Chapter 3

PCC Agent based assay for the detection of anti-HIV antibodies from human sera

3.1 INTRODUCTION

HIV diagnostic assays typically use chimeric proteins comprised of immunogenic and conserved antigens from the HIV to capture specific anti-HIV antibodies from the body fluid of potentially infected patients. However, the biologically produced chimeric recombinant proteins are susceptible to thermal, chemical, and biological degradation, as well as batch to batch variability. These limitations can adversely influence the performance of a diagnostic test [1,2,3], especially when used outside of controlled laboratory settings.

This chapter explores the ability of the PCC Agent cocktail described in Chapter 2 to detect anti-HIV antibodies from human sera as compared to that of a standard recombinant chimeric antigen like those typically used in anti-HIV antibody detection assays. The chimeric protein used contained a fragment of HIV-1 gp41 (residues 546-692), the "O" group HIV-1 gp41 immunodominant region (residues 580-623), and a fragment of HIV-2 gp36 (residues 591-617). A good performance of this chimeric antigen has been reported elsewhere [4]. Two sets of samples were assayed, one clinical and one commercial. In both cases the PCC Agent cocktail showed increased average signal to noise compared to the chimeric antigen.

3.2 PATIENT SAMPLE ASSAY

The PCC Agent cocktail and the standard antigen were co-evaluated against a panel of clinical samples using sandwich ELISAs. Serum samples were collected from nine HIV-1-positive patients in Southern California. For the comparison assays,

streptavidin-coated 96-well plates were saturated with the PCC Agent cocktail or chemically biotinylated chimera in triplicates. Serum samples were diluted to 1% v/v in Tris-buffered saline (TBS) supplemented with 0.1% w/v bovine serum albumin (BSA), and incubated in the wells for 1 hr at room temperature. Unbound proteins were washed off and captured IgG was detected with a peroxidase-conjugated mouse monoclonal anti-human IgG-Fc antibody. The comparison assay results for the patient serum samples along with a healthy control are shown in Figure 3.1. The signal-to-noise (S/N) ratio in these assays is defined as the measured ELISA signal for a given patient sample, divided by that for the healthy control. The PCC Agent cocktail performed at least as well, and typically much better, than the standard chimeric protein antigen. The average S/N improvement by the cocktail PCC agent over the chimeric antigen was a factor of 2.5.

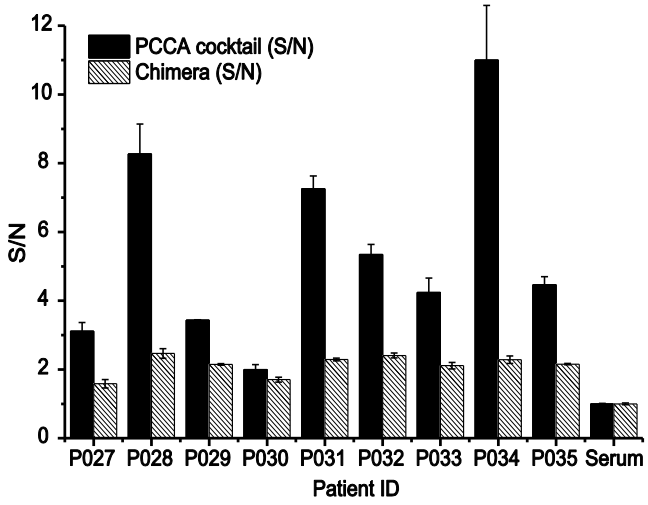

Figure 3.1. Comparative performance of the PCC Agent cocktail versus the commercial chimeric protein, using sandwich ELISAs to detect anti-HIV-1 IgGs from a panel of sera samples collected from nine HIV-positive patients. The absorbance at 450 nm (A450) for each sample is normalized against the A450 for the healthy control, to yield a measurement of the signal-to-noise ratio of the assay. The PCC Agent cocktail, which is designed to capture a subset of anti-gp41 IgGs, exhibits superior performance for every sample, even though the chimeric protein is designed to capture antibodies against multiple HIV-1-associated epitopes (those containing fragments of HIV-1 gp41, "O" group HIV-1 gp41 immunodominant region, and HIV-2 gp39). For the assays, the PCC agent cocktail and the biotinylated chimeric antigen were immobilized on a streptavidin-coated 96-well plate and incubated with diluted patient serum (1% v/v). Captured anti-HIV antibodies were detected using peroxidase-conjugated anti-human IgG antibody.

