A Microtiter Plate-Based Assay for Inhibitors of Penicillin-Binding Protein 2a from Methicillin Resistant Staphylococcus aureus

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Short Title: Microtiter plate assay for PBP2a.

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Abstract: PBP2a, the molecular determinant for high level β-lactam resistance in Methicillin-resistant *Staphylococcus aureus* (MRSA), is intrinsically resistant to most β-lactam antibiotics. The development and characterization of new inhibitors targeting PBP2a would benefit from an effective and convenient assay for inhibitor binding. This study was directed towards the development of a fluorescently detected β-lactam binding assay for PBP2a from MRSA. Biotinylated ampicillin and biotinylated cephalexin were tested as tagging reagents for fluorescence detection using a strepavidin-HRP conjugate. Both bound surprisingly well to PBP2a, with binding constants of 1.6 ± 0.4 µM and 13.6 ± 0.8 µM respectively. Two forms of the assay were developed, a one step direct competition form of the assay and a two step indirect competition form of the assay, and both forms of the assay gave comparable results. This assay was then used to characterize PBP2a binding to ceftobiprole which gave results consistent with previous studies of ceftobiprole-PBP2a binding. This assay was also demonstrated for screening for PBP2a inhibitors by screening a set of thirteen randomly selected β-lactams for PBP2a inhibition at 750 µM. Meropenem was observed to give substantial inhibition in this screen, and a follow-up titration experiment determined its $K_{app}$ to be 480 ± 70 µM. The availability of convenient and sensitive microtiter-plate based assays for the screening and characterization of PBP2a inhibitors is expected to facilitate the discovery and development of new PBP2a inhibitors for use in combating the serious public health problem posed by MRSA.
Keywords: β-lactam; assay; penicillin-binding protein; antibacterial; cell wall; peptidoglycan; MRSA; drug resistance, PBP2a.
INTRODUCTION

Bacterial infections were the major cause of death and morbidity prior to the development of modern antibiotics, and the increasing resistance of pathogenic bacteria to commonly used antibacterial agents is of major public health concern. One organism of particular concern is methicillin-resistant *Staphylococcus aureus* (MRSA) (1, 7, 11). The high level of β-lactam resistance seen in MRSA, as compared to methicillin-sensitive *S. aureus* (MSSA), is due to the presence of a novel acquired penicillin-binding protein (PBP) – PBP2a ((10), recently reviewed in (13, 14, 19)). PBP2a is a high molecular mass (HMM) PBP which is intrinsically resistant to most β-lactam antibiotics. Assay methods for inhibitor binding to PBP2a have been described based on radiolabeled β-lactam binding (17), or on nitrocefin (9) (a chromogenic cephalosporin derivative) or BOCILIN-FL (a fluorescently tagged penicillin derivative) binding (8, 20). However, these assays are incompatible with a microtiter plate format required for efficient high-throughput inhibitor screening and characterization. Given the high intrinsic resistance of PBP2a to β-lactams it was uncertain if a microtiter plate β-lactam binding assay of the type we have described recently for other HMM PBPs (16), e.g. based on biotinylate β-lactams, would work with PBP2a. In the study reported here we investigate the use of biotinylated β-lactams in microtiter plate assays for PBP2a-inhibitor screening and characterization.

A common approach to determining PBP binding affinities is with a two step assay, where the β-lactam test agent is preincubated with the PBP for a short period (10 - 30 min) to allow complex formation, followed by addition of a saturating concentration
of a β-lactam probe agent (such as a radiolabeled, fluorescently labeled, or biotin
labeled β-lactam) for a second short period (10-30 min), which reacts with and labels
the uncomplexed PBP (c.f. (3, 4, 15)). This approach is based on relatively slow β-
lactam release kinetics from the PBP target, so that addition of probe cannot shift the
equilibrium between the test agent-PBP complex substantially in the incubation time
used. The slow off-rates observed for β-lactam probes bound to PBP2a indicates that
such a kinetic approach is appropriate for measuring test agent β-lactam binding to
PBP2a (9). To provide a basis for comparing the one step steady-state approach we
described previously (16) with a classic two step kinetic approach, a microtiter plate-
based two step assay was also implemented.
MATERIALS and METHODS

