CPS 2024 RFP – FINAL PROJECT REPORT

Project Title:
An immunomagnetic separation method for concentrating and increasing the recovery efficiency of *Cyclospora*

Project Period:
January 1, 2025 – December 31, 2025 (extended to January 31, 2026)

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Objectives:

1. Develop immunomagnetic separation (Cyclo-IMS) directly from environmental samples using magnetic beads linked with antibodies against a principal oocyst wall protein (COWP2 and/or TA4 antigen-like surface protein) of *C. cayetanensis*.

2. Evaluate the value of Cyclo-IMS to concentrate oocysts of *C. cayetanensis* from environmental samples.

3. Evaluate improvements to DNA isolation by coupling Cyclo-IMS to extraction methods, previously optimized on *Eimeria* surrogates, for *C. cayetanensis* (including freeze-thaw, bead beating, and osmotic shock to lyse oocysts).

Funding for this project was provided partly through the CPS Campaign for Research.

FINAL REPORT

Summary of Findings and Recommendations

Our research provides strong evidence that new antibody-based tools can significantly improve the detection of *Cyclospora cayetanensis* in produce and environmental samples. We successfully identified and produced two highly specific antibodies that recognize proteins on the surface of *Cyclospora* oocysts. These antibodies demonstrated excellent sensitivity—able to detect very small amounts of oocyst material—and showed no cross-reactivity with closely related organisms typically found in agricultural settings. Importantly, the antibodies continued to perform well even after standard bleach treatments used during sample preparation, indicating they are compatible with routine produce testing workflows.

These results support the development of Cyclo-IMS, an immunomagnetic separation (IMS) method designed using these two antibodies to pull *Cyclospora* oocysts out of complex matrices such as leafy greens, herbs, berries, irrigation water, and wash water. This type of enrichment is a critical missing step in current testing: without it, oocysts remain too rare and too difficult to extract for reliable polymerase chain reaction (PCR) detection. By improving recovery before PCR, Cyclo-IMS could greatly enhance sensitivity, reduce false negatives, and provide a more dependable tool for outbreak investigations and routine monitoring.

Recommendation:
The produce industry should consider adopting antibody-based enrichment tools, once validated, as part of routine surveillance programs. Integrating Cyclo-IMS into existing testing workflows could:

- Improve detection of low-level contamination.
- Strengthen verification of agricultural water quality.
- Support compliance with food safety regulations and emerging guidance.
- Enhance outbreak response by enabling faster and more accurate source-tracking.

As development continues, we will ensure that the final method is practical, scalable, and aligned with operational needs throughout the fresh-produce supply chain.

Abstract

Cyclospora cayetanensis is an important foodborne pathogen associated with recurrent outbreaks linked to fresh produce. Current detection methods are hindered by low recovery efficiencies and polymerase chain reaction (PCR) inhibition caused by complex environmental matrices. To address these limitations, we proposed the development of Cyclo-IMS, an antibody-based immunomagnetic separation platform targeting the oocyst wall proteins COWP and TA4. Recombinant antigens were expressed and purified in *Escherichia coli*, and high-titer rabbit polyclonal antibodies were generated and validated using an enzyme-linked immunosorbent assay (ELISA) and dot blot assays. The antibodies demonstrated strong specificity for *Cyclospora* with minimal cross-reactivity. We are currently coupling these antibodies to magnetic beads to evaluate oocyst-capture efficiency across diverse sample matrices. Based on these preliminary findings, we anticipate that Cyclo-IMS will provide an efficient and scalable approach to improving *Cyclospora* detection in food safety and environmental monitoring applications.

Background

Cyclospora cayetanensis is an emerging coccidian parasite responsible for recurrent foodborne illness outbreaks in the United States and globally. Every year, diarrheal diseases account for approximately 1.7 billion cases worldwide, and foodborne illness alone costs the U.S. economy over $15.6 billion annually. The Centers for Disease Control and Prevention estimate that one in six Americans contracts a foodborne pathogen each year, resulting in roughly 3,000 deaths. Among the more than 30 pathogens linked to diarrheal disease, *C. cayetanensis* is a particularly challenging enteric parasite due to its environmental persistence, low infectious dose, and difficulty of detection in fresh produce and environmental samples.

