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Development of homogeneous immunoassays based on protein fragment complementation

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ABSTRACT

We demonstrate a functional *in vitro* proof-of-principle homogeneous assay capable of detecting small (<1 kDa) to large (150 kDa) analytes using TEM-1 β-lactamase protein fragment complementation. In the assays reported here, complementary components are added together in the presence of analyte and substrate resulting in colorimetric detection within 10-min. We demonstrate the use of functional mutations leading to either increased enzymatic activity, reduced fragment self-association or increased inhibitor resistance upon analyte driven fragment complementation. Kinetic characterization of the resulting reconstituted enzyme illustrates the importance of balancing increased enzyme activity with fragment self-association, producing diagnostically relevant signal-to-noise ratios. Complementation can be utilized as a homogeneous immunoassay platform for the potential detection of a range of analytes including, antibodies, antigens and biomarkers.

Heterogeneous enzyme immunoassays (EIAs) such as the enzyme-linked immunosorbent assay (ELISA) have dominated the diagnostic industry due to their high sensitivity and specificity. Major limitations of heterogeneous EIAs include lengthy processing times and multiple wash and incubation steps, necessitating specialized instrumentation. Homogeneous EIAs offer an alternative and possibly superior disease diagnosis platform in that these require no washing steps and are compatible with high throughput and point-of-care immunoassays. One approach is to use the split reporter protein system in a homogeneous EIA format.

Split reporter proteins were developed for the simple and rapid detection of *in vivo* protein–protein interactions, in protein (or enzyme) fragment complementation assays (PCAs; Fig. 1) for drug discovery and biochemical pathway mapping using various enzymes [1–6]. In proof-of-principle experiments, two partner proteins with high affinity for each other (e.g., Bad and Bcl2T apoptotic proteins [2] or Fos and Jun leucine zippers [3]) were engineered as fusions with one or other fragment of the "split" enzyme (Fig. 1). Upon co-transformation in cells, the interaction between the two partner proteins resulted in reassembly of the fused enzyme fragments forming an active reporter enzyme. Reassembly of reporter fragments through the interaction of fused partner proteins is the fundamental principle underlying PCA.

The development of an *in vitro* PCA generates unique challenges, aside from the usual problems associated with immunoassay development (cross-reactivity, interference and inhibition from components present in biological samples). First, the enzyme fragments must be produced in a soluble, active and stable form. Second, the fragments must be attached (by molecular engineering or conjugation) to analyte recognition moieties, such as a disease-specific antigen or antibody. Third, the fragments must have negligible affinity for each other in the absence of analyte, to prevent false positives or high background signals. Fourth, the analyte size and complexity must be taken into account to avoid steric effects that could interfere with complementation. Finally, the extracellular conditions of the *in vitro* diagnostic assay must be made conducive to protein folding.

Here, we have applied the *in vivo* PCA technology to an *in vitro* setting. TEM-1 β-lactamase (EC 3.5.2.6) was selected for fragmentation and development of a prototype homogeneous assay, because it is monomeric, has a high turnover rate, and minimal post-translational modifications (a single disulfide bond) facilitating its production in recombinant form [7]. Suitable break-point termini have been described and the resulting fragments were reported to have low intrinsic affinity for each other producing negligible background signals *in vivo* [2,3]. In addition, β-lactamase is simple to assay with colorimetric, fluorescent or luminescent substrates [8–10].

We demonstrate the successful application of functional proof-of-principle *in vitro* PCA assays for diagnostic purposes. Kinetic characterization of the reconstituted enzyme illustrates the effects of mutations used to modify activity, self-association and inhibitor resistance.

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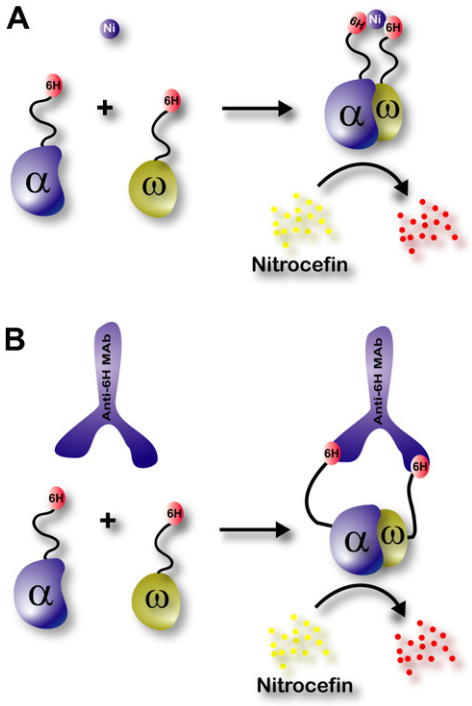