Cloning of a truncated mecA gene. Chromosomal DNA of MRSA (ATCC 3300) was used as a template for PCR. Primers were designed based on the published mecA sequence from NCBI and the primers being a forward primer 5’-PBP2a-EcoRI, BamHI: 5’-GGATCCGAATTCCTGGAAGTTCTGTTCCAGGGCCCATGGGCTTTAAGATAAA-3’ and a reverse primer 3’-PBP2a-XhoI, Hind III: 5’-AAGCTTCTCGAGTTATTCATCTATCGTA-3’. The primers were designed so that the first 23 amino acids at the N-terminus were deleted. The resulting DNA fragment (~2 kb) was gel purified and then extracted using a gel purification kit (Invitrogen) according to the manufacturer’s protocol. The gene was ligated using T4 ligase into the pGEM®-T vector (Promega, Madison, WI), and transformed into competent XL1 Blue cells. The mecA gene in the pGEM®-T vector was sequenced using T7 and SP6 promoter primers. The verified insert DNA and the pGEX-4T1 vector (GE Healthcare, Piscataway, NJ) were both digested with same restriction enzymes (EcoRI and XhoI) and then ligated together to give the expression vector pGEX-PBP2a.

PBP2a expression. The recombinant vector pGEX-PBP2a was transformed into Escherichia coli BL21 (DE3) cells (Invitrogen) for protein expression. Cells were grown in Luria–Bertani broth containing 100 µg/mL ampicillin at 37 °C, until the culture reached 0.6 OD600. The culture was chilled in an ice bath for 10 min, then placed in a shaker at 18 °C and protein expression induced by adding 0.5 mM IPTG. Cells were then grown overnight (16 hrs) at 18 °C with shaking and then harvested by centrifugation at 4 °C. For large scale production, 3 one-liter flasks each containing 350 mL of culture were used.
**GST-PBP2a purification.** All purification steps were done at 4 °C. The bacterial cell pellet was resuspended in 60 mL cold lysis buffer (40 mM Na$_2$HPO$_4$, 10 mM KH$_2$PO$_4$, 300 mM NaCl at pH 7.4). Bacterial cells were lysed using a Microfluidizer (model M 100L, Newton MA). The bacterial extract was centrifuged for 30 min at 30,000g the supernatant was collected, spun for an additional 20 min, and the supernatant was again collected and stored frozen at -80 °C. The GST-PBP2a fusion protein was purified on GST Resin (GenScript Cat. No. L00206) following manufacturers (GenScript) instructions.

**Cleavage of the GST tag.** Thrombin (GE Healthcare, Cat. No. 27-0846-01) was reconstituted with PBS to give a final solution of 1 unit/µL. Small aliquots were stored at -80 °C. To cleave the GST tag from purified GST-PBP2a, 35 µg of GST-PBP2a was treated with 70 units of thrombin at 4 °C for overnight. SDS-PAGE was used to confirm cleavage. The free GST tag and uncleaved GST-PBP2a were removed from the cleaved product PBP2a by passage over the GST resin as described above. Purified untagged PBP2a passed though the column in the flow through, whereas GST and GST-PBP2a were retained. Fractions were collected analyzed for purity by SDS-PAGE, for protein concentration by Bradford assay, and then concentrated.

**Biotinylated ampicillin (BIO-AMP) and biotinylated cephalexin (BIO-CEPH) preparation.** BIO-AMP (Fig. 1) was prepared by a modification of the method of Dargis and Malouin (4) as described previously (16). BIO-CEPH (Fig. 1) was prepared using the same procedure except that the final stock concentration was 4.2 mM due to the lower solubility of BIO-CEPH.
General procedure for GST-PBP2a loading onto microtiter plates, labeling, and detection. The protocol was similar to that described previously (16). Briefly, for GST-PBP2a attachment, black walled microtiter plate wells (Costar, product no. 3631) were treated with 0.5-1 µg of GST-PBP2a in 50 µL of PBS/20% glycerol at 25 °C for 30 min with gentle rocking, followed by treatment (3x) with 150 µL/well of blocking buffer (PBS/0.2% Tween-20), and then washing (3x) with 200 µL/well of washing buffer (PBS/0.05% Tween-20). To label GST-PBP2a in initial proof-of-principle experiments, 50 µL of 100 µM BIO-AMP or BIO-CEPH in PBS was added to the wells. After 15 min the biotin labeled GST-PBP2a was denatured with heating at 80 °C for 3 min in a water bath, followed by quick cooling on ice. The plates were then washed (3x) with washing buffer (250 µL PBS/0.05% Tween-20). Streptavidin-horse radish peroxidase (HRP) conjugate (Pierce #21126) (50 µL of 0.4 µg/mL) was then added to each well. After 30 min the wells were washed (3x) with washing buffer, and 100 µL of a fluorescent HRP substrate mixture (1 mM H₂O₂, 20 µM Amplex Red (Molecular Probes) in 100 mM Tris pH 8.5) was added to each well. After 30-60 min the fluorescence signal was read (Excitation: 546 nm, Emission: 595 nm) in a Tecan Spectrafluor Plus microtiter plate reader. An identical protocol was also followed using untagged PBP2a for comparison.