Although more than 20 *Cyclospora* species have been described, only three—including *C. cayetanensis*—infect humans. In recent years, domestic outbreaks in the United States have risen sharply, with 1,241 laboratory-confirmed cases in 2020, and over 2,300 cases annually in 2018 and 2019. Outbreak investigations repeatedly implicate fresh herbs, berries, and leafy greens irrigated or washed with contaminated water, highlighting the vulnerability of produce supply chains. Contamination can occur at extremely low levels; ingestion of only a few oocysts can lead to infection.

Despite the pathogen's significance, detection remains notably difficult. *Cyclospora* oocysts occur at low abundance in environmental samples and food matrices, and standard workflows—such as flotation followed by qPCR—are hampered by PCR inhibitors (e.g., soil particulates, microbes, plant material) found in water, soil, and produce. These inhibitors can reduce quantitative detection by several orders of magnitude. Additionally, due to rigid oocysts wall of these parasite and presence of inhibitors, deoxyribonucleic acid (DNA) isolation commonly results in <30% DNA recovery even when using purified oocyst preparations. Currently, there are no internationally accepted testing standards for *C. cayetanensis* in environmental matrices, nor are there commercial antibodies available for antibody-based detection.

A central barrier to sensitive *Cyclospora* detection is the absence of a robust, selective method to separate oocysts from complex matrices without significant oocyst loss. Immunomagnetic separation (IMS) has proven highly effective for concentrating other protozoan pathogens—including *Giardia* and *Cryptosporidium*—with reported recoveries exceeding 70–90%, even in turbid water. Commercial IMS kits employ antibodies targeting cysteine-rich oocyst wall proteins, enabling selective immunocapture with magnetic beads. However, no such system exists for *Cyclospora*, despite the clear need for an enrichment method that improves recovery prior to PCR-based detection.

Comparative genomic studies have identified promising antigenic targets for *Cyclospora* IMS development, including the oocyst wall protein COWP (cyc_01725) and the TA4 antigen-like surface protein. These antigens exhibit structural similarities to well-characterized cysteine-rich oocyst wall proteins in *Eimeria* and *Cryptosporidium*, which are successfully used in commercial IMS kits. Recent studies—such as DNA aptamer-based staining—demonstrate strong binding affinity to COWP and TA4, supporting their value as immunocapture targets.

The complexity of environmental sample matrices and the increasing frequency of cyclosporiasis outbreaks emphasize the urgent need for a sensitive, rapid, and cost-effective enrichment method to detect *Cyclospora*. An antibody-based IMS workflow—Cyclo-IMS—enables improved recovery efficiency, reducing matrix inhibition, and enhancing DNA extraction performance. The development of such tools is essential to strengthen outbreak response, environmental surveillance, and produce safety practices across the agricultural sector.

Research Methods and Results

1) Antigen Design, Synthesis, and Cloning

Targets. Two *C. cayetanensis* oocyst surface antigens were selected: (i) the cysteine-rich oocyst wall protein (COWP; cyc_01725) and (ii) a TA4 antigen-like surface protein (National Center for Biotechnology Information RefSeq XP_026191783.1), both supported by comparative genomics and aptamer-based staining data indicating strong and selective binding to *Cyclospora* oocyst walls.

Gene synthesis and vector construction. Codon-harmonized and native open reading frames for COWP and TA4 were synthesized (GenScript) with BamHI/EcoRI ends and inserted into pTrcHisA for high-level expression in *E. coli*. Ligations were transformed first into DH5α for cloning, then into BL21 for expression. Constructs were confirmed by restriction digest, agarose gel excision, QIAquick Gel Extraction, and Sanger sequencing to verify correct reading frames.

2) Recombinant Expression and Protein Purification

Expression. BL21 transformants were grown in LB + ampicillin (100 µg/mL) at 37°C to OD_{600} = 0.5, then induced with 1 mM Isopropyl-D-1-thiogalactopyranoside (IPTG) for 4 h.