3.3 PATH SAMPLE ASSAY

The PCC Agent cocktail and the standard antigen were also co-evaluated against a panel of commercial samples. Serum samples from individual patients containing high, medium, or low titers of anti-HIV antibodies were obtained from SeraCare, through PATH of POC diagnostics program funded by the Bill and Melinda Gates Foundation. For the comparison assays, streptavidin-coated 96-well plates were saturated with the PCC Agent cocktail or chemically biotinylated chimera in triplicates. Serum samples were diluted to 1% v/v in Tris-buffered saline (TBS) supplemented with 0.1% w/v bovine serum albumin (BSA), and incubated in the wells for 1 hr at room temperature. Unbound proteins were washed off and captured IgG was detected with a peroxidase-conjugated mouse monoclonal anti-human IgG-Fc antibody. The comparison assay results for the serum samples are shown in Figure 3.2. The signal-to-noise (S/N) ratio in these assays is defined as the measured ELISA signal for a given high, medium, or low titer sample, divided by that for the respective healthy control. The PCC Agent cocktail generally outperformed the standard chimeric protein antigen, with the exception of the low titer samples. The reasons for this are not entirely clear. However, the average S/N improvement by the cocktail PCC agent over the chimeric antigen was a factor of 2.8.

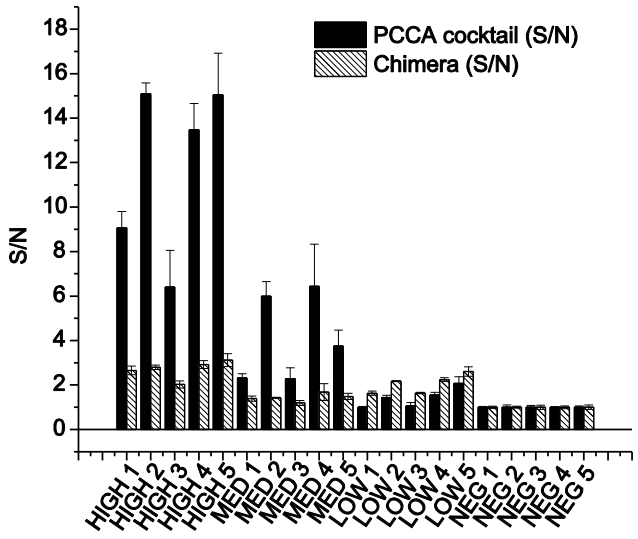

Figure 3.2. Comparative performance of the PCC Agent cocktail versus the commercial chimeric protein, using sandwich ELISAs to detect anti-HIV-1 IgGs from a panel of HIV-1 specific IgG containing plasma samples provided by PATH. The samples were rated HIGH, MED, or LOW in accordance with the titer of anti-HIV antibodies, and pooled HIV negative sera samples were used as a control (NEG). The absorbance at 450 nm (A450) for each sample is normalized against the A450 for the healthy control, to yield a measurement of the signal-to-noise ratio of the assay. The PCC Agent cocktail, which is designed to capture a subset of anti-gp41 IgGs, exhibits superior performance for every sample, even though the chimeric protein is designed to capture antibodies against multiple HIV-1-associated epitopes (those containing fragments of HIV-1 gp41, "O" group HIV-1 gp41 immunodominant region, and HIV-2 gp39). For the assays, the PCC agent cocktail and the biotinylated chimeric antigen were immobilized on a streptavidin-coated 96-well plate and incubated with diluted patient serum (1% v/v). Captured anti-HIV antibodies were detected using peroxidase-conjugated anti-human IgG antibody.

3.4 STABILITY ASSAY

We then tested the PCC Agents for thermal stability. The PCC Agent cocktail component **(iii)** were synthesized at a large scale for an academic setting (~7 mg, Figure 3.3) and the lyophilized samples were stored under N_2 at 25°C, 37°C, or 57°C for 58 days. The samples were then analyzed by HPLC to determine the presence of any degradation product. The traces of the peptide at each temperature are nearly identical, indicating little to no degeneration at these temperatures (Figure 3.3). The performance of these stored PCC Agents was then also tested in an ELISA, with no detectable loss of performance (Figure 3.3).

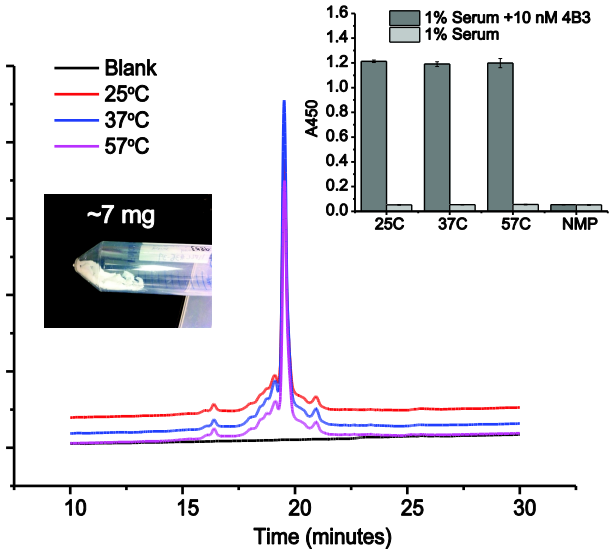

Figure 3.3. Samples of **(iii)** were stored as a powder (inset photo), under N_2 at temperatures up to 57°C for ~2 months, and resolved by analytical HPLC to determine the presence of any degradation product. The HPLC traces reveal that the fingerprint of the PCC Agent is unchanged. The inset shows that the assay performance of the PCC Agent is also unaffected.