Determination of BIO-AMP and BIO-CEPH $K_{m}^{app}$ for binding to PBP2a. To assess BIO-AMP and BIO-CEPH binding to PBP2a, microtiter plate bound PBP2a was treated with serially diluted (steps of 2) concentrations of BIO-AMP or BIO-CEPH (each concentration tested in triplicate), and the remaining steps of the assay performed as described above. To assess the effect of labeling reaction incubation time (in the GST-PBP2a + BIO-AMP reaction) on the detected signal, an alternative incubation time of 30
min for labeling was also tested. Data (relative fluorescence units; RFU) were analyzed for the $K_m^{\text{app}}$ (the apparent steady-state binding constant to PBP2a, since PBPs turn over $\beta$-lactams, albeit generally very slowly) by fitting the data to Eqn. 1 (16). No protein blanks were also included in these experiments.

$$RFU = RFU_0 + \frac{(RFU_{\text{max}} \times [I])}{(K_m^{\text{app}} + [I])}$$

Eqn. 1

**Solution-phase SDS-PAGE-based BIO-AMP binding assay to confirm untagged PBP2a BIO-AMP binding activity, and to provide a solution phase $K_m^{\text{app}}$ for BIO-AMP.** As described further below, GST-PBP2a gave good signals in the above described assay, but untagged PBP2a failed to give any detectable signal, even when the amount of PBP2a loaded into each well was increased up to 8 $\mu$g/well. This observation suggested that either untagged PBP2a was unable to bind BIO-AMP (e.g., it was inactive), or that untagged PBP2a was unable to bind to the microtiter plate wells. It was also desirable to determine if the affinity of GST-PBP2a for BIO-AMP in solution was the same as in the microtiter plate-based assay, and also if GST-PBP2a (in solution) had the same affinity as PBP2a (in solution, and also if active). To address these issues, GST-PBP2a and untagged PBP2a were labeled in solution with BIO-AMP, the complex denatured and resolved by SDS-PAGE, and BIO-AMP labeled proteins detected using a procedure similar to that described by Dargis and Malouin (4). A range of concentrations of BIO-AMP were used in the labeling reaction to assess affinity ($0xK_m^{\text{app}}$, $\frac{1}{8}xK_m^{\text{app}}$, $\frac{1}{2}xK_m^{\text{app}}$, $2xK_m^{\text{app}}$, $8xK_m^{\text{app}}$).

**Characterization of inhibition of PBP2a using a one step approach.** For inhibitor screening and characterization the concentration of BIO-AMP was used at a
fixed concentration equal to its determined $K_m^{\text{app}}$ for PBP2a (1.6 µM). This was selected since it is high enough to give $\frac{1}{2}$ of the maximum possible assay signal and low enough to still allow inhibition to be readily detected. GST-PBP2a was first attached to the wells of a microtiter plate as described above. Serially (steps of 2) diluted solutions of the prospective inhibitor, plus BIO-AMP at a fixed concentration of 1.6 ± 0.4 µM, in 100 µL PBS were added to the wells, with each concentration tested in triplicate. After 15 minutes the binding reactions were stopped and the plates developed as described above. With $[\text{BIO-AMP}] = K_m^{\text{app}}$, and taking into account the background (blank) fluorescence, the competitive (with BIO-AMP) inhibitor binding isotherm will be described by Eqn. 2 (16)

$$RFU = RFU_0 + RFU_{\text{max}} / ([I] / K_I^{\text{app}} + 2)$$

Eqn. 2

Inhibitor binding data were plotted, and the saturation curves were analyzed for the $K_I^{\text{app}}$ of binding by fitting the data to Eqn. 2.

Application to screening of potential PBP2a inhibitors using a one step approach. To demonstrate the potential of this assay for inhibitor screening and characterization, a demonstration screening of 13 randomly selected β-lactam antibiotics (Table 1) against GST-PBP2a was performed. Screening was done with a high concentration of each antibiotic (750 µM), with the BIO-AMP concentration fixed at its $K_m^{\text{app}}$. Each determination was done in triplicate.

