



EDGEWOOD CHEMICAL BIOLOGICAL CENTER

U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-CR-171

DEVELOPMENT AND TESTING OF ENHANCED AFFINITY REAGENTS FOR USE IN ENVIRONMENTAL DETECTION ASSAYS

Jeff D. Ballin

EXCET, INC.
Springfield, VA 22150-2519

Stacey Broomall

RESEARCH AND TECHNOLOGY DIRECTORATE

June 2018

Approved for public release: distribution unlimited.

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 h per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) XX-06-2018		2. REPORT TYPE Final		3. DATES COVERED (From - To) Mar 2010 – Dec 2011	
4. TITLE AND SUBTITLE Development and Testing of Enhanced Affinity Reagents for Use in Environmental Detection Assays				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Ballin, Jeff D. (Excet, Inc.) and Broomall, Stacey (ECBC)				5d. PROJECT NUMBER CB3646	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Director, ECBC, ATTN: RDCB-DRB-M, APG, MD 21010-5424 Excet, Inc.; 6225 Brandon Avenue, Suite 360, Springfield, VA 22150-2519				8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-CR-171	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Defense Threat Reduction Agency, 8725 John J. Kingman Road, MSC 6201, Fort Belvoir, VA 22060-6201				10. SPONSOR/MONITOR'S ACRONYM(S) : DTRA	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release: distribution unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: Current affinity reagent development methodologies generally rely on costly and slow antibody production that is based on animal inoculations with attenuated, inactivated, or surrogate biothreat agents. Recent literature has demonstrated that the de novo computer design of recombinant affinity candidates, followed by affinity maturation, can bind to viral targets. In this study, analogous methods were used to generate recombinant binders to a surface antigen of the <i>Vaccinia</i> virus. Several lead candidates were identified using two different approaches; however, these were later abandoned due to either off-target affinity or their absence in affinity maturation selection pools. In addition, in collaboration with AxioMx, Inc. (Branford, CT), a phage-based library and the methods needed for isolation and production of thermostable antibodies were developed. These techniques leveraged the Defense Advanced Research Projects Agency (Arlington, VA)-initiated Antibody Thermostability Program Immunoglobulin G (IgG) framework to be a scaffold of the antibody production pipeline. The library was then transferred to the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) to enable in-house production of thermostable antibodies against targets of interest.					
15. SUBJECT TERMS Surface antigen Phage Thermostability de novo computer design Immunoglobulin G (IgG) Affinity reagent Antibody <i>Vaccinia</i> virus					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Renu B. Rastogi
U	U	U	UU	76	19b. TELEPHONE NUMBER (include area code) (410) 436-7545

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Blank

PREFACE

The work described in this report was authorized under project no. CB3646. This work was started in March 2010 and completed in December 2011.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

Blank

EXECUTIVE SUMMARY

The Defense Threat Reduction Agency (DTRA; Fort Belvoir, VA) Enhanced Affinity Reagents Program was directed at the adaption and improvement of existing affinity reagent technologies to enhance operational characteristics such as thermal stability and binding affinity. The U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD) served as an independent testing laboratory for this program, provided specific technical support on immune reagents, and assisted in defining specific government-supplied antibody–antigen pairs. Additional efforts will (1) guide the establishment of a highly extensible library that will allow for the conversion of existing and new antibodies into thermostable variants, (2) adapt and evaluate use of nanoparticle tags to supplement or replace existing gold nanoparticles in immunoaffinity assays, and (3) provide biophysical characterization of existing affinity reagents that are relevant to DTRA.

Blank

CONTENTS

	PREFACE	iii
	EXECUTIVE SUMMARY	v
1.	INTRODUCTION	1
2.	TASK 1: AFFINITY MATURATION, PRODUCTION, AND VALIDATION OF ROSETTA-DESIGNED AFFINITY REAGENT CANDIDATES	2
2.1	Validation of Binding Performance for Selected Rosetta-Designed Sequences.....	2
2.2	Candidate Cloning, Protein Production, and Characterization	4
2.2.1	Cloning of SPxx Sequences from Selection Vector to Protein- Expression Vector	4
2.2.2	Protein Expression, Production, and Purification	6
2.2.3	Purity Assessed Using Experion Lab-on-a-Chip	7
2.2.4	Measurement of Protein Concentration	8
2.2.5	Solubility Analysis by Dynamic Light Scattering (DLS)	8
2.2.6	Thermal Stability Assessment by Differential Scanning Calorimetry (DSC).....	9
2.3	Quantification of Equilibrium Binding Affinity to Recombinant Antigen.....	9
2.4	Characterization of Binding Affinity to Intact <i>Vaccinia</i> virus.....	9
2.5	Candidate Characterization for Inclusion into the Defense Biological Products Assurance Office Antibody Repository	9
2.6	Redesign of Ligand Scaffolding for Binding to L1R.....	9
2.7	Two Candidates Were Identified from Initial Screens	12
3.	TASK 2: CREATION OF A THERMOSTABLE IgG SCAFFOLD- BASED SINGLE-CHAIN VARIABLE FRAGMENT (scFv) LIBRARY	14
3.1	Adapt AnaptysBio Thermostable IgG Backbone to Build a scFv → IgG Splicing Pair.....	15
3.2	Develop an scFv Library with a Diversity of at Least 1×10^{10} Clones	17
4.	TASK 3: CRYSTALLOGRAPHY DATA COMPILATION	22
4.1	Crystal Structure of RiVax Complex with AxioMx IgX 2691 at 2.7 Å	22
4.1.1	Summary of RiVax–IgX 2691 Fab Structure	22
4.1.2	IgX 2691 Fab Preparation.....	25
4.1.3	RiVax–IgX 2691 Fab Size-Exclusion Chromatography (SEC).....	25
4.1.4	RiVax–IgX 2691 Fab Crystallization	26
4.1.5	RiVax–IgX 2691 Fab Data Collection and Processing.....	26
4.1.6	RiVax–IgX 2691 Fab Structure Phasing, Model Building, and Refinement.....	27