Fig. 1. Cartoon representation of β-lactamase-based PCA. (A) Enzyme fragments, α and ω, linked to analyte binding moieties (histidine tag; 6H). In the presence of a small analyte (Ni^{2+}), the fragments are forced into close proximity (right), thereby initiating the hydrolysis of nitrocefin, visible as a color change from yellow to red. (B) Complementation in the presence of a large analyte (anti-His MAb).

Materials and methods

Plasmid construction and site-directed mutagenesis. General schematics of the β-lactamase constructs, primer sequences and detailed methods for the construction of all DNA can be found in Supplementary material.

Protein expression and purification. Starter cultures of BL21 (DE3; Stratagene) *Escherichia coli* transformants were grown in Overnight Express medium (Novagen) at 37 °C for 26 h with shaking. Detailed purification methods are described in Supplementary material.

In vitro protein fragment complementation assays. Enzyme activity assays were performed at ~23 °C with 10 nM of each fragment (unless stated otherwise) in 50 mM sodium phosphate, 150 mM NaCl pH 7 in the presence of 200 μM $NiSO_4$ (Ni^{2+}) or 10 nM anti-His MAb (R&D sytems: clone AD1.1.10) analyte, to a final volume of 100 μL. Control reactions without analyte were combined in the same way, in the presence of 5 mM EDTA. Assay components were added in a 96-well plate (Greiner), mixed and incubated for 5 (Ni^{2+}) or 10 min (anti-His MAb) prior to the addition of substrate. Nitrocefin (Merck; 100 μL of a 200 μM stock in 5% DMSO, 50 mM sodium phosphate, 150 mM NaCl pH 7) was then added to give final concentrations of 100 μM nitrocefin, 2.5% DMSO, 50 mM sodium phosphate, 150 mM NaCl pH 7 in a 200 μl reaction volume. The rate of nitrocefin hydrolysis was measured at 492 nm over 10 min using a SpectraMax190 (Molecular Devices, USA) plate reader. The initial rates of reaction (first 10–30 points at 10 s intervals) were analyzed using SoftMaxPro software (Molecular Devices) to determine activity rates (mAU min^{-1}).

Determination of kinetic parameters. Kinetic parameters (V_{max} and K_m) were determined by observing enzymatic activity towards nitrocefin at 492 nm ($\varepsilon = 20{,}500\ M^{-1}\ cm^{-1}$) over 10 min. Activity assays were performed as outlined above with the exception of the αV74T and ωM211Q mutants, included at a final concentration of 50 nM and full-length TEM-1 was assayed at a final concentration of 1 nM. Assays containing fragments and analyte were pre-incubated for 5 min followed by addition of 100 μL of a twofold dilution series of nitrocefin (12.5–800 μM). The kinetic parameters were determined by plotting initial rates of reaction (first 10 points) versus [*S*]. Data were fitted to the classical Michaelis–Menten equation with GraphPad Prism 4.0 (GraphPad Software, CA, USA) to determine the V_{max} and K_m for each α and ω fragment pair. The k_{cat} parameter was determined using the equation $k_{cat} = V_{max}/[E]$, where the concentration of fragments was determined using the Coomassie Plus™ Protein Assay (Pierce).

Determination of IC_{50} values. Nitrocefin (100 μM) containing a twofold dilution of tazobactam (Sigma; 0.125–10 μM), clavulanic acid (Molekula; 0.156–100 μM) or sulbactam (Molekula; 0.156–100 μM) was assayed as previously described with 25 nM fragments and 200 μM $NiSO_4$. The initial rate of reaction (first 10 points) was then plotted (GraphPad Prism 4.0) against each inhibitor concentration to determine the inhibitor IC_{50}.

