



Jenkins, E., Adam, E., Ah-San Tang, A., Wilcox, A., Räuber, C., McMorran, L., Johnson, M. and Basran, A.

## Abstract

Affimer reagents and therapeutics are a class of non-antibody binding proteins that have been engineered for a wide range of applications where antibodies and aptamers have limitations. They are produced **without the use of animals**, can be used to detect difficult targets, can easily be formatted for a wide range of applications and can be easily and cost effectively manufactured. Using phage display, we have identified competitive binders to a range of targets, including the immune check point, Programmed death-ligand 1 (PD-L1). PD-L1 plays an important role in immune homeostasis and blockade of the PD-L1/PD-1 pathway using antibodies has demonstrated impressive anti-tumour responses in cancer patients. Our inhibitors have been shown to be highly selective for PD-L1 with  $K_D$ 's of single digit nM as determined by BIAcore. We have shown that the scaffold is amenable to being engineered to make multimers (dimers, trimers and tetramers) as well as being formatted to extend the serum half-life.

## The Affimer Technology and Phage Selections

The Affimer therapeutic scaffold is based on the human protein Stefin A which has been engineered not to bind to its native target, cathepsin (Figure 1). The scaffold is  $\sim 1/10^{\text{th}}$  the size of a antibody.

Large peptide libraries ( $1 \times 10^{10}$ ) have been created by engineering two 9 amino acid loops into the surface. By using phage display (Figure 2), the libraries can be efficiently screened for binders against protein targets.

The library was panned against human PD-L1-Fc (R&D Systems) with 3 rounds of selections and the outputs cloned into a protein expression vector for small-scale screening using the iQue flow cytometry platform (IntelliCyt Corporation). Positive PD-L1 binding clones were then DNA sequenced.

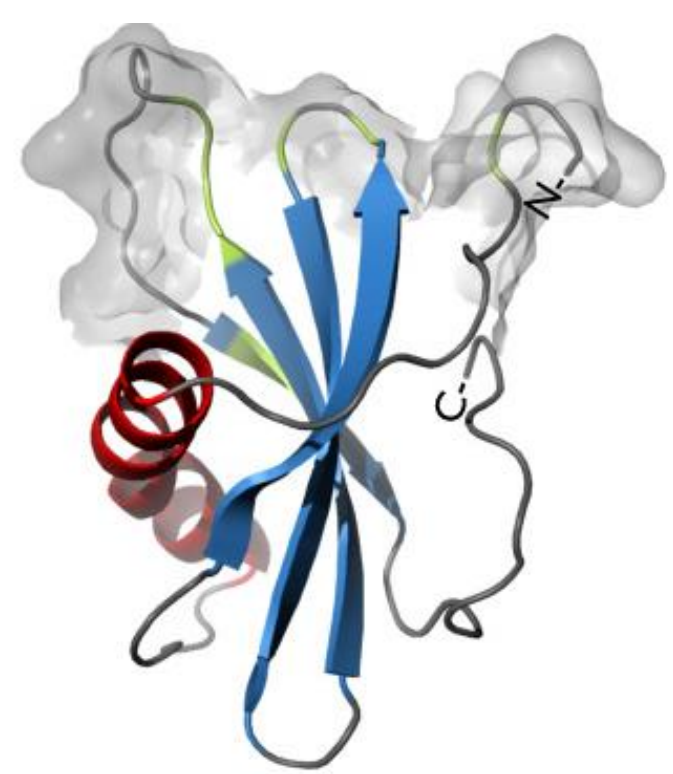


Figure 1. Structure of a Stefin A on which the Affimer biotherapeutic platform is based upon