Verification. Lysates were fractionated (native vs denaturing/urea-soluble) and analyzed by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by either visualization of protein gel migration with Coomassie Blue or transblotting to Immobilon membrane (Millipore-Sigma, Burlington, MA) followed by immunoprobing with mouse anti-His sera (1:5000 dilution, Invitrogen), biotinylated anti-mouse IgG (1:1000 dilution, Sigma Aldrich, St. Louis MO) and alkaline-phosphatase-labeled avidin (1:25,000 dilution, Sigma Aldrich), followed by incubation with 5-Bromo-4-Chloro-3-Indolyl-Phosphate/NitroBlue Tetrazolium (NBT-BCIP - Thermo-Scientific, Rockford IL). COWP appeared at ~66kDa, whereas A4 appeared at ~30–33 kDa, predominantly in the denaturing (urea-soluble) fraction, consistent with cysteine-rich wall proteins (**Figures 1** and **2**).

Purification. Proteins were purified by Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography; when yield was limiting, Coomassie-stained gel bands were excised to recover antigen for immunization and assay development (**Figures 3** and **4**).

3) Rabbit Polyclonal Antibody Production and Purification

Immunization schedule. Two New Zealand White rabbits were immunized with 100 µg Ni-NTA-purified COWP or TA4, using complete Freund's Adjuvant (CFA) for priming and incomplete Freund's adjuvant (ICFA) for boosts at 3, 6, and 10 weeks; bleeds were collected 1 and 3 weeks after the final boost (**Figure 5**).

Protein A purification. Sera (1 mL) were diluted (1:10) in 20 mM sodium phosphate, 0.45 µm filtered, and passed three times over a HiTrap Protein A HP column equilibrated with 10 mL buffer. Antibodies were eluted with 2.5 mL 0.1 M citrate, pH 3.5, immediately neutralized with 200 µL 0.1 M Trizma, pH 9.0, desalted on PD-10 pre-equilibrated with 25 mL Phosphate-buffered saline (PBS), and eluted in 3.5 mL PBS; concentration was determined by Qubit.

4) Antibody Characterization: Indirect ELISA

Plate coating. Recombinant COWP was serially diluted (1 µg/mL–0.001 µg/mL) in carbonate/bicarbonate coating buffer (14 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and 90 µL/well was incubated overnight at 4°C.

Blocking and probing. Plates were washed (3×, 300 µL Tris-Buffered Saline with Tween-20 (TBST)) and blocked with SuperBlock (300 µL/well). Purified rabbit anti-COWP was added (1:100–1:2000, 100 µL/well, 1 h, room temperature), followed by HRP-conjugated goat anti-rabbit Immunoglobulin G (IgG) (1:1000, 100 µL, 1 h, RT). Detection used 1-Step Ultra TMB (100 µL, 15 min); reactions were stopped and A_{450} recorded from three technical replicates with blank subtraction.

COWP Antibody ELISA Sensitivity. To assess the sensitivity of the COWP antibody, we performed an indirect ELISA assay. Serial dilutions of recombinant *Cyclospora* COWP (1 µg/mL to 0.001 µg/mL) were coated onto 96-well plates and probed with rabbit COWP primary antibody at four dilutions (1:100, 1:500, 1:1 000, 1:2 000). All primary antibody dilutions produced a saturation plateau when the coating concentration reached 1 µg/mL, indicating maximal surface coverage. The lowest antigen concentration that yielded a signal significantly above background was 0.005 µg/mL for every dilution. These data suggest that a primary antibody concentration of 1:500 could be optimal for subsequent immunomagnetic separation experiments, providing sufficient sensitivity while minimizing reagent use (**Figure 6**).

5) Antibody Specificity: Dot Blot on Oocyst Lysates

Oocyst preparation. ~8.4×10^4 sucrose-floated oocysts were pelleted (16,000×g, 4 min), washed (3× PBS), and bead-beaten (TissueLyzer II, 5 min, 30 Hz) to prepare lysates.

Membrane prep and probing. Polyvinylidene difluoride (PVDF) was activated (100% methanol, 1 min), rinsed (PBS, 10 min), and 10 µL of recombinant COWP or lysate was applied. Membranes were blocked (5% non-fat milk in TBST, 1 h, RT), probed with rabbit anti-COWP (1:500, 1 h, RT), then with Alexa Fluor 488 goat anti-rabbit (1:1000, 1 h, RT), washed (3×, 10 min), and imaged (Bio-Rad ChemiDoc MP).