4.2	Crystal Structure of the Fab of Anaptys 7d11 at 2.64 Å.....	28
4.2.1	Summary of AnaptysBio 7d11 Fab Structure.....	28
4.2.2	AnaptysBio 7d11 Fab Preparation.....	30
4.2.3	AnaptysBio 7d11 SEC.....	30
4.2.4	AnaptysBio 7d11 Fab Crystallization.....	30
4.2.5	AnaptysBio 7d11 Fab Data Collection and Processing.....	31
4.2.6	AnaptysBio 7d11 Fab Phasing, Model Building, and Refinement.....	32
4.3	Crystal Structure of the AnaptysBio 7d11 Graft Fab in Complex with L1R at 2.15 Å.....	33
4.3.1	Summary of L1R–AnaptysBio 7d11 Fab Structure.....	33
4.3.2	AnaptysBio 7d11 Fab Preparation.....	34
4.3.3	L1R–AnaptysBio 7d11 Fab Complex Formation and SEC.....	34
4.3.4	L1R–AnaptysBio 7d11 Fab Crystallization.....	36
4.3.5	L1R–AnaptysBio 7d11 Fab Data Collection and Processing.....	36
4.3.6	L1R–AnaptysBio 7d11 Fab Structure Phasing, Model Building, and Refinement.....	37
5.	TASK 4: USING UCNPS TO CREATE MORE SENSITIVE AND MULTIPLEX-CAPABLE LFIS.	38
5.1	Immobilize Antibodies on UCNPs and Assay Affinity.....	40
5.2	Estimate LOD for IgG–Nanoparticle Adducts in LFI-like Sandwich Assays.....	42
5.2.1	UCNP-Only LOD on LFI Strips.....	42
5.2.2	ELISA Using UCNP Emission Detection in 96-Well Format.....	44
5.2.3	Detection of Antibody Conjugates in LFI Format.....	45
5.3	Construction of an LFI UCNP Detection System.....	46
5.4	Construction of Anti-Ricin LFIs Using Gold or UCNP Reporters.....	48
5.5	Quantification of Anti-Ricin Gold or UCNP LFI Response.....	48
6.	METHODS.....	51
6.1	Reagents.....	51
6.2	Antibody-Reporter Conjugation.....	51
6.3	ELISA Studies.....	51
6.4	LFI Construction.....	52
6.5	Gold LFI Scans.....	52
6.6	Prototype Construction.....	52
6.7	Nanocrystal LFI Scans.....	53
	LITERATURE REFERENCES.....	55
	ACRONYMS AND ABBREVIATIONS.....	59

FIGURES

1.	Rosetta modeling software (RosettaCommons.org) candidate sequences, as excised from the pETCON vector (provided by the Baker Laboratory) and expressed from pET-21b.....	3
2.	Cloning strategy to transfer SPxx constructs from pETCON to pET-21b.....	5
3.	Overexpression of first-generation binding candidates	6
4.	Validation of SP4 and SP16 purity by denaturing polyacrylamide gel electrophoresis after Ni ²⁺ -affinity purification and cation-exchange chromatography that was visualized using (A) Coomassie stain or (B) enhanced chemiluminescence probing with anti-His ₆ antibody.....	7
5.	Experion Lab-on-a-Chip analysis of first-generation candidates	7
6.	Scaffold strategy derived from mini-proteins based on scorpion toxins	10
7.	Computational strategies to design L1 affinity reagents.....	11
8.	Rosetta candidate selection protocol.....	12
9.	Heat map of enrichment values for Rosetta candidates that bind to L1R and negative-control proteins, which are listed across the top as bait.....	13
10.	Structural models of the two Rosetta candidates that advanced to affinity maturation, which are indicated by arrows in Figure 9	14
11.	Axiomx affinity maturation of phage-displayed scFv sequences via emulsion screening.....	15
12.	An scFv affinity enhancement from (A) the starting scFv with K _D = 7.6 nM to (B) the lead scFv after affinity maturation with K _D = 0.96 nM, as determined using the PerkinElmer AlphaScreen technology	16
13.	IgG affinity enhancement from (A) the original IgG with K _D = 0.128 nM to (B) the lead IgG after affinity maturation with K _D = 0.076 – 0.092 nM across several different lots of material, as determined using the PerkinElmer AlphaScreen technology	16
14.	Library construction strategy	17
15.	Library construction by modified Kunkel mutagenesis.....	18
16.	scFv mutagenesis oligonucleotides.....	19
17.	Example of library manufacturing workflow.	20
18.	Library validation.....	21
19.	Calculated phage library diversity	21
20.	Ribbon diagram of the Fab–RiVax complex	23
21.	Superimposition of the Fab–RiVax complex and PDB ID: 4KUC showing both Fabs (A) binding the same region of the antigen (coloring scheme as shown in panel B)	24
22.	Refined 2F _o – F _c electron density of RiVax–IgX 2691 Fab	24
23.	Axiomx IgX 2691 and anti-RiVax Fab preparation	25
24.	RiVax–IgX 2691 Fab co-crystals.....	26
25.	CDR superimposition of Fab 7d11 (PDB ID: 2I9L) versus AnaptysBio 7d11	29
26.	Refined 2F _o – F _c electron density of Anaptys 7d11 Fab surrounding the six CDRs (blue), which are contoured at 1.0 σ.....	29