3.5 MATERIALS AND METHODS

3.5.1 Patient serum ELISA

Peripheral blood was obtained from HIV-1 infected patients at the University of California, Los Angeles (UCLA) Medical Center between July and August of 2012. Sera were stored at -80°C before subsequent analysis. Streptavidin-coated 96-well plates were saturated with the biotinylated biligand cocktail or chimera using 1 uM solutions in TBS. The plates were blocked with 5% w/v dry milk in TBS, and then the capture reagents were incubated with patient serum samples diluted to 1% v/v in TBS containing 0.1% w/v bovine serum albumin (BSA). Additionally, both cocktail and chimera capture reagents were incubated with 1% v/v commercial healthy human serum in TBS. Bound antibody was probed with HRP-conjugated mouse monoclonal antibody to human IgG Fc, diluted 1:10,000 in TBS with 0.1% w/v BSA. The colorimetric assay was developed with TMB substrate, then quenched with 1 M H_2SO_4 and read at 450 nm (A450). The A450 for each sample is normalized against the A450 for the healthy control, to yield a measurement of the signal-to-noise ratio of the assay.

3.5.2 PATH samples ELISA

Serum samples were obtained from SeraCare, through PATH of POC diagnostics program funded by the Bill and Melinda Gates foundation. Five groups of four samples were tested, where each group contained high, medium, and low titer samples, along with a negative sample that did not contain anti-HIV antibodies. Sera were stored at -80°C before subsequent analysis. Streptavidin-coated 96-well plates were saturated

with the biotinylated biligand cocktail or chimera using 1 uM solutions in TBS. The plates were blocked with 5% w/v dry milk in TBS, and then the capture reagents were incubated with patient serum samples diluted to 1% v/v in TBS containing 0.1% w/v bovine serum albumin (BSA). Additionally, both cocktail and chimera capture reagents were incubated with 1% v/v commercial healthy human serum in TBS. Bound antibody was probed with HRP-conjugated mouse monoclonal antibody to human IgG Fc, diluted 1:10,000 in TBS with 0.1% w/v BSA. The colorimetric assay was developed with TMB substrate, then quenched with 1 M H_2SO_4 and read at 450 nm (A450). The A450 for the samples in each group are normalized against the A450 for the negative sample in that group.

3.5.3 Stability assay

Small amounts of **(iii)** were stored under N_2 at 25°C, 37°C, or 57°C for 58 days. Samples were diluted in 1:1 ACN/H_2O with 0.1% v/v TFA to an equal concentration determined by measuring the absorbance values at 280 nm using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) and resolved by analytical RP-HPLC. The remaining samples were lyophilized and used in a single point sandwich ELISA. Streptavidin-coated 96-well plates were saturated with the samples of **(iii)** recovered from the storage experiments using 1 uM solutions in TBS. The plates were blocked with 5% w/v dry milk in TBS, then incubated with either 1% v/v human serum or 10 nM 4B3 spiked in 1% v/v human serum in 0.5% w/v dry milk in TBS at room temperature for 1 hr. Bound antibody was probed with HRP-conjugated mouse

monoclonal antibody to human IgG Fc diluted 1:15,000 in 0.5% w/v dry milk in TBS. The colorimetric assay was developed with TMB substrate, then quenched with 1 M H_2SO_4 and read at 450 nm.

3.6 CONCLUSION

This thesis demonstrated the successful application of this method for HIV-1 diagnostics by producing a cocktail of three PCC Agents that detected the presence of anti-HIV antibodies in clinical samples with a significantly enhanced signal-to-noise relative to the standard, recombinant protein-based chimeric antigen. In a recent report, an antigenic peptide cocktail comprised of synthetic peptides derived directly from gp120/V3-I (HIV-1 Indian isolate), gp41 (HIV-1), and gp36 (HIV-2), as well as the recombinant protein rp24 (HIV-1) was shown to also provide superior performance relative to the chimeric antigen [5]. This points to the possibility that expanding the current approach by developing multiple cocktails of PCC Agents, each directed against a distinct HIV epitope, would likely provide superior performance to that reported here. The strategy presented provides a promising approach for developing assays for detecting the immune response to other infectious agents, especially where challenges associated with the polyclonal nature of a humoral immune response can compromise assay sensitivity.

3.7 ACKNOWLEDGEMENTS

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3.8 REFERENCES

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