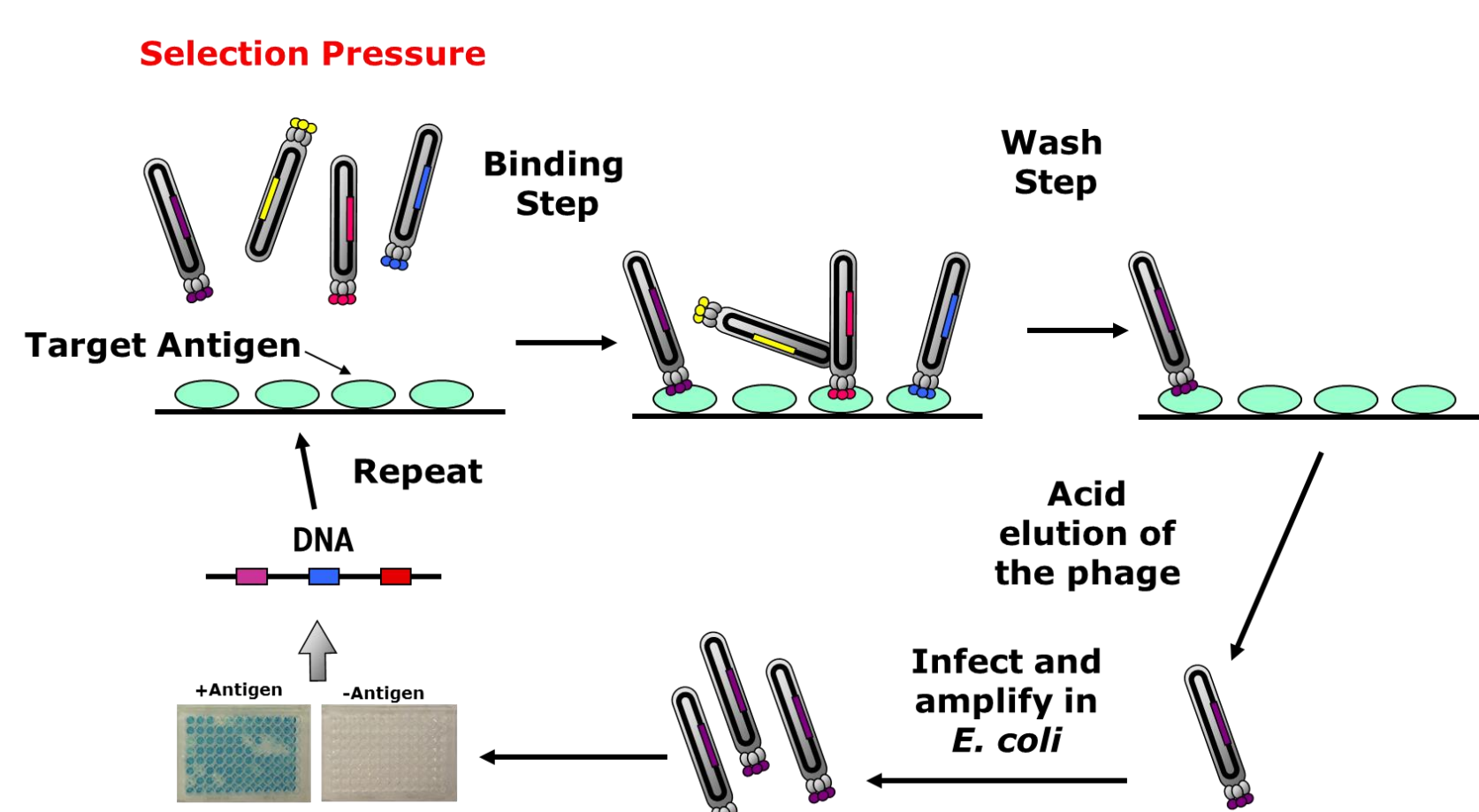


Figure 2. Scheme showing how the Affimer biotherapeutic is selected for against a target antigen using phage display. After 3 rounds of selection the output is cloned into a *E. coli* expression vector for screening to identify positive binders.