27.	AnaptysBio 7d11 Fab size-exclusion chromatogram showing (left) peak fractions of the 7d11 Fabs that were pooled and concentrated, and (right) SDS-PAGE gel confirming that the fractions contain high-purity Fab	30
28.	Image of optimized AnaptysBio 7d11 Fab crystal.	31
29.	Ribbon diagram showing superimposition of the AnaptysBio 7d11–L1R complex and PDB ID: 2I9L	33
30.	Refined $2 F_o - F_c $ electron density of L1R–AnaptysBio 7d11 Fab structure	34
31.	Purification of the L1R-anti–L1R Fab complex	35
32.	Image of initial positive result for L1R–AnaptysBio 7d11 Fab complex from condition G6 of the Hampton Research Index screen.....	36
33.	The visible emissions of YF ₃ nanocrystals, which were stimulated by infrared excitation at ~980 nm, are tuned by doping with erbium (Er), thulium (Tm), or both as the fraction of Yb ³⁺ increases from 10–90% (2)	39
34.	LFI process.....	39
35.	Affinity for UCNP-coupled antibodies for antigen or nonspecific target.....	41
36.	Comparison of affinity for titrating UCNP–antibody-coupling ratios.....	42
37.	Time-dependent emission from UCNPs at (A) 625 ng/μL and (B) 40 pg/μL	43
38.	UCNP titration curve from LFI strips after normalizing for additional gain on the low end (lowest three data points)	44
39.	UCNP distribution on LFI membrane coated with 625 ng/μL UCNP per stripe.....	44
40.	Visualization of antibody-coupled UCNPs.....	45
41.	Validation of LFI production process	46
42.	Optical (A) schematic and (B) breadboard implementation of the UCNP detection system that highlights the primary components.....	47
43.	Proportionality of UCNP emission to laser intensity for UCNPs striped onto an LFI membrane at 200 μg/mm	48
44.	Performance of nanocrystals relative to gold anti-ricin LFIs (black and orange filled circles, respectively) that were constructed with identical reagents and materials, and tested with recombinant ricin A chain protein	49
45.	Signal intensity of UCNP LFI detection of 20 pg analyte relative to background observed for buffer alone	50

TABLES

1.	Candidate-Predicted Molecular Weight (MW) and Isoelectric Point (pI).....	8
2.	Replicate DLS Characterization of SPxx Candidate Solubility.....	8
3.	Affinity Reagent Thermostability	16
4.	RiVax–IgX 2691 Fab Crystal Data Collection Details.....	27
5.	RiVax–IgX 2691 Fab Crystal Data Collection Statistics.....	27
6.	RiVax–IgX 2691 Fab Structure Refinement and Model Statistics	28
7.	AnaptysBio 7d11 Fab Data Collection Details	31
8.	AnaptysBio 7d11 Fab Data Collection Statistics.....	32
9.	AnaptysBio 7d11 Fab Refinement and Model Statistics	32
10.	L1R–AnaptysBio 7d11 Fab Data Collection Details.....	37
11.	L1R–AnaptysBio 7d11 Fab Data Collection Statistics.....	37
12.	L1R–AnaptysBio 7d11 Fab Structure Refinement and Model Statistics	38
13.	Integrated Peak Areas of Test Line Intensity for Gold and UCNP-based LFIs	50

Blank

DEVELOPMENT AND TESTING OF ENHANCED AFFINITY REAGENTS FOR USE IN ENVIRONMENTAL DETECTION ASSAYS

1. INTRODUCTION

Current affinity reagent development methodologies generally rely on costly and slow antibody production that is based on animal inoculations using an attenuated, inactivated, or surrogate biothreat agent. Recent literature has shown that the de novo computer design of recombinant affinity candidates, followed by affinity maturation, demonstrates binding to viral targets (1). In this study, analogous methods were used to generate recombinant binders to a surface antigen of the *Vaccinia* virus. Several lead candidates were identified using two different approaches; however, these were later abandoned due to either off-target affinity or their absence in affinity maturation selection pools. Antibody fragments (Fabs) alone or in complex with their cognate antigen were crystallized for structure determination. Three structures were solved: the anti-ricin AxioMx IgX 2691 (AxioMx, Inc.; Branford, CT) bound to recombinant ricin vaccine (RiVax) at 2.7 Å, the anti-*Vaccinia* AnaptysBio 7d11 graft Fab (AnaptysBio, Inc.; San Diego, CA) alone at 2.64 Å, and the anti-*Vaccinia* AnaptysBio 7d11 graft bound to recombinant L1R at 2.15 Å.

The development of a thermally stable antibody library would provide the Department of Defense with significant savings for the production of novel, thermostable antibodies that can operate without dependence on a cold-chain infrastructure. In collaboration with AxioMx, Inc., a phage-based library and the methods needed for isolation and production of thermostable antibodies were developed. These techniques leveraged the Defense Advanced Research Projects Agency (DARPA; Arlington, VA)-initiated Antibody Thermostability Program Immunoglobulin G (IgG) framework to be a scaffold of the antibody production pipeline. The library was then transferred to the U.S. Army Edgewood Chemical Biological Center (ECBC; Aberdeen Proving Ground, MD) to enable in-house production of thermostable antibodies against targets of interest.

Using conventional gold nanoparticle or upconverting nanoparticle (UCNP) reporters (2) and otherwise identical antibodies and construction, lateral flow immunoassays (LFIs) were manufactured to detect the ricin A chain toxin surrogate. Estimated limits of detection (LODs) were quantified relative to a threshold that was determined based on replicate measurements of analyte-free buffer runs for both gold- and UCNP-based LFIs. UCNP LFIs demonstrated a 4- to 10-fold increase in sensitivity to analyte relative to analogous gold LFI detection limits, which indicated that UCNPs offer comparable, if not better, sensitivity for a common target. Because UCNPs are amenable to the same coupling chemistries and manufacturing handling processes that are already in widespread use for the production of gold LFIs, the use of UCNPs can offer a low-cost improvement of immunoassay performance that can be easily integrated into existing production streams.

2. TASK 1: AFFINITY MATURATION, PRODUCTION, AND VALIDATION OF ROSETTA-DESIGNED AFFINITY REAGENT CANDIDATES

This task was aimed at validating the binding performance of selected Rosetta-designed sequences formulated to bind the recombinant *Vaccinia*-derived protein target, L1R. Candidate cloning, protein production, characterization, expression, purification, and analysis were performed.