Characterization of inhibition of PBP2a using a two step assay approach. GST-PBP was bound to microtiter plate wells as described above. In the first step, various concentrations of ceftobiprole (Fig. 1) in 50 µL PBS were added to the wells. For
the second step, after 15 min 50 µL of BIO-AMP at 16x the $K_m^{\text{app}}$ for BIO-AMP (25.6 µM) (final concentration of 8x the $K_m^{\text{app}}$) was added to each well. After an additional 15 min the reactions were stopped by heat denaturation and the plates developed as described above. Taking into account the background (blank) fluorescence, the inhibitor binding isotherm in this experimental design will be described by Eqn. 3, which can be derived following the same general procedure as previously described for Eqn. 2 (16).

$$RFU = RFU_0 + \left[ RFU_{\max} \times \frac{K_i^{\text{app}}}{(K_i^{\text{app}} + [I])} \right]$$

Eqn. 3

Data collected using the two step assay were analyzed by fitting with Eqn. 3.

RESULTS and DISCUSSION

**Assay development.** Given the intrinsic resistance of PBP2a to most β-lactam antibiotics, it was uncertain at the outset if BIO-AMP and/or BIO-CEPH would bind to PBP2a. PBP2a was therefore overexpressed as a GST fusion protein, and the potential of a BIO-AMP- or BIO-CEPH-based assay approach tested. In initial tests strong signals were obtained with either 100 µM BIO-AMP or BIO-CEPH as the detection reagent, demonstrating the feasibility of this approach.

GST-PBP2a was next titrated with both BIO-AMP and BIO-CEPH to determine their respective apparent binding constants ($K_m^{\text{app}}$s). Both gave classic saturation binding curves (Fig. 2). Analysis of these curves demonstrated binding constants of 1.6 ± 0.4 µM for BIO-AMP and 13.6 ± 0.8 µM for BIO-CEPH (± standard errors). These are both surprisingly good binding constants, and demonstrate that the biotinyl group can apparently form favorable interactions within the active site of PBP2a. That these two
biotinylated β-lactams can effectively bind to PBP2a provides a foundation for their use in characterizing other inhibitors of PBP2a in competitive binding experiments.

The same effort using untagged PBP2a (obtained by cleaving the GST tag using thrombin) gave no signals. At first it was suspected that the untagged PBP2a sample had lost its ability to bind BIO-AMP. However, a follow-up solution phase binding experiment using the BIO-AMP reagent followed by SDS-PAGE, blotting, and detection revealed that both the GST-PBP2a and untagged PBP were capable of binding to BIO-AMP (Fig. 3). This experimental observation indicates that GST-PBP2a can bind efficiently to microtiter plates, whereas untagged PBP2a appears unable to bind to microtiter plates under the assay conditions used here. As also illustrated in Fig. 3, the midpoint for the affinity of BIO-AMP to GST-PBP2a and untagged PBP2a in solution is similar to the $K_{m}^{app}$ as determined for GST-PBP2a as determined in the microtiter plate-based assay (Fig. 2A).

The one step assay is very precise. For the BIO-AMP titration at 15 min incubation (Fig. 2A), the standard deviation for a set of replicate (n=3) samples averaged 4% of the total signal change over the titration (a standard deviation of 2,004 RFU on average for each set of replicates, and with a total RFU change over the entire BIO-AMP titration RFU change of 53,000 RFU).

**Steady-state binding isotherm for BIO-AMP and lack of time dependence.** To determine if the reaction of BIO-AMP with PBP2a was reaching steady-state under the conditions used here, the results of BIO-AMP + PBP2a incubations for 15 min and 30 min were determined and compared, and the data and analysis results are shown in...
Fig. 2A. It is clear that both the 15 min and 30 min incubations gave virtually identical results, and demonstrate that the reaction of BIO-AMP and PBP2a reached steady-state within 15 min. This is consistent with the time course for approach to steady-state for β-lactam binding in solution phase as reported by Graves-Woodward and Pratt (9). The excellent Michaelis-Menten saturation binding curves observed for both BIO-AMP and BIO-CEPH (Fig. 2) are also consistent with a steady-state reaction system.