COWP antibody specificity. We performed a dot-blot assay to assess the specificity of the rabbit COWP antibody. Fluorescent signal was detected on the PVDF membrane for the recombinant *Cyclospora* COWP (0.1 ug/mL) and for lysates of *Cyclospora* oocysts, indicating that the antibody recognized the native COWP present in the oocyst wall (**Figure 7**). Bleaching is frequently used during oocyst isolation to remove contaminant bacteria but can denature surface antigens. The intensity of the signal from bleached oocysts was comparable to that from unbleached oocysts, indicating that the bleaching procedure did not appreciably remove or denature the surface exposed COWP epitope. In contrast, lysates from the three *Eimeria* species examined (*E. tenella*, *E. acervulina*, and *E. maxima*) showed no detectable fluorescence above background. These findings demonstrate that the anti COWP antibody selectively binds *Cyclospora* COWP while tolerating the bleach treatment.

6) Future directions

Building on the successful generation of highly specific anti-COWP and anti-TA4 antibodies and the preliminary establishment of the Cyclo-IMS platform, future work will focus on completing bead–antibody optimization, expanding recovery profiling across diverse food and environmental matrices, and integrating the system into a fully validated detection pipeline. Upcoming efforts will refine coupling chemistry to enhance bead stability, maximize antibody orientation, and further reduce non-target binding under high-turbidity conditions. We will conduct systematic matrix trials using produce washes, irrigation waters, and concentrated environmental samples to quantify recovery efficiencies at low oocyst inputs and operational volumes relevant to regulatory testing. Parallel optimization of oocyst elution conditions and downstream DNA extraction will continue, with an emphasis on evaluating freeze–thaw, mechanical lysis, and chemical workflows to ensure maximal template yield following IMS enrichment. Finally, qPCR benchmarking using the BAM 19b assay will be expanded to determine limits of detection, reproducibility, and performance relative to current gold-standard methods. Together,

these next steps will enable full analytical validation of Cyclo-IMS and position the platform for deployment in food safety laboratories and environmental monitoring programs.

Outcomes and Accomplishments

The project achieved several key milestones that collectively establish a strong foundation for developing an immunomagnetic separation (IMS) platform for *C. cayetanensis*. Two high-confidence oocyst surface antigens, COWP and TA4, were successfully designed, synthesized, cloned, and expressed in *E. coli*, with construct integrity confirmed through molecular validation. Recombinant proteins were purified under denaturing conditions, consistent with the biochemical properties of cysteine-rich wall proteins, and were used to generate high-titer rabbit polyclonal antibodies through a multi-stage immunization schedule. Purified antibodies demonstrated robust sensitivity by ELISA, detecting recombinant antigen at concentrations as low as 0.005 μg/mL across multiple dilutions. Specificity assays further confirmed selective recognition of native *Cyclospora* COWP in oocyst lysates, with no cross-reactivity detected against three *Eimeria* species. Notably, the antibody maintained strong binding to both bleached and unbleached oocysts, underscoring its suitability for use in standard oocyst-purification workflows. Collectively, these accomplishments validate the antigen design strategy, confirm the generation of sensitive and highly specific antibodies, and position the project for the next phase: coupling antibodies to magnetic beads to evaluate oocyst-capture efficiency across food and environmental matrices.

APPENDICES

Publications and Presentations

None

Budget Summary

The total project budget was $55,433, which included $15,000 allocated for personnel. However, we submitted a request to CPS for a line-item shift to repurpose the personnel funds for reagent purchases, and this adjustment was approved. To date, we have expended $51,356.26, leaving a remaining balance of $4,076.74 that we plan to use to support participation in the CPS Research Symposium in 2026.