## Identification/characterisation of Lead PD-L1 Binders

Unique PD-L1 binding clones identified from the iQue screen were scaled to 15 ml *E. coli* cultures (terrific broth media, at 30°C with overnight induction) for further characterisation. The Affimer biotherapeutic is engineered with a C-terminal HIS tag that allows for single step purification using nickel NTA-agarose with >95% purity (Figure 3).

The majority of the clones expressed at 200-300 mg/L culture and were further characterised by PD-1/PD-L1 competition ELISA and BIAcore (Figure 4 A and B).

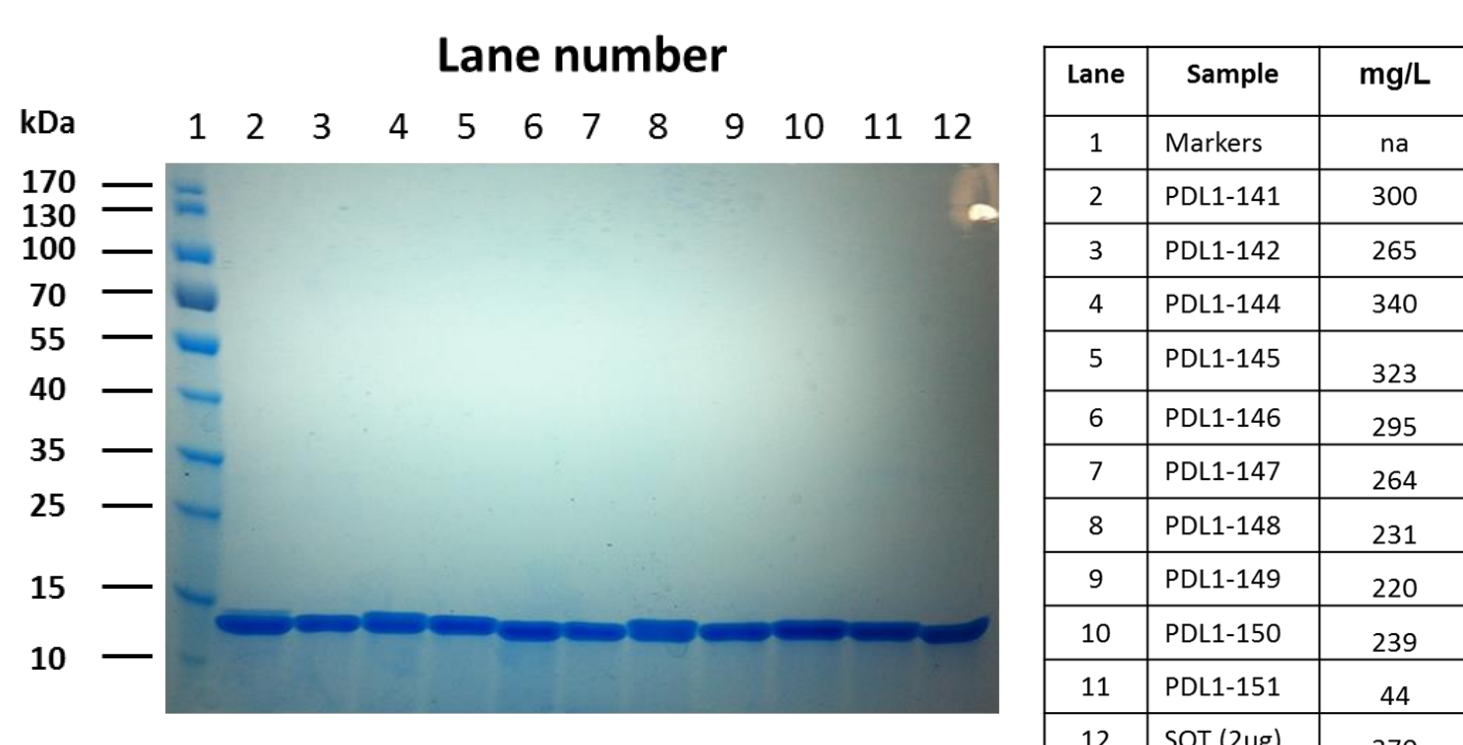


Figure 3. SDS-PAGE showing the purified Affimer biotherapeutic proteins that were positive binders to human PD-L1 as determined by the iQue. The proteins were expressed in *E. coli* (BL21 cells) in terrific broth. Following harvesting, the cells were lysed, cellular debris removed by centrifugation before the supernatants processed using batch binding to Ni-NTA agarose. The eluted proteins were buffer exchanged into PBS.