2.1 Validation of Binding Performance for Selected Rosetta-Designed Sequences

Results of the initial work executed by the staff at the University of Washington School of Medicine (Seattle, WA) David Baker Laboratory were reported in fiscal year (FY) 2014. To expand on that work, three candidates were selected through yeast display screens of a larger pool of “SPxx” designs using biotinylated recombinant *Vaccinia*-derived protein target L1R, as bait-identified by the Rosetta modeling software (Figure 1). Although the binding target was a structurally known tertiary structural epitope on L1R, affinity maturation efforts by the Baker group found that these three candidates preferentially bound the streptavidin reporter instead (Section 2.7). The ECBC characterization pipeline included purity (Experion Lab-on-a-chip [Bio-Rad Laboratories, Inc.; Hercules, CA]), concentration (NanoDrop system [Thermo Fisher Scientific, Inc.; Waltham, MA]), and behavior in solution (dynamic light scattering). This pipeline was used to discover that purified SP4 and SP16 proteins aggregate and that SP1 protein expression was extremely low across multiple production and purification strategies. Further work with the SPxx series was suspended in favor of an alternative design and screening strategy using new binders that were based on scorpion toxin motifs (Section 2.7). No binders were able to meet the subsequent criteria for enrichment and optimization screening.



Figure 1. Rosetta modeling software (RosettaCommons.org) candidate sequences, as excised from the pETCON vector (provided by the Baker Laboratory) and expressed from pET-21b. (A) DNA sequences, and (B) expressed protein sequence that includes residues as originally designed using the Rosetta software (black font), the C-terminal 6-histidine tag (underline blue) encoded by the pET-21b after insertion via NdeI (underline green), and the XhoI (underline brown) restriction sites. The first two bases (cc) of the NdeI site are upstream of the start codon ATG.

2.2 Candidate Cloning, Protein Production, and Characterization

The three candidates identified in FY14, SP1, SP4, and SP16, were cloned into a protein-expression system, produced and isolated, and characterized as the next-generation affinity designs being developed by the Baker Laboratory. The first-generation candidates were produced to allow head-to-head comparison of biophysical characteristics and binding behavior to later-generation designs that were identified downstream. One design, SP1, showed poor expression in bacteria, whereas SP4 and SP16, although they were produced in high yield and purity, were prone to aggregation under standard buffer conditions.

2.2.1 Cloning of SPxx Sequences from Selection Vector to Protein-Expression Vector

The Rosetta candidates were encoded between the NdeI and XhoI restriction sites of the pETCON vector that was used for the yeast display, thus providing a direct route for cloning these constructs into the pET-21b protein-expression vector (Figure 2A). The SP1, SP4, and SP16 plasmids, along with the commercially available pET-21b vector, were produced and purified using standard approaches. Double digestion of plasmids and gel purification were used to liberate the desired inserts and linearized vector (Figure 2B). Ligation and transformation of SPxx-pET-21b into DH5 α competent cells were successfully generated and archived as glycerol stocks.