Figures

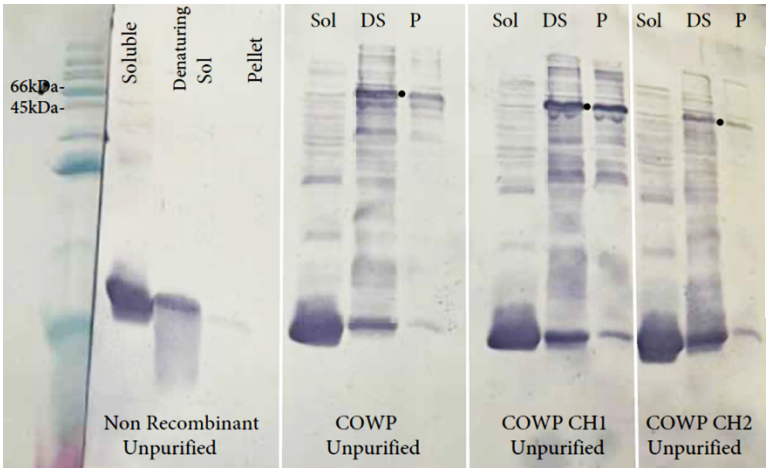

Figure 1. Expression of COWP. There were 2 codon harmonized clones (CH1 & CH2) synthesized and cloned in addition to CycNF1 COWP sequence. The expression level of CH2 was considerably lower than COWP and COWP CH1.

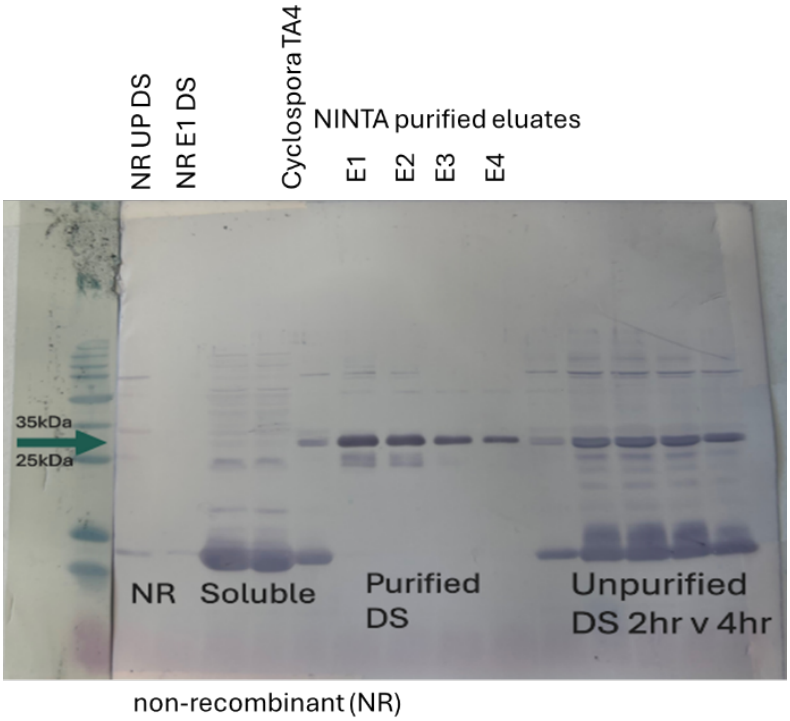

Figure 2. Expression of T4A. Anti-His probed Western Blot showed protein around 30kDa that is not present in NR or soluble.

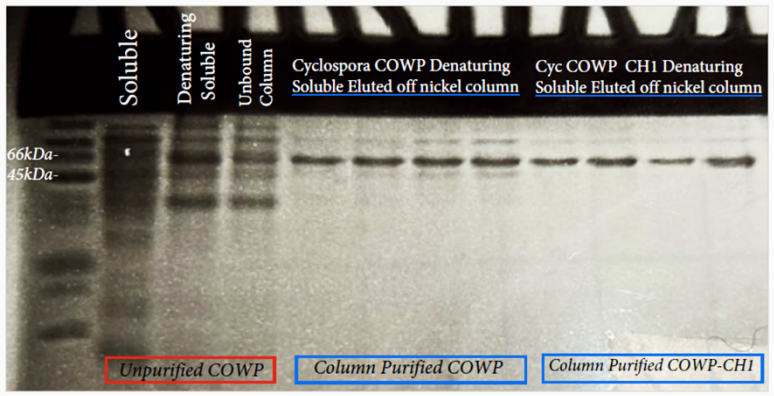

Figure 3. Purification of COWP. The unpurified denaturing soluble protein was excised from SDS-Page gel, Coomassie stained, and destained in water.