**Competitive one step binding assay for characterization of ceftobiprole binding to PBP2a.** To demonstrate the utility of the one step microtiter plate-based assay described here for the characterization of inhibitors of PBP2a, it was used to characterize PBP2a inhibition by ceftobiprole (Fig. 4) – a new β-lactam designed to inhibit PBP2a and provide a β-lactam based treatment option for MRSA infections (2, 12, 18). The results from the assay of variable concentrations of ceftobiprole vs. a fixed concentration of BIO-AMP are shown in Fig. 4A. Both 15 min and 30 min incubations were used to determine if equilibrium was being achieved. Analysis of this competitive binding data by fitting with Eqn. 2 gave a $K_{i}^{app}$ of 6 ± 2 µM after 15 min incubation, and 7 ± 2 µM after 30 min incubation. The close similarity in $K_{i}^{app}$ values at the two different incubation times indicates that steady-state was reached within 15 min. Also, the competitive binding data fits very well to competitive binding equation (Eqn, 2), further supporting this conclusion.

This $K_{i}^{app}$ of 6 µM is above the MIC of ceftobiprole against MRSA of 1 µg/mL (reviewed in (18)) (equal to 1.9 µM). This value is also slightly higher than range of
IC50s reported for ceftobiprole binding to PBP2a in membrane extracts of from 0.6 - 1.7 µM (5, 6).

**Determination of Ceftobiprole binding to PBP2a using a two step assay for comparison with one step assay results.** In order to compare the results from a microtiter plate-based two step assay for characterizing ceftobiprole binding to PBP2a to those form the one step binding assay described above (Fig. 4A), a microtiter plate-based two step assay was performed. The results from this two step microtiter plate-based assay are shown in Fig. 5. Several features are notable. First, the $K_{i}^{app}$ is lower than that determined using the one step assay, but the observed difference is not significant at the P<0.05 level. However, the lower $K_{i}^{app}$ obtained with the two step assay is more consistent with the MIC of ceftobiprole against MRSA of 1 µg/mL (reviewed in (18)) (equal to 1.9 µM). This value is also more consistent with the range of IC50s reported for ceftobiprole binding to PBP2a in membrane extracts of from 0.6 - 1.7 µM (5, 6).

**Demonstration inhibitor screening experiment and follow-up inhibitor characterization.** This assay (one step) was then tested for its utility for screening for PBP2a inhibitors. A random set of β-lactams was selected, and screened for inhibition of PBP2a at 750 µM. This provided a ranked list of inhibitors (Table 1). As expected given PBP2a’s intrinsic resistance to β-lactams, most of these β-lactams did not inhibit even at the high concentration of 750 µM – only meropenem (Fig. 1) gave greater that 50% inhibition (Table 1). As a follow-up, the $K_{i}^{app}$ for meropenem inhibition was determined using the one step assay as described above for ceftobiprole, including with
both 15 min and 30 min incubations, which gave $K_{\text{app}}$s of 550 ± 90 (15 min incubation) and 480 ± 70 µM (30 min incubation) for meropenem (Fig. 4B). This value of meropenem is approximately twice the IC50 value of 260 µM determined in membrane extracts (17), approximately a two-fold difference.

In conclusion, this study develops and demonstrates a sensitive and convenient microtiter plate-based assay approach for the screening and characterization of inhibitors for PBP2a. The primary focus was on using a single step assay with a relatively short incubation time of 15 min. This approach was validated against a solution phase β-lactam assay detected by SDS-PAGE, which demonstrated that GST-tagged PBP2a, necessary for binding to microtiter plates, bound BIO-AMP with the same affinity as untagged PBP2a, and that the solution phase and microtiter plate assays gave the same apparent binding constants within the resolution of these different approaches. The one step assay was further validated by using a longer (30 min) incubation time to demonstrate that the results using this assay were the same with the 15 min incubation time. Finally, a two step assay protocol was also implemented in the microtiter plate format. The $K_{\text{app}}$ for ceftobiprole with the two step approach (2.9 µM) was lower that with the one step protocol (6 µM) but not by a statistically significant factor. The value for ceftobiprole binding obtained with the two step microtiter plate-based approach were very similar to other studies using a two step SDS-PAGE-based approach as cited in the text. The one step approach gave more precise results that the two step approach, but the two step approach may provide a more accurate estimate, or at least an estimate more consistent with previous studies.