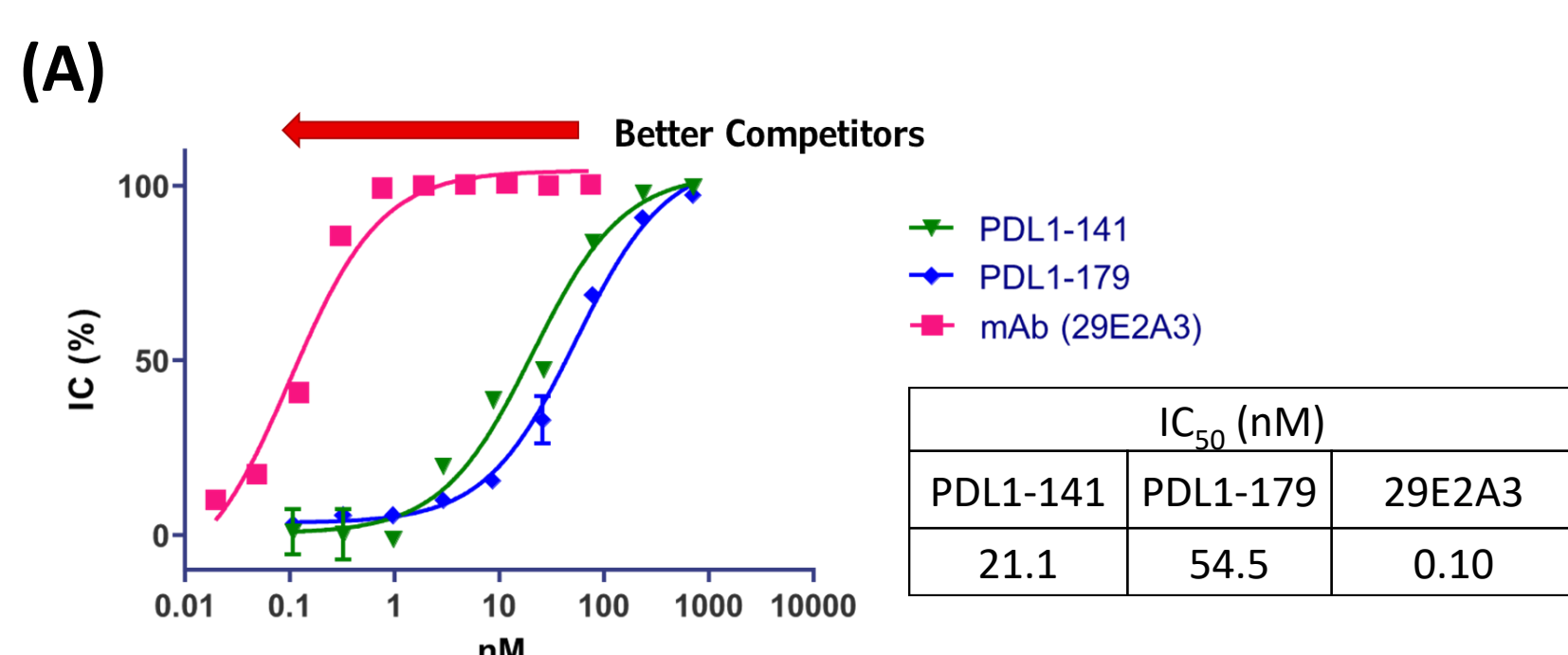
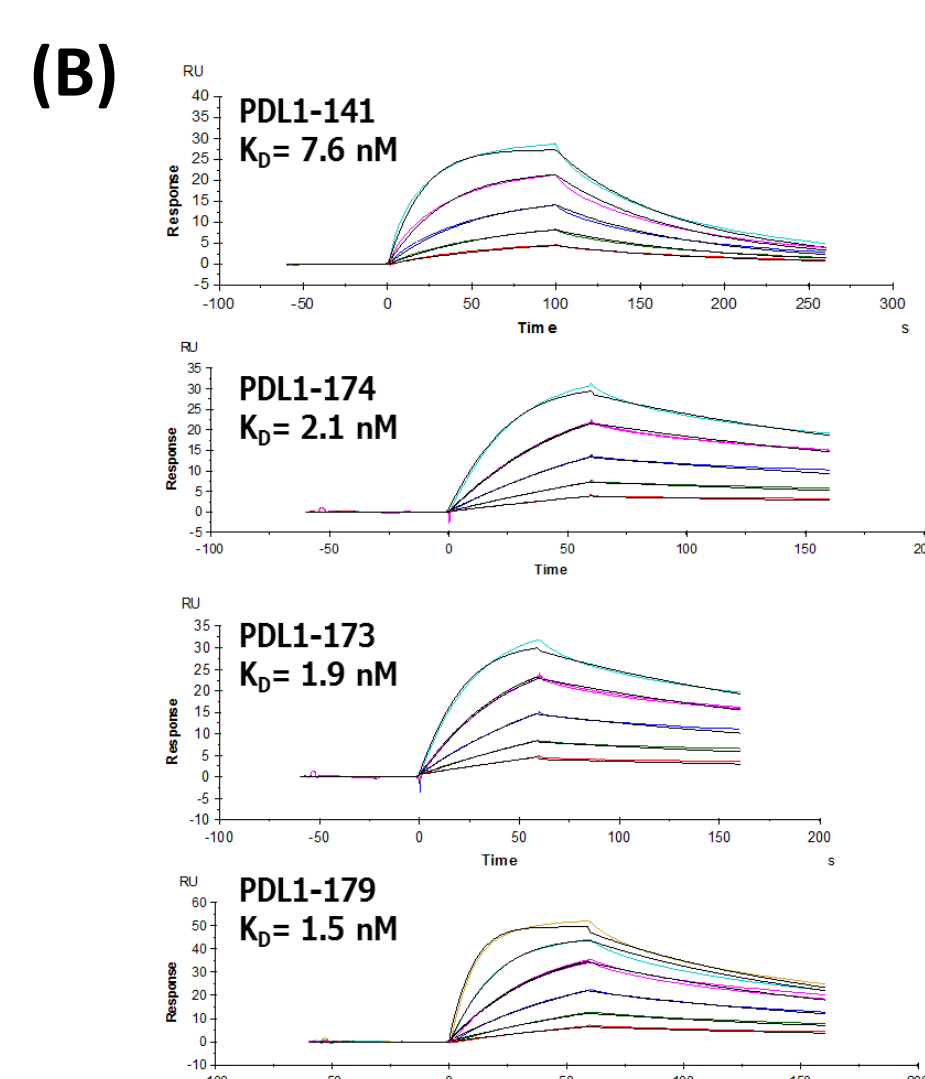


Figure 4. (A) PD-1/PD-L1 competition ELISA showing that the selected Affimer biotherapeutics are able to bind and compete for the PD-1 epitope on PD-L1. (B) The  $K_D$  of several of the lead clones were then measured using BIAcore. Several of the lead Affimer biotherapeutics had  $K_D$ 's in the low single digit nM region.