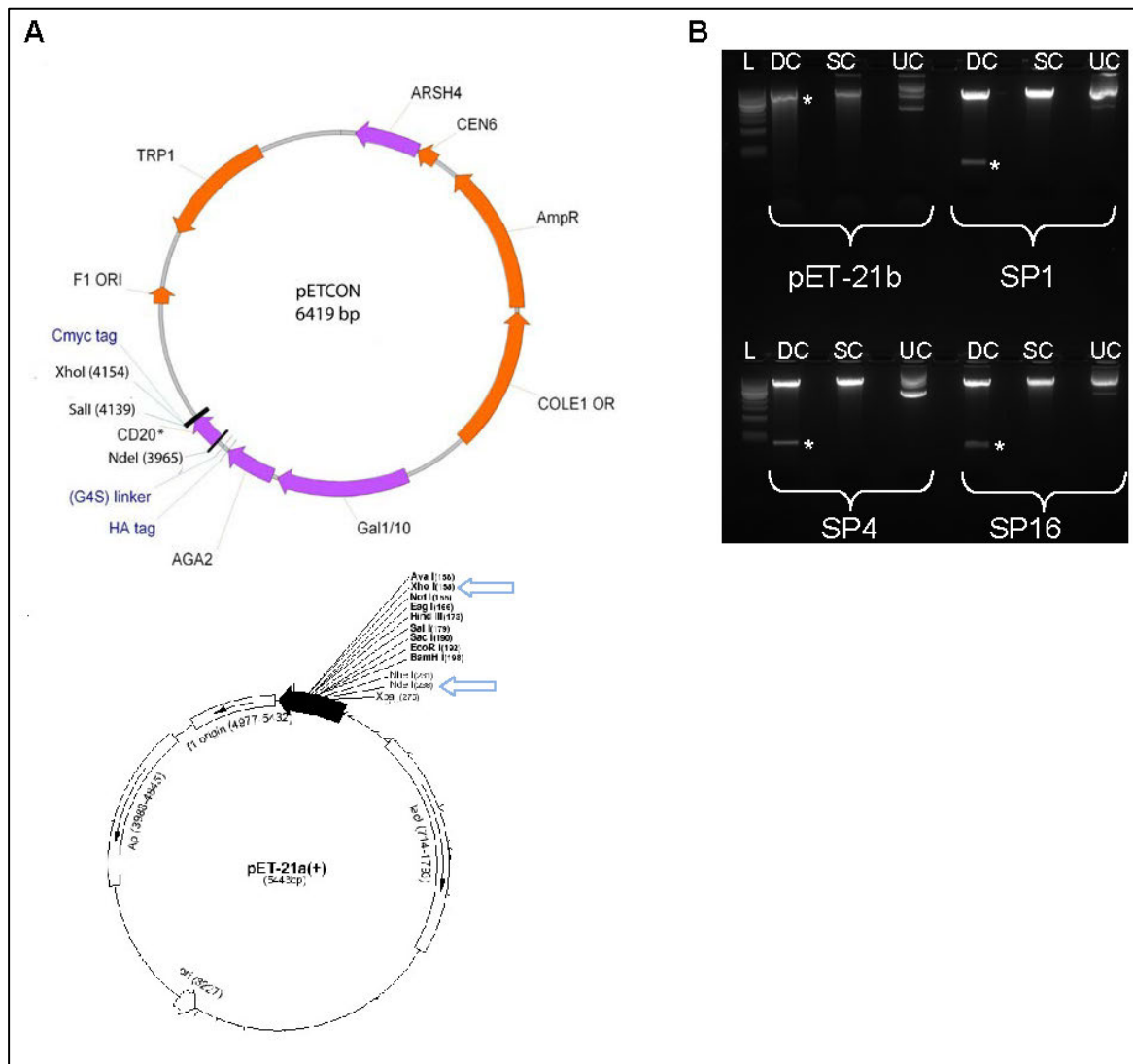


Figure 2. Cloning strategy to transfer SPxx constructs from pETCON to pET-21b. (A) Rosetta-designed constructs were cloned from their source pETCON yeast display vector (top left) into the bacterial protein-expression vector pET-21b (bottom left) via standard restriction digest and ligation protocols. (B) Each Rosetta candidate design (SP1, SP4, and SP16) was excised from its respective pETCON vector by NdeI/XhoI double digestions, along with the target pET-21b protein-expression vector. Bands indicated by asterisks (*) (top right) were excised from the agarose gel and gel-purified for subsequent ligation and transformation into DH5 α cells. “L” indicates the 1 Kb size ladder, “DC” is the double digest, “SC” is a single digest with XhoI, and “UC” is the undigested source material.

2.2.2 Protein Expression, Production, and Purification

An optimal expression of the SP4 construct was obtained in the BL21(DE3) Star expression system that was grown in auto-induction media, and the SP16 overexpression was best obtained in a T7 Express cell line. Evaluation of the sodium dodecyl sulfate (SDS) gel visualization indicated that the use of Superior Broth yielded slightly better SP4 expression; however, the auto-induction media yielded similar results and was selected for follow-on production (Figure 3A). Sufficient yields of SP4 protein were isolated from the soluble fraction of a 1 L preparation, despite a significant proportion that was also seen in the insoluble fraction (Figure 3B). Unfortunately, SP1 production was universally poor in several cell lines, including BL21 (DE3) Star (Figure 3A), BL21-AI (Figure 3B), T7 Express (not shown), or Rosetta2 (not shown). No further attempts were made to produce and isolate SP1 after the Baker group established that it was a poor L1R binder during affinity maturation efforts.

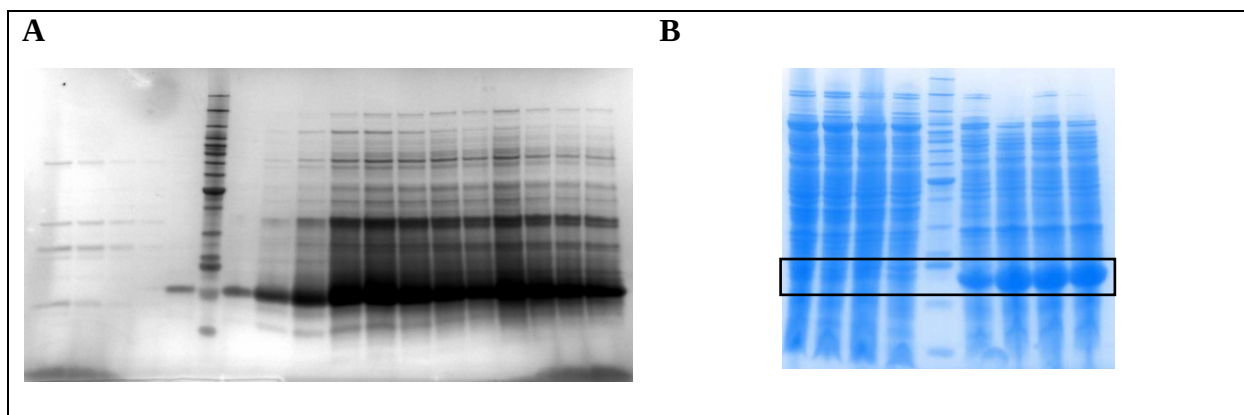


Figure 3. Overexpression of first-generation binding candidates.

Figure 3A shows the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of His-tagged SP1 and SP4 fractions that were eluted from an ÄKTAexpress HisTrap affinity column (GE Healthcare Life Sciences; Pittsburgh, PA). SP1-pET-21b transformed into BL21-AI cells, which are often used for production of toxic proteins, and it expressed poorly when induced with 1% arabinose and 1 mM isopropyl β -D-1-thiogalactopyranoside. SP4-pET-21b transformed in BL21 Star (DE3) shows good overexpression (lanes 5 and 7–18). Figure 3B shows SP1-pET-21b in BL21 Star (DE3), which expressed poorly (lanes 1–4), in contrast with the strong overexpression of SP16-pET-21b in T7 Express cells (lanes 6–9). SP1 and SP4 products are indicated by a rectangular outline.

Recombinant SP4 and SP16, produced as described herein, were purified from cell paste by Ni^{2+} -affinity purification followed by cation-exchange chromatography. The purity of recombinant SP4 and SP16 were verified by Coomassie staining (Figure 4A), and an anti-His₆ Western blot demonstrated that the C-terminally histidine-tagged proteins were fully translated (Figure 4B).

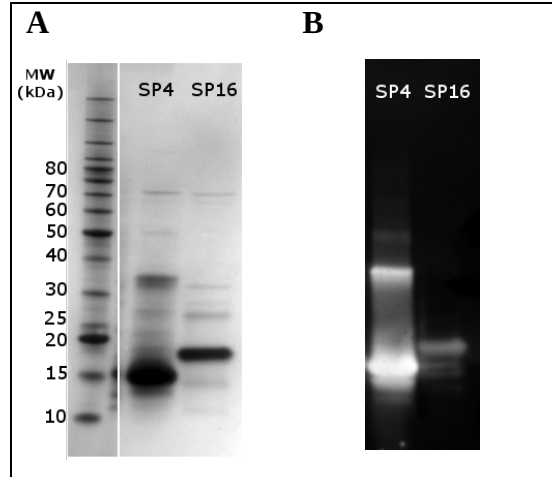


Figure 4. Validation of SP4 and SP16 purity by denaturing polyacrylamide gel electrophoresis after Ni^{2+} -affinity purification and cation-exchange chromatography that was visualized using (A) Coomassie stain or (B) enhanced chemiluminescence probing with anti-His₆ antibody.