Results

Recombinant β-lactamase purification and determination of kinetic parameters

Recombinant β-lactamase lacking its signal sequence (residues 1–25) and incorporating a C-His tag was expressed in *E. coli* and found to accumulate in insoluble inclusion bodies. The protein was extracted under denaturing conditions and refolded on His-Trap™ columns using a urea gradient. Table 1 shows the kinetic parameters (k_{cat} and K_m) determined for refolded β-lactamase using nitrocefin as substrate. The K_m for refolded β-lactamase was 61 μM, similar to that previously reported for wild-type β-lactamase (55 μM) purified from the periplasm under native conditions [11,12]. The k_{cat} was eightfold slower and may to some extent reflect a less than quantitative yield upon refolding.

β-Lactamase fragmentation and purification

We split the enzyme between residues 196 and 197 to give α (residues 26–196) and ω (residues 197–290) fragments. To facilitate purification, a histidine tag was introduced at the break-point termini of all fragments. Finally, fragments were engineered to include either a short (G_4S) or long ($G_4S)_3$ linker between the enzyme fragment and the histidine tag, to allow flexibility during the complementation process. We purified both α and ω fragments under native conditions but yields were low, so we adopted the same purification procedure used to refold and purify the non-fragmented parental enzyme. All refolded fragments were eluted in a highly purified form as observed by SDS–PAGE and were predominantly monomeric (>90%; gel-filtration data, not shown). The purification protocol routinely yielded 2–5 mg of >95% pure protein per gram of cells.

In vitro protein fragment complementation proof-of-principle assay

The histidine affinity tags on the fragments also served as binding moieties for small (Ni^{2+}) or large (anti-His MAb) analytes, to induce complementation and hence enzymatic activity. Both short and long linker fragments resulted in β-lactamase activity upon

Table 1
Kinetic parameters of TEM-1 β-lactamase complementation hydrolysis of nitrocefin[a]

Enzyme	Fragment pair	K_m (μM)	k_{cat} (s^{-1})
TEM-1[b]		55	930
TEM-1		61	122
	α/ω	35	6.5
	α/ω [c]	45	0.7
	αM182T/ω	59	20.0
	α$(G_4S)_3$/ω	31	3.9
	α/ω$(G_4S)_3$	39	11.0
	α$(G_4S)_3$/ω$(G_4S)_3$	34	5.8
	α$(G_4S)_3$/ω$(G_4S)_3$ [c]	53	2.6
	αV74T/ω	73	4.3
	α/ωM211Q	104	0.1
	αV74T/ωM211Q	138	0.2
	αV74T.M182T/ω	74	11.7
	αM182T/ωM211Q	26	1.0
	αV74T.M182T/ωM211Q	129	8.7

[a] Standard errors were determined from triplicate experiments and were ±5% for k_{cat}, ±15% for K_m.
[b] Published TEM-1 β-lactamase kinetic parameters cited here were obtained using wild-type full-length enzyme [12].
[c] Complementation done using anti-His MAb.

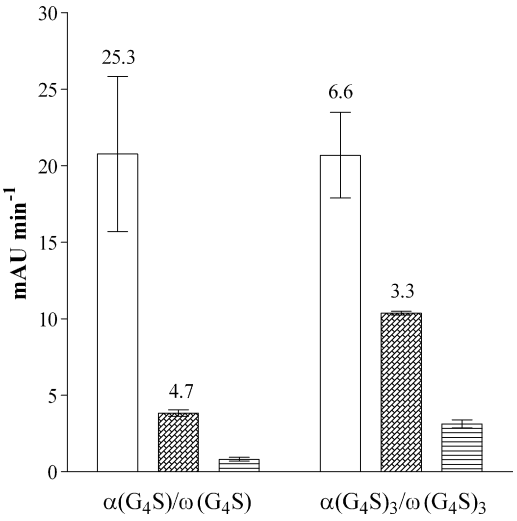

Fig. 2. β-Lactamase based *in vitro* PCA. Assays were performed by combining 10 nM of each fragment with 200 μM $NiSO_4$ (no fill), 10 nM His MAb (cross-hatch fill) or no analyte (buffer containing 5 mM EDTA; horizontal line fill) with short (G_4S) or long ($(G_4S)_3$ linker fragments. Reactions were pre-incubated for 5-min followed by addition of nitrocefin. Substrate hydrolysis (mAU min^{-1}) was determined with a 10-min kinetic reading at 492 nm. Numbers above bars indicate the *S*:*N* ratio. Error bars represent the standard deviations from duplicate experiments.

inclusion of either analyte (Fig. 2). It is important to note that these proof-of-principle assays also allowed the formation of inactive homodimers of α/α and ω/ω complexes. Theoretically, the functional α/ω complexes represent ~50% of the population of the analyte-induced complexes. Therefore, the observed k_{cat} values are likely to represent an underestimate, probably less than half, the true substrate turnover rates of functional complexes.

