CHS, 2% DMSO), a baseline zero-concentration measurement was taken in fresh assay buffer. To run a single-concentration kinetic measurement, we selected a concentration of 100 μM for Compound A. This concentration is thought to be in the middle of the linear titration range between Compound A and CR. The compound was applied to the biosensor chip containing immobilized CR and the kinetic binding experiment was performed 3 times. The association was measured for 5 minutes, and then fresh assay buffer was added to allow the dissociation of Compound A from immobilized CR for 5 minutes.

**CR and Compound A Standard Curve**

The biosensor chips were calibrated in fresh assay buffer. A 3-fold dilution series of small molecule Compound A ranging from 0.3 μM to 2000 μM was prepared in assay buffer, and each concentration was applied to a separate biosensor chip for measurement. The association was measured for 5 minutes, and then fresh assay buffer was added to allow the dissociation of Compound A from immobilized CR for 5 minutes. Each small molecule concentration was performed with 2 or 3 independent replicates to generate a standard curve.

**A2A and TH Single-Concentration Kinetic Measurement**

The GPCR A2A measurement was performed on a bed of ice to preserve the stability of the immobilized target. A baseline zero-concentration measurement was taken in fresh assay buffer (50 mM HEPES pH 7.5, 0.005% DDM, 0.025% CHS, 0.1% DMSO). After the biosensor chip was calibrated, a 100 μM sample of TH in assay buffer was added to the chip. After 5 minutes of TH association, the fluid on the chip was removed and fresh assay buffer was applied to the biosensor chip. Dissociation of TH was monitored for 5 minutes. Two independent replicates of A2A and TH binding were performed.
Analysis

Agile Plus Software Description
Agile R100 includes Agile Plus software for data acquisition and real-time data analysis. Agile Plus software contains an analysis suite that integrates FEB sensing technology with classic kinetic algorithms derived from the Hill-Langmuir equation. The sensor response is measured in two ways: I-Response indicates changes in charge distribution at the biosensor surface, and C-Response indicates water displacement symptomatic of conformational changes during binding. The response curve is measured in biosensing units (BU), which is the percent change in conductance measured by the system, multiplied by 10.

Curve fits are applied to the sensor response versus time to generate association rates (k_on), dissociation rates (k_off), and dissociation constants (K_D). In Agile Plus software, k_on is reported in units of M^-1s^-1, k_off is reported in s^-1, and K_D is reported in µM. Note that in this study, the units of k_off have been adjusted for notation, and k_on is reported in ks^-1 (per kiloseconds). Agile Plus software also graphs sensor response versus time to generate single-concentration kinetic measurement data and sensor response versus concentration for standard curve data.

CR and Compound A Single-Concentration Kinetic Measurements
A K_D value from the single-concentration kinetic measurement of the GPCR CR and Compound A interaction was calculated by curve fitting the association and dissociation steps for each I-Response (n = 3). Individual association and dissociation fits were applied to each sensor response versus time and averaged to generate kinetic binding data for the GPCR CR and Compound A interaction. The association and dissociation rates (K_D, K_off) from the individual fits at 100 µM Compound A were then averaged, and the standard deviation calculated. The K_D value reported from the single-concentration kinetic measurement is the average of K_D values of individual measurements calculated by Agile Plus software, not the average K_off divided by the average K_on.

CR and Compound A Standard Curve
Standard curves are one of the most used analytical methods to determine K_D values. Here, I-Response magnitudes calculated from the Agile Plus software were plotted against the user-input Compound A concentration interacting with GPCR CR. Concentrations of Compound A in the lower saturated binding region, the linear region of binding, and the upper saturated binding region were tested and graphed in the standard curve to calculate the K_D and standard deviation using the Hill-Langmuir fit. The replicate independent measurements were plotted with standard deviations for each concentration graphed. The K_D average and standard deviation determined from a Hill-Langmuir fit of the standard curve and the K_off average and standard deviation from each concentration in the linear range (30 to 100 µM) determined from curve fitting the dissociation were used to calculate average K_on, and the K_on standard deviation was determined using error propagation from K_D and K_off. Concentrations in the linear range of the Hill-Langmuir fit were selected to calculate K_off as this range represents the most accurate kinetic binding.

RESULTS AND DISCUSSION

CR and Compound A Single-Concentration Kinetic Measurements
Stacked sensorgrams for each independent replicate (n = 3) of the single-concentration kinetic measurements of 100 µM Compound A are shown in Figure 1, depicting reproducible kinetics and low inter-assay variability. The k_on and k_off values were calculated from the fits applied to individual measurements. The k_on average across independent measurements was determined as k_on = 66.8 ± 16.4 M^-1s^-1, and the K_D was calculated as K_D = 7.38 ± 3.33 ks^-1. The calculated K_D from the single-concentration kinetic measurement was then calculated as K_D = 93.9 ± 24.9 µM. Average K_on, K_off, and K_D values for CR sensing 100 µM Compound A are listed in Table 1, showing reliable preliminary kinetic characterization using a single concentration.