2.2.3 Purity Assessed Using Experion Lab-on-a-Chip

Purified SP4 and SP16 were characterized using the Experion Lab-on-a-Chip system (Figure 5). For both proteins, the observed molecular weights were higher than those predicted using the ExPASy Swiss Institute of Bioinformatics (Lausanne, Switzerland) resource portal (Table 1). The presence of multiple higher molecular weight bands, even under denaturing conditions, suggested that SP4 and SP16 may have aggregated in solution.

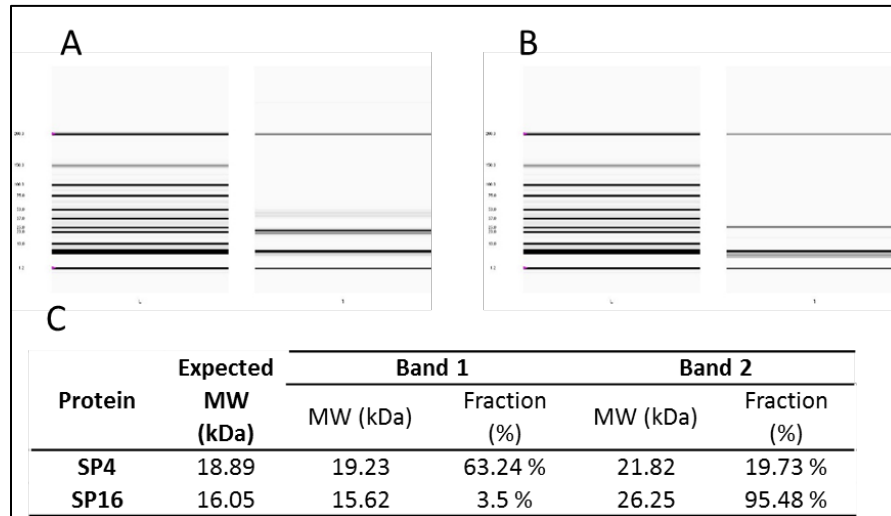


Figure 5. Experion Lab-on-a-Chip analysis of first-generation candidates. (A) SP4 and (B) SP16. (C) The fraction of total mass found in the two most prevalent protein bands.

Table 1. Candidate-Predicted Molecular Weight (MW) and Isoelectric Point (pI)

Rosetta Construct	Predicted MW (Da)	Predicted pI
SP1	14294	7.06
SP4	18887	4.55
SP16	16047	5.95

Note: These values were calculated using ExPASy for SPxx sequences that cloned into pET-21b via the NdeI and XhoI restriction sites. These calculations include the C-terminal amino acid sequence LEHHHHHH that was introduced by cloning into the pET-21b vector.

2.2.4 Measurement of Protein Concentration

Absorbance measurements of purified SP4 and SP16 were quantified using a NanoDrop system at 280 and 260 nm (A_{280} and A_{260} , respectively), as per the routine protocol.

2.2.5 Solubility Analysis by Dynamic Light Scattering (DLS)

Evaluation of a DLS analysis of purified SP4 and SP16 in phosphate-buffered saline (PBS) at pH 7.4 found that both preparations had a large estimated particulate radius and high polydispersity (Table 2), which indicated that these preparations aggregated in solution. By comparison, a monoclonal antibody with an approximate molecular weight of 150 kDa typically has a peak radius between 4 and 6 nm.

Table 2. Replicate DLS Characterization of SPxx Candidate Solubility

Candidate	Peak Radius (nm)	Peak Polydispersity (nm) (% of radius)	Peak MW-R (kDa)	Peak Mass (% total)
SP4	8.1	6.1 (75.5)	450	100
	8.2	6.5 (79.4)	463	99.7
	8.4	5.4 (64.8)	585	99.9
SP16	13.4	13.2 (99.1)	1451	100
	11.5	9.0 (77.8)	1022	100
	17.8	25.3 (142)	2839	100
	7.7	19.7 (111)	2815	100

Note: This analysis was performed in PBS at pH 7.4, and it calculated the particle radius, the polydispersity (the peak width at half-height of an assumed Gaussian particle size distribution), the predicted molecular weight assuming a Raleigh spherical model (MW-R), and the fraction of mass associated with a given peak.

2.2.6 Thermal Stability Assessment by Differential Scanning Calorimetry (DSC)

DSC measurements provide information about the robustness of a protein when exposed to changes in temperature, which offers insight into the interconversion between protein conformations, and by extension, the robustness of binding activity over time and temperature fluctuations. No DSC was performed because the candidates did not bind the desired L1R target, and they were prone to aggregation (i.e., conformational instability [Section 2.2.3]).

2.3 Quantification of Equilibrium Binding Affinity to Recombinant Antigen

The binding kinetics to the L1R recombinant target were to be quantified using surface plasmon resonance or bio-layer interferometry for affinity candidates that were established as pure and thermodynamically stable. However, no candidates passed this decision point to proceed forward.

2.4 Characterization of Binding Affinity to Intact *Vaccinia* virus

Verification binding studies using intact *Vaccinia* virus, such as by enzyme-linked immunosorbent assay (ELISA), would have been performed if any SPxx candidate had demonstrated recognition of the recombinant L1R protein. However, no candidates progressed to this stage of analysis.

2.5 Candidate Characterization for Inclusion into the Defense Biological Products Assurance Office Antibody Repository

No SPxx candidates identified were deemed to be suitable for inclusion into the Defense Biological Product Assurance Office Antibody Repository. No other characterization beyond that described herein was pursued.