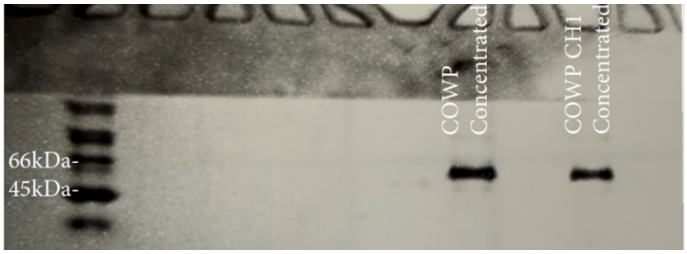

Figure 4. 2 mg of denaturing soluble concentrated protein of COWP and COWP CH1 was pooled for antibody production.

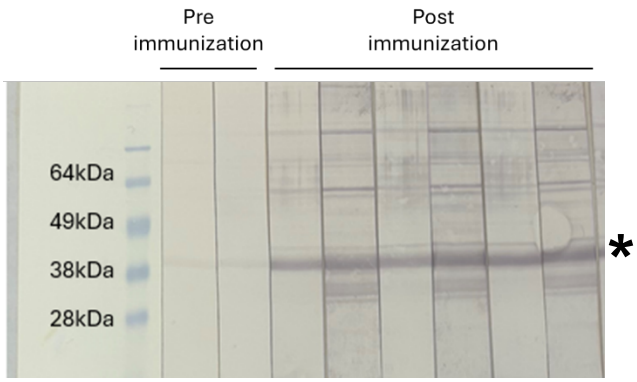

Figure 5. Recognition of the rabbit *Cyclospora* TA4 sera against the recombinant protein. * indicates the T4A band.

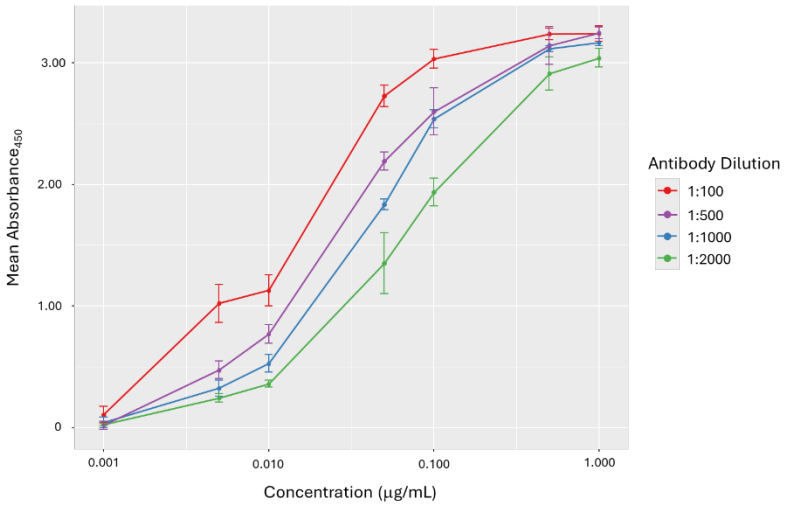

Figure 6. COWP antibody ELISA sensitivity. Serial dilutions of recombinant *Cyclospora* COWP (1-0.001 ug/mL) were coated onto microplate wells and blocked with SuperBlock. Absorbance was measured at 450 nm. Absorbance values were corrected by subtracting the blank, averaged from three technical replicates, and error bars represent the standard error of the mean.

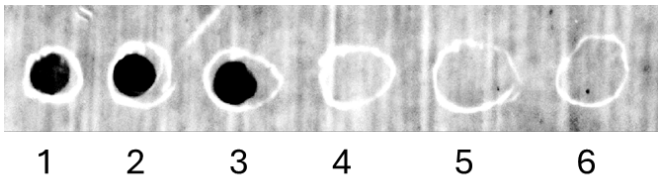

Figure 7. COWP antibody specificity. Lysates from oocysts were dotted onto a PVDF membrane: (1) 0.1 ug recombinant *Cyclospora* COWP; (2) unbleached *Cyclospora* oocysts; (3) bleached *Cyclospora* oocysts; (4) *E. tenella*; (5) *E. acervulina*; and (6) *E. maxima*. After blocking, the membrane was probed with rabbit COWP primary antibody (1 : 500) and subsequently with Alexa-Fluor 488-conjugated goat rabbit secondary antibody (1 : 1000). Fluorescence was captured on a Bio-Rad ChemiDoc MP imager.