## PD-L1 Cell Based Assay

Lead molecules PDL1-141 and -179 were tested in the cell based reporter assay (Promega) to confirm that the proteins would be able to inhibit the PD-1/PD-L1 interactions on the cell surface (Figure 5A and B). The potency of the Affimer biotherapeutic is lower than the control mAb 29E2A3. This may be due to the larger bi-valent nature of the antibody as the corresponding Fab has a similar (although incomplete) competition profile as the Affimer biotherapeutic.

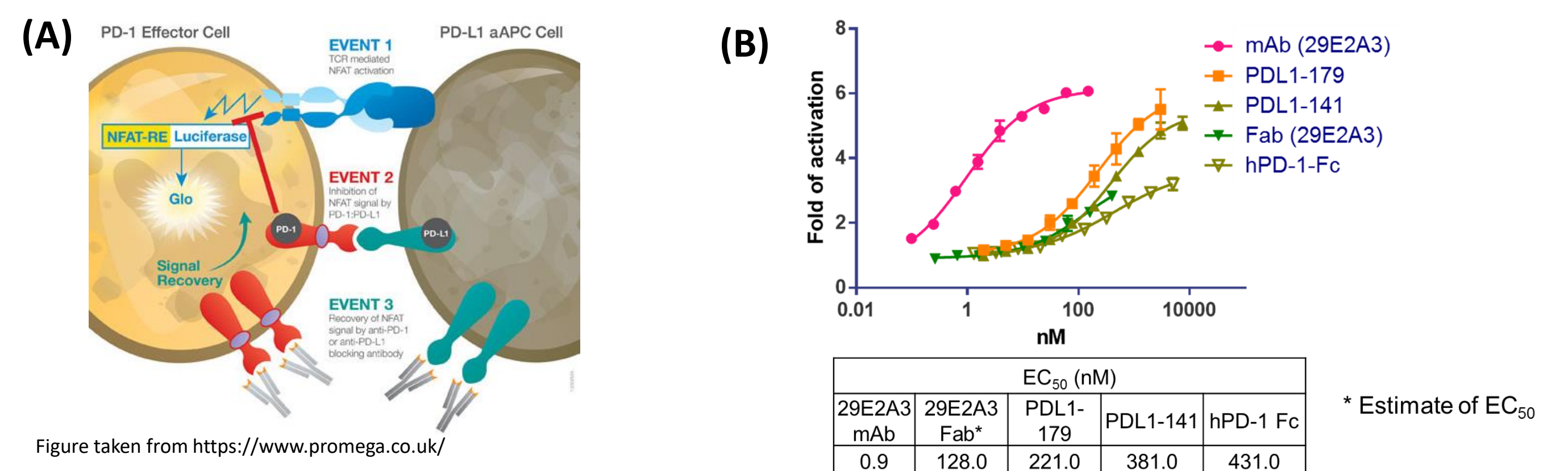


Figure 5. (A) PD-L1 cell based assay: Event 1. T Cell Receptor engagement induces luciferase activity. Event 2. Co-engagement of an immune checkpoint receptor-ligand pair inhibits luciferase activity. Event 3. Antibody-mediated blockade of the immune checkpoint inhibitory signal restores luciferase activity. (B) PDL1-141 and -179 were tested in the cell based assay and shown to be able to bind to PD-L1 on the cell surface and blockade the signal restoring luciferase activity.

## Affimer Biotherapeutic Formatting

There is a great deal of interest in combining immunomodulatory targets for improved efficacy in cancer patients. This has already been demonstrated in the clinic by the co-administration of two approved mAbs, Nivolumab (anti-PD-1) with Ipilimumab (anti-CTLA-4) in patients with advanced melanoma. The administration of more than one mAb therapy will be expensive due to the high costs of the drug. Ideally, a single drug product that could target multiple pathways would be a more viable option. To demonstrate that Affimer biotherapeutics have the potential to bind multiple targets we genetically fused PDL1-141 to create dimer, trimer and tetramers (Figure 6). The binding characteristics of each format was measured using the PD-1/PD-L1 competition ELISA and BIAcore (Figure 7).