Figure 1: I-Response overlaid sensorgrams for single-concentration kinetic measurements (n = 3) using 100 µM Compound A interacting with GPCR CR in assay buffer. K_D = 93.9 ± 24.9 µM.

CR and Compound A Standard Curve
Another analysis method offered in Agile Plus is kinetic binding characterization via a standard curve. Using the equilibrium method (i.e. a Hill-Langmuir fit of the standard curve), the K_D value was determined (Figure 2 and Table 1). Each concentration is shown as an average of 2 or 3 independent replicates with standard deviations depicting quantitative inter-assay consistency. The K_D value determined from the standard curve was K_D = 68.5 ± 17.7 µM.
demonstrate concentration kinetic posed by the instability of A. Despite restrictions with GPCR A at temperatures, employed to analyze the A concentration small molecule.

Table 1: Summary of the kinetic binding data for the GPCR CR and small molecule Compound A interaction using both single-concentration kinetic measurements and standard curve quantification with Agile R100.

<table>
<thead>
<tr>
<th>Method</th>
<th>$k_{\text{on}}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{\text{off}}$ (ks$^{-1}$)</th>
<th>$K_D$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-Concentration</td>
<td>66.8 ± 16.4</td>
<td>7.38 ± 3.33</td>
<td>93.9 ± 24.9</td>
</tr>
<tr>
<td>Standard</td>
<td>149 ± 41.6</td>
<td>10.2 ± 1.10</td>
<td>68.5 ± 17.7</td>
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**A$_{2A}$ and TH Single-Concentration Kinetic Measurement**

Single-concentration kinetic measurements were also employed to analyze the GPCR A$_{2A}$ and TH compound interaction. Since GPCR A$_{2A}$ demonstrates instability at ambient temperatures, only a single concentration of TH interacting with GPCR A$_{2A}$ was tested ($n = 2$) to generate kinetic data from the association and dissociation rates. Despite restrictions posed by the instability of A$_{2A}$, the successful single-concentration kinetic measurements of A$_{2A}$ and TH (Figure 3) demonstrate Agile R100’s ability to characterize even challenging targets. The average $k_{\text{on}}$, $k_{\text{off}}$, and $K_D$ of the A$_{2A}$ and TH interaction on Agile R100 are $k_{\text{on}} = 56.7 ± 0.20$ M$^{-1}$s$^{-1}$, $k_{\text{off}} = 61.1 ± 0.46$ ks$^{-1}$, and $K_D = 1.08 ± 0.089$ μM (Table 2). Using a single concentration of compound, reproducible kinetic values give insight to the behavior of an early stage compound with its target. With this valuable information delivered earlier in the drug discovery process, better decisions can be made on which compounds to pursue for particular targets, saving time and money and potentially increasing the success rate of drugs.

Table 2: Agile Plus kinetic binding data summary for GPCR A$_{2A}$ binding with TH small molecule.

<table>
<thead>
<tr>
<th>$k_{\text{on}}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{\text{off}}$ (ks$^{-1}$)</th>
<th>$K_D$ (μM)</th>
</tr>
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<tbody>
<tr>
<td>56.7 ± 0.20</td>
<td>61.1 ± 0.46</td>
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**CONCLUSIONS**

With GPCRs dominating the drug market, kinetic characterization advances are necessary to accommodate impediments presented by this challenging target class. Agile R100 is a kinetic characterization system for quantifying GPCR and small molecule interactions with reproducible results across multiple independent measurements. Due to the thermal stability of GPCR CR, we were able to complete a full standard curve that included a range of Compound A concentrations. The CR and Compound A interaction was analyzed with both single-concentration kinetic and standard measurements, which generated consistent $K_D$ values within a factor of 1.4 across methods, demonstrating high reproducibility.
reproducibility. With single-concentration kinetic measurements highly indicative of full standard curve results, researchers can potentially reduce the number of total measurements required to obtain accurate binding data, cutting material costs and experimental time. Agile R100 also shows successful characterization of unstable GPCRs such as A2A using single-concentration kinetic measurements. The system’s small size and electrical sensing mechanism (that is unaffected by temperature) enables experiments to be performed on a bed of ice to preserve the structural integrity of these temperature-sensitive targets. Agile R100 delivers advanced quantitative kinetic characterization and is not impeded by challenges such as measuring in detergents and solvents, fluid-flow stress, temperature sensitivity, and sample volume. These advancements open new doors by providing easier real-time, label-free kinetic characterization of GPCR targets.

REFERENCES


For questions, please contact techsupport@nanomedicaldiagnostics.com.