Also, for inhibitors (β-lactams) which reveal particularly slow acylation kinetics, a two
step assay with inhibitor binding in the absence of competing probe may be preferable. For rapid screening and characterization of prospective new inhibitors the one step protocol will likely be preferred. Also, for the discovery and characterization of novel non-covalent PBP2a inhibitors, a slow off rate is not expected and the one step assay will be required for such efforts. Given that PBP2a is a key molecular determinant for high-level \(\beta\)-lactam resistance in MRSA, and that new inhibitors for PBP2a could provide new agents effective against MRSA, the microtiter plate-based assay approach described here is expected to facilitate the discovery, development, and characterization of new inhibitors against this important drug target.
ACKNOWLEDGEMENTS

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**FIGURE LEGENDS**

**Fig. 1:** Structures of key β-lactams used in this study.

**Fig. 2:** Panel A; Data, best fit curves, and best fit parameter values for titration of GST-PBP2a with BIO-AMP with 15 min and 30 min incubations. Data points obtained after 15 min incubations of are denoted by diamonds (◊), and those after 30 min incubations are denoted by boxes (□). The best fit curve through data points obtained after 15 min incubation is denoted by the solid line, and after 30 min incubation is denoted by the dashed line. Panel B; Data and results for BIO-CEPH with a 15 min incubation. For all values ± standard errors are given.

**Fig. 3:** SDS-PAGE gel of BIO-AMP labeled GST-PBP2a (left) and untagged PBP2a (right), as a function of the BIO-AMP concentration relative to the $K_{m}^{app}$ for BIO-AMP (as determined against GST-PBP2a in the microtiter plate based assay). Some untagged PBP2a was present in the GST-PBP2a preparation due to the presence of endogenous proteases during GST-PBP2a purification.

**Fig. 4:** Competitive titration of GST-PBP2a by ceftobiprole (Panel A) and meropenem (Panel B) in the presence of a fixed concentration ($K_{m}^{app} = 1.6 \mu M$) of BIO-AMP. including data points, error bars, best fit line and best fit parameter values. Data points obtained after 15 min incubation are denoted by diamonds (◊), and those after 30 min incubation are denoted by boxes (□). Best fit curve through data points obtained after 15 min incubation is denoted by the solid line, and after 30 min incubation is denoted by the dashed line. For all values ± standard errors are given.

**Fig. 5:** Results from two step binding assay result for ceftobiprole vs GST-PBP2a.
Table 1: Screening results in terms of percent inhibition of BIO-AMP binding in order of increasing potency. Given as value ± standard error.

<table>
<thead>
<tr>
<th>β-Lactam (750 µM)</th>
<th>% Inhibition</th>
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<tr>
<td>Penicillin G</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>0 ± 10</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>0 ± 8</td>
</tr>
<tr>
<td>Carbenicillin</td>
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</tr>
<tr>
<td>Cephaloridine</td>
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</tr>
<tr>
<td>Cefotaxime</td>
<td>0 ± 7</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0 ± 6</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>Meropenem</td>
<td>80 ± 2</td>
</tr>
</tbody>
</table>
REFERENCES


A

B

\( K_{M, \text{app}} = 1.6 \pm 0.4 \mu M \)

\( [RU]_0 = 4,000 \pm 3,000 \mu M \)

\( [RU]_{\text{max}} = 53,000 \pm 4,000 \mu M \)

\( K_{M, \text{app}} = 13.6 \pm 0.8 \mu M \)

\( [RU]_0 = 700 \pm 500 \mu M \)

\( [RU]_{\text{max}} = 53,600 \pm 700 \mu M \)
<table>
<thead>
<tr>
<th>GST-PBP2a</th>
<th>Untagged PBP2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km&lt;sub&gt;app&lt;/sub&gt;</td>
<td>0  ⅛ ½ 2    8 0  ⅛ ½ 2     8</td>
</tr>
</tbody>
</table>

[BIO-AMP] as a multiple of the microtiter plate based Km<sub>app</sub>
A

B

<table>
<thead>
<tr>
<th>Ceftobiprole (µM)</th>
<th>15 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFU</td>
<td>9,000 ± 3,000</td>
<td>8,000 ± 3,000</td>
</tr>
<tr>
<td>RFU_{max}</td>
<td>65,000 ± 6,000</td>
<td>68,000 ± 5,000</td>
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<table>
<thead>
<tr>
<th>Meropenem (µM)</th>
<th>15 min</th>
<th>30 min</th>
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</thead>
<tbody>
<tr>
<td>RFU</td>
<td>7,900 ± 900</td>
<td>7,400 ± 800</td>
</tr>
<tr>
<td>RFU_{max}</td>
<td>38,500 ± 1,600</td>
<td>44,800 ± 1,400</td>
</tr>
</tbody>
</table>
$K_{m, app} = 2.9 \pm 1.5 \mu M$

$RFU_0 = 31,000 \pm 1,200$

$RFU_{max} = 16,000 \pm 2,000$