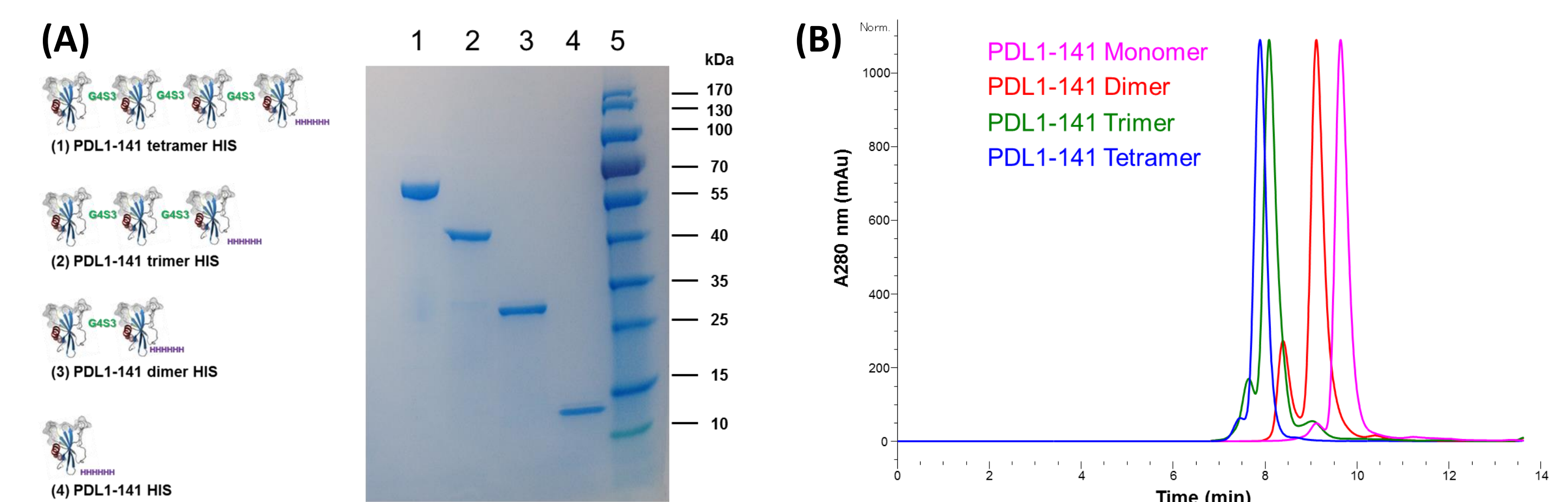


Figure 6. (A) SDS-PAGE showing the final purified monomer, dimer, trimer and tetramer of PDL1-141. Following expression in *E. coli* and purification using Ni-NTA agarose, the purity as estimated by SDS-PAGE was >95%. No significant breakdown products of the fused Affimer biotherapeutics were observed. (B) The purified proteins were run on size exclusion HPLC (Acclaim Sec-300, 0.7 mL/min in PBS pH 7.2). The majority of the Affimer biotherapeutics were monomeric in solution (as determined by SEC MALS), with low levels of dimers present. No higher ordered aggregates were observed under these unoptimised buffer storage conditions.