A low level of activity was observed in the absence of analyte, resulting in signal-to-noise (*S*:*N*) ratios of 25.3 for Ni^{2+} and 4.7 for anti-His MAb using the short linker fragments. Likewise, *S*:*N* ratios of 6.6 and 3.3 were obtained using the long linker fragments with Ni^{2+} and anti-His MAb, respectively. The *S*:*N* ratios are based on the absolute rates of nitrocefin hydrolysis (mAU min^{-1}) with no correction for background (short linker, 0.82 ± 0.13 mAU min^{-1} and long linker; 3.12 ± 0.26 mAU min^{-1}). We observed spontaneous complementation hydrolysis rates of up to 3 mAU min^{-1} in the absence of analyte using 10 nM fragments which is comparable to that observed previously for *in vitro* β-lactamase based PCA detection of double-stranded DNA [13].

Kinetic parameters for protein fragment complementation

The kinetic parameters of complementation were investigated for fragments with different mutations and linker lengths (Table 1). We chose to characterize the kinetic properties rather than the complementation process itself, since substrate turnover is the end result of successful α/ω fragment interaction, proposed to be driven by analyte concentration and affinity.

The k_{cat} of reconstituted β-lactamase driven by Ni^{2+} forced complementation of short linker fragments was 6.5 s^{-1} compared with 122 s^{-1} for intact refolded β-lactamase, and 930 s^{-1} for natively purified β-lactamase [12]. The K_m values for all three were similar (Table 1) indicating that reassembly of the enzyme fragments preserved the basic structure of the active site, implying that the lower k_{cat} observed for the reassembled enzyme fragments is a consequence of inefficient hydrolysis and/or complementation. We generated a mutant of the α fragment designed to overcome the poor substrate turnover. The mutation, M182T, acts as a global suppressor of TEM β-lactamase missense mutations that cause folding and stability defects, restoring function to mutated enzymes [14,15]. Inclusion of the M182T mutation in the α fragment did not affect K_m, but it increased k_{cat} threefold to 20 s^{-1}, representing 16.4% of the activity of intact refolded β-lactamase (Table 1).

Determination of k_{cat} and K_m using the large analyte (150 kDa anti-His MAb) highlighted the importance of engineering fragments with optimized linker lengths. The fragments with short linkers to the analyte-binding moiety gave poorer k_{cat} results for the large analyte (0.7 s^{-1}) than for the small analyte (6.5 s^{-1}). By contrast, the long linker fragments had a fourfold higher k_{cat} than the short linker fragments (2.6 s^{-1} and 0.7 s^{-1}, respectively; Table 1). The K_m value for all combinations of fragments was largely unchanged indicating that the steric constraints of the large analyte did not impact on binding affinity of the nitrocefin ligand.

Reduction of in vitro fragment self-association

We anticipated that high enzyme fragment concentrations would be advantageous in capturing as much of the available analyte as possible, leading to formation of active reporter enzyme complexes. In practice, however, we observed high self-association levels that reduced *S*:*N* ratios when fragment concentrations exceeded 10 nM (Fig. 3A). We therefore typically used 10 nM of each fragment in the proof-of-principle assays. In an attempt to limit fragment self-association *in vitro*, we introduced two mutations at the fragment complementation interface (V74T in α and M211Q in ω) to reduce the hydrophobicity of the exposed surface that resulted from enzyme fragmentation. Previously, fragmentation studies have shown that interactions among hydrophobic core residues are major determinants of affinity between fragments and stability of the resulting complex [16].

Generally, introduction of V74T and M211Q mutations decreased the binding affinity for substrate (K_m values 73–138 μM compared with 35 μM for the original enzyme fragments; Table 1) and the M211Q mutation also had a negative impact on k_{cat}. The hydrophobic mutants appear to have successfully decreased the affinity of the α/ω fragments resulting in lower spontaneous reassembly (Fig. 3B) yet their adverse impact on substrate turnover limited their utility. Interestingly, incorporation of the M182T restorative mutation with the V74T mutant doubled the turnover rate observed with the original enzyme fragments (k_{cat} values of 11.7 s^{-1} and 6.5 s^{-1}, respectively; Table 1).