2.6 Redesign of Ligand Scaffolding for Binding to L1R

Studies during affinity maturation revealed that the SPxx series had greater affinity for streptavidin than for the L1R target. The Rosetta candidates were expressed on the yeast surface bound to the streptavidin of the preincubated L1R-streptavidin complex; therefore, the candidate showed high enrichment during the initial yeast display screening. In light of this discovery, a new approach was pursued that was similar to the Koga Baker de novo design (3). Permutations were made of extremely stable mini-proteins, which were each made up of less than 42 amino acids designed with a disulfide bond stabilized simple topology of three β -sheets and one α -helix. The Baker Laboratory (Chris D. Bahl) pioneered this approach and has since structurally verified some of the Rosetta computational designs that were made (Figure 6).

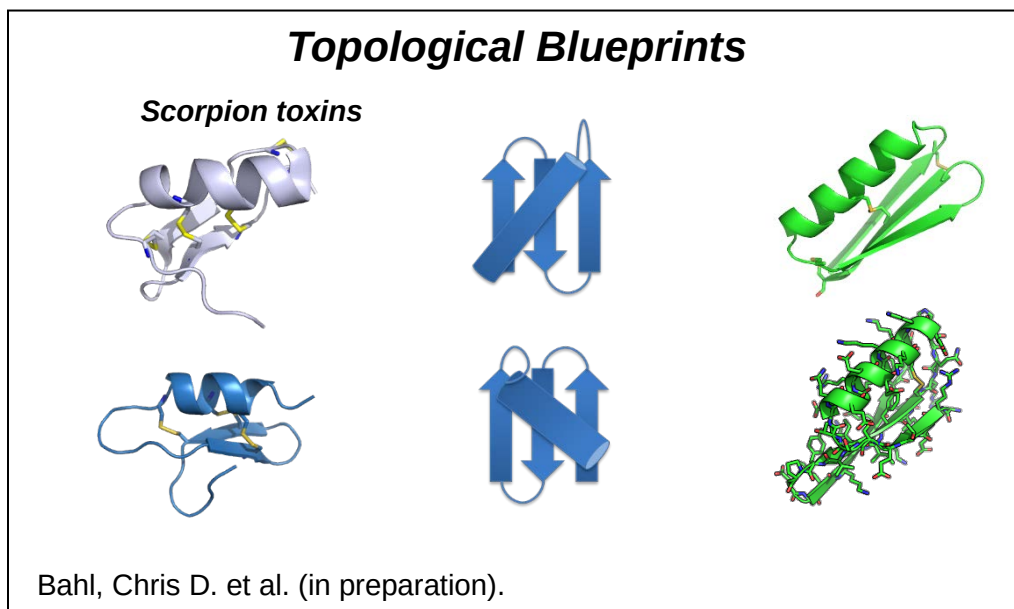


Figure 6. Scaffold strategy derived from mini-proteins based on scorpion toxins.

Because the disulfide-linked mini-proteins are small, their genes can be synthesized as a single strand by chip oligonucleotide synthesis. The mini-genes were amplified and linker regions were added to allow recombination into a yeast-surface display expression vector for screening and selection.

Two parallel approaches were pursued to develop the next generation of L1 binders. First, the original library of decoys, which were developed as part of the first generation of L1 candidates, was used to source 18,000 backbones that contained disulfide bonds with topology variations analogous to the scorpion toxins (i.e., “blueprint-based backbone” in Figure 7). Roughly 2000 candidates were ordered and synthesized by chip oligonucleotide synthesis, and flanking sequences were appended to barcode the candidates and to allow them to be introduced into the yeast display screening pipeline (Figure 8). Another 75 designs were made using a second computational approach that was built around critical interactions in the L1R binding site and was analogous to those seen in the 7d11-L1 crystal structure epitope (4) (i.e., “blueprint-like definition of topology components” shown in Figure 7).

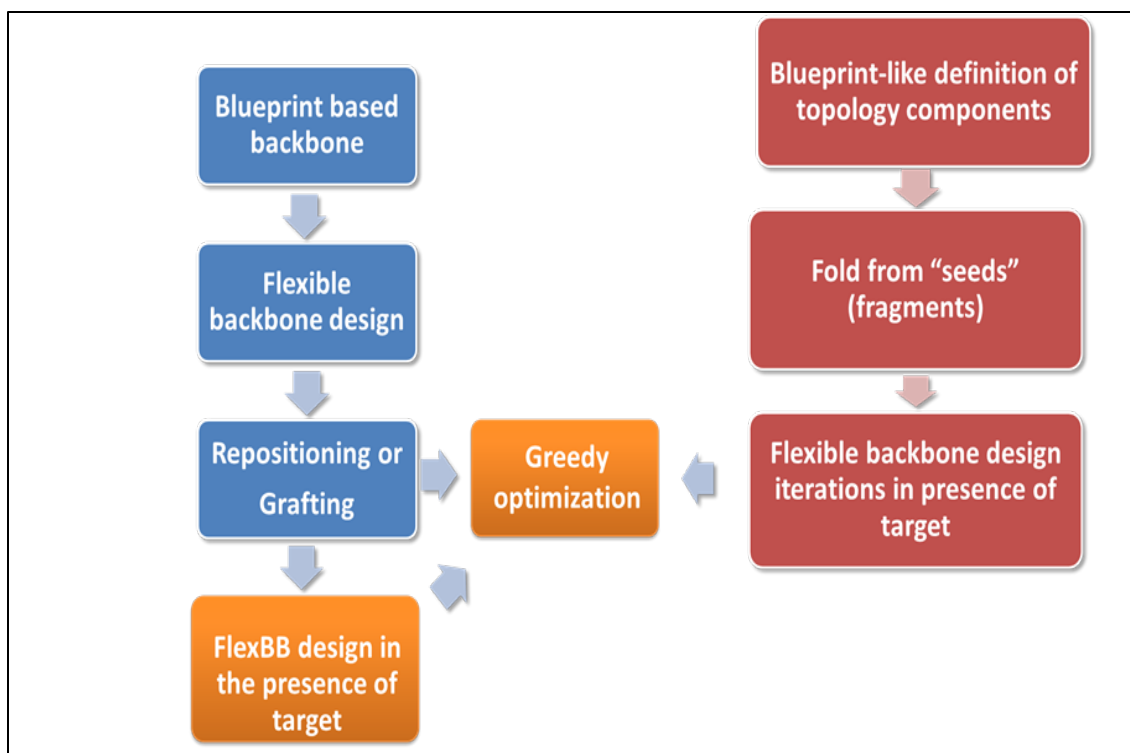


Figure 7. Computational strategies to design L1 affinity reagents.