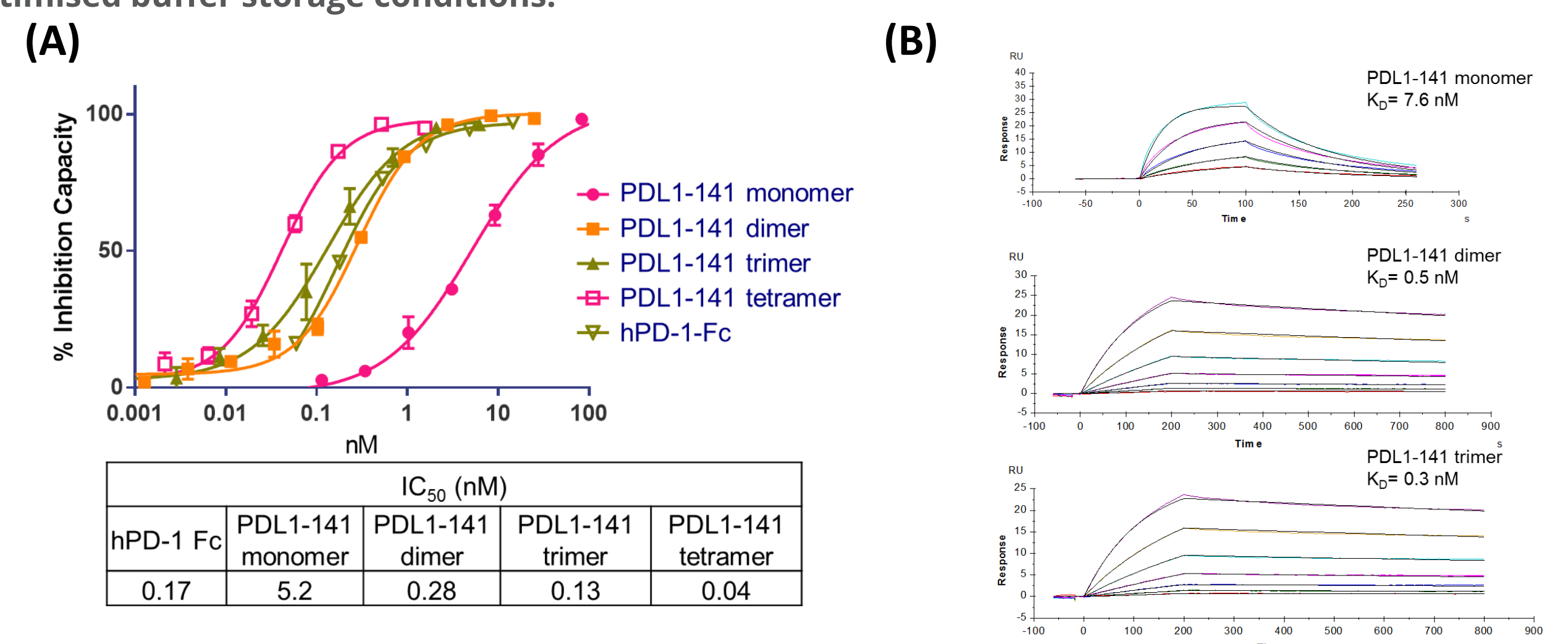


Figure 7. (A) All formats of PDL1-141 were tested in the PD-1/PD-L1 competition ELISA. As the number of binding domains increases in the molecule, there is a decrease in the  $IC_{50}$  due to avidity effects. The PD-L1 and PD-1 used in the assay are Fc-fusions and so dimeric in nature. The avidity effect could only be occurring if each Affimer biotherapeutic in the protein could engage the target. (B) The  $K_D$ 's of the PDL1-141 formats were determined using BIAcore, again an increase in binding affinity was observed as the Affimer biotherapeutic was multimerised.

## Summary

The Affimer technology is a versatile, animal free platform that allowed the rapid identification of monomer binders to PD-L1 with single digit  $K_D$ 's as determined by BIAcore. We have demonstrated that the Affimer biotherapeutics can compete for the PD-1 binding site on PD-L1 when present on the cell surface. The molecules are easily expressed and amenable to formatting to create multimers, which will allow the generation of bi-, tri and tetra- specific binding molecules, with the potential of bringing improved efficacy to the patient. Affimer binders can be generated for a broad variety of *in vitro* assays.