Engineering resistance to β-lactamase inhibitors

A potential drawback of using β-lactamase in PCA-based serological testing is that it is susceptible to β-lactamase inhibitors in the serum of individuals treated with antibiotics. Clavulanic acid, sulbactam, and tazobactam are commonly used, and serum levels are sufficient to inhibit β-lactamase reporter enzyme activity [17–19]. As a precautionary measure for future *in vitro* diagnostic serology testing, we introduced inhibitor resistance mutations [20,21] into α and ω fragments. The natural isolates TEM-32 (M69I, M182T), TEM-33 (M69L), and TEM-35 (M69L, N276D) provide good resistance to the β-lactamase inhibitors and maintain reasonable catalytic activity [21]. We therefore generated fragments incorporating mutations (M69L, M182T, and N276D) alone or in combination and assessed their ability to overcome chemical β-lactamase inhibition during complementation (Table 2). Our results suggest that the more effective a mutant enzyme is at resisting the β-lactamase inhibition, the less effective it is at hydrolyzing substrate, in agreement with the previously described trade-off between increased β-lactamase resistance and decreased enzyme activity [22].

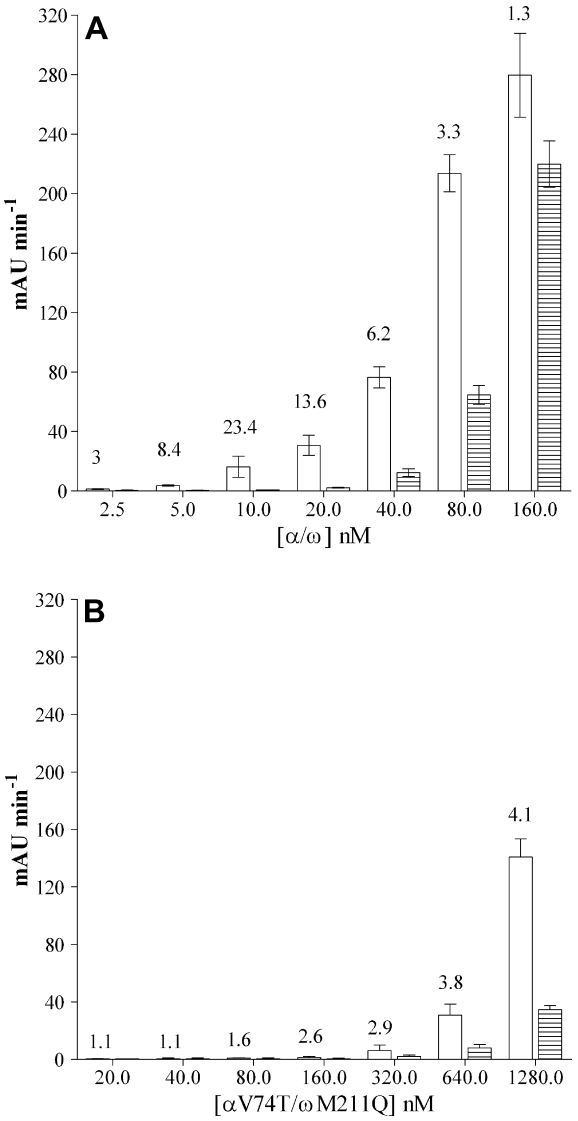

Fig. 3. Increasing fragment concentration results in increasing spontaneous complementation of a β-lactamase based *in vitro* PCA. (A) Assays were performed by combining equimolar concentrations of α and ω fragments with 200 μM $NiSO_4$ analyte (no fill) and no analyte (buffer containing 5 mM EDTA; horizontal line fill). (B) Assays using hydrophobic interface mutants, αV74T and ωM211Q. Reactions were pre-incubated for 5-min followed by addition of nitrocefin. Substrate hydrolysis (mAU min^{-1}) was determined with a 10-min kinetic reading at 492 nm. Numbers above bars indicate the *S:N* ratio. Error bars represent the standard deviations from duplicate experiments.

Discussion

Whilst significant advances have been made with the utilization of PCA for *in vivo* applications [23], little has been reported for its use *in vitro*. Of particular interest is the oligonucleotide [13,24] and single-chain Fv [4,5] driven *in vitro* enzyme fragment complementation models for the detection of sequence specific double-stranded DNA and small to medium (66 kDa) sized antigens, respectively. Here, we present a proof-of-principle *in vitro* application for PCA, which we plan to further develop as a platform technology for the development of a homogeneous EIA for the purpose of detecting analytes in a diagnostic setting. We have shown that *in vitro* PCA can be used to successfully detect small to large antibody analytes. This has been achieved by producing enzyme fragments that: (1) can be purified in a stable and active form; (2) produce low background in the absence of analyte; (3) overcome potential steric constraints imposed by large complex analytes; and (4) generate a diagnostically relevant *S:N* ratio.

Interestingly, it has been proposed that PCA relies on protein folding during complementation rather than docking of prefolded subunits [23]. In this study we refolded all our recombinant fragments independently, as fragment stability and solubility is critical to the successful application of PCA to an *in vitro* homogeneous EIA. We have demonstrated functional PCA using two analytes (Ni^{2+} and anti-His MAb) and obtained K_m values, which indicate that, the complementation process we have observed recreates the native catalytic site.

Kinetic characterization of enzyme reconstitution upon complementation has highlighted several key limitations, including low activity of the reconstituted enzyme complex, that most likely reflects poor complementation efficiency [25]. Given that the full-length circular permutant enzyme (196/198 split without a hexahistidine tag) retains 20% wild-type activity *in vitro* as determined from lysates [2] and that our reconstructed enzyme retains 5% activity we should be able to improve our activity up to fourfold with efficient complementation. Equally problematic is the observation of measurable levels of background enzyme activity due to spontaneous assembly of fragments in the absence of analyte at fragment concentrations exceeding 10 nM. This is contrary to *in vivo* results [2,3,6], and highlights one key difference in the underlying nature of *in vivo* assays and those performed in a defined *in vitro* environment. *In vivo* PCA is used to detect protein–protein interactions in cells, where the concentration of enzyme fragments may be as low as 25 molecules per cell (~fM range [2]). In contrast, our *in vitro* assays use higher fragment concentrations (~low nM range), thereby increasing the possibility of spontaneous re-association. Both of these key limitations have been addressed by incorporating desired traits into the reconstituted

Table 2
TEM-1 β-lactamase inhibitor resistant complementation IC_{50} values[a]

Enzyme	Fragment pair	K_m (μM)	k_{cat} (s^{-1})	Tazobactam IC_{50} (nM)	Clavulanic Acid IC_{50} (μM)	Sulbactam IC_{50} (μM)
TEM-1[c]		55[b]	930[b]	30	0.08	
TEM-1M69L[c]				500	2	
TEM-1M69L.N276D[c]				600	12	
	α/ω	35	6.5	102.3	1.7	2.7
	α/ωN276D	36	5.6	175.9	2.1	2.9
	αM69L/ω	259	2.5	1200	30.9	24.6
	αM69L/ωN276D	58	1.5	828.6	246.6	47.1
	αM69L.M182T/ω	106	4.4	1529	46.7	32.5
	αM69L.M182T/ωN276D	84	4.6	1208	59.2	52

[a] Standard errors were determined from triplicate experiments using nitrocefin as substrate and were ±10% for k_{cat}, ±30% for K_m except for TEM-1 which had a standard error of ±5% for k_{cat}, ±15% for K_m. IC_{50} determinations had standard errors of ±15% for tazobactam and ±30% using either clavulanic acid or sulbactam.
[b] Published TEM-1 K_m and k_{cat} data using nitrocefin [12].
[c] Published TEM-1, TEM-1^{M69L}, and TEM-$1^{M69L.N276D}$ IC_{50} data using nitrocefin [20].

enzyme using well-characterized mutations. In both instances the mutations successfully enhanced function; the M182T mutant increased the k_{cat} value threefold; the hydrophobic interface mutants (V74T and M211Q) increased the fragment concentration at which measurable spontaneous activity was observed 16-fold, however, in both instances the mutations had a negative impact on self-association and activity, respectively. We have yet to completely understand the effect of these mutations on the complementation process *per se*; however, the ease with which desired traits can be engineered into PCA as further evidenced with the β-lactamase inhibitor resistant mutations (Table 2) suggests that *in vitro* PCA is a robust process, mimicking very well the non-fragmented parental enzyme.

We are currently working on developing a herpes simplex virus type-1 and herpes simplex virus type-2 specific assay based on β-lactamase *in vitro* PCA for the differential diagnosis of type-1 and -2 in human serum.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.03.057.

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