The original library of decoys that was developed during the first L1 binder generation was used to source 18,000 backbones that contained disulfide bonds with different topology variations but still had three β -sheets and one helix motif. This library was used to design an interface with L1, along with the redesign of the core of the starting decoys (blue path on the workflow in Figure 7). Additionally, a second computational approach (red path on the workflow in Figure 7) that builds a mini-protein around the binding epitope taken from the neutralizing antibody 7d11 is concurrently being pursued.

Figure 8 depicts the protocol used for candidate selection as follows: Genes encoding the Rosetta designs were transformed as a pool into yeast cells and evaluated for binding as a pool. Cells were induced for expression, mixed with 1 μ M biotinylated L1R protein that was preincubated with the chromophore streptavidin–phycoerythrin (SAPE), washed, and probed with fluorescein-labeled (fluorescein isothiocyanate [FITC]) anti-Myc antibody. These chromophores were used to interrogate surface-displayed Rosetta designs by flow cytometry, which sorts cells based on their capability to retain fluorophore conjugate. FITC fluorescence reports expression levels via anti-Myc antibody binding to the c-Myc sequence tagging each Rosetta design, whereas SAPE-based fluorescence monitors binding through the L1R–biotin–streptavidin interaction. Cells were sorted once to collect the subset that expressed designs that were displayed on the yeast surface and then sorted again for L1R binders based on an increased SAPE signal. After selecting for binding, deoxyribonucleic acid (DNA) from each pool was isolated and polymerase chain reaction (PCR)-amplified to attach adapter sequences that would allow next-generation sequencing of the barcodes. The frequency of a given gene found within a sample pool was tracked by the number of occurrences of its barcode. The propensity of a design

to bind L1R was evaluated by its “enrichment value,” which is defined as the ratio of a construct’s surface display frequency versus the subpopulation that displays and binds to SAPE.

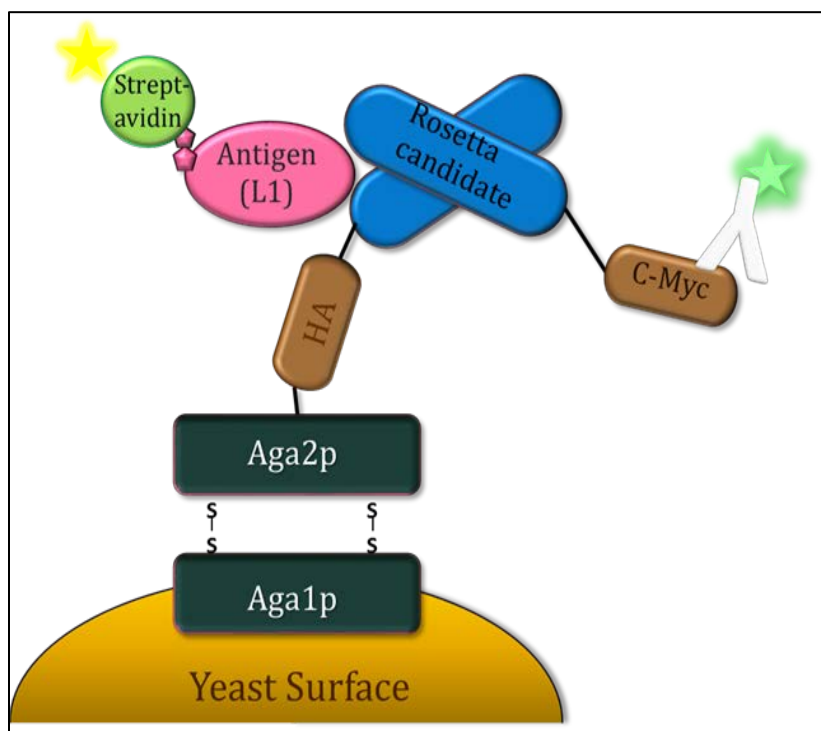


Figure 8. Rosetta candidate selection protocol.

2.7 Two Candidates Were Identified from Initial Screens

A number of additional controls were introduced into the screening approach to reduce the likelihood of selecting false positives in the yeast display pipeline (Figure 8). Yeast display candidates were biopanned against preincubated streptavidin complexes with nontarget proteins, such as a gp120S2–streptavidin adduct. Binding to these nontarget controls would suggest a nonspecific interaction; therefore, disqualifying the candidate for further consideration. Conversely, a ternary complex, L1Ab–streptavidin, which was formed by preincubating L1R with both 7d11 and streptavidin, was used as a negative screening control. This capitalized on the fact that the Rosetta candidates were designed to bind the same L1R epitope that is tightly bound by the 7d11 antibody (dissociation constant, K_D , of ~5.5 nM, data not shown). Candidates with a specific affinity for the desired L1R epitope will be blocked from binding by the tightly bound 7d11 antibody. Therefore, Rosetta candidates that (1) bind free L1R, (2) have poor affinity for the L1Ab complex, and (3) have little-to-no association with non-L1R streptavidin complexes are expected to be especially promising candidates. Using these criteria, two candidates were selected in FY15 quarter (Q)1 for affinity maturation (Figures 9 and 10). In FY15Q2, the Baker group reported promising screening results from pooled collections of most recent designs, with cell sorter signals that were much higher than anything obtained thus far in this study. Unfortunately, analysis of sequencing efforts in FY15Q3 found that the enriched pools had little correlation with the original designs, which suggested a multitude of

pseudorandom binders to either L1R or to the SAPE reporter. Resynthesizing and rescreening in FY15Q4 found that the designs represented <1% of the genes in the synthesized pool. As a result of these findings, the Task 1 initiative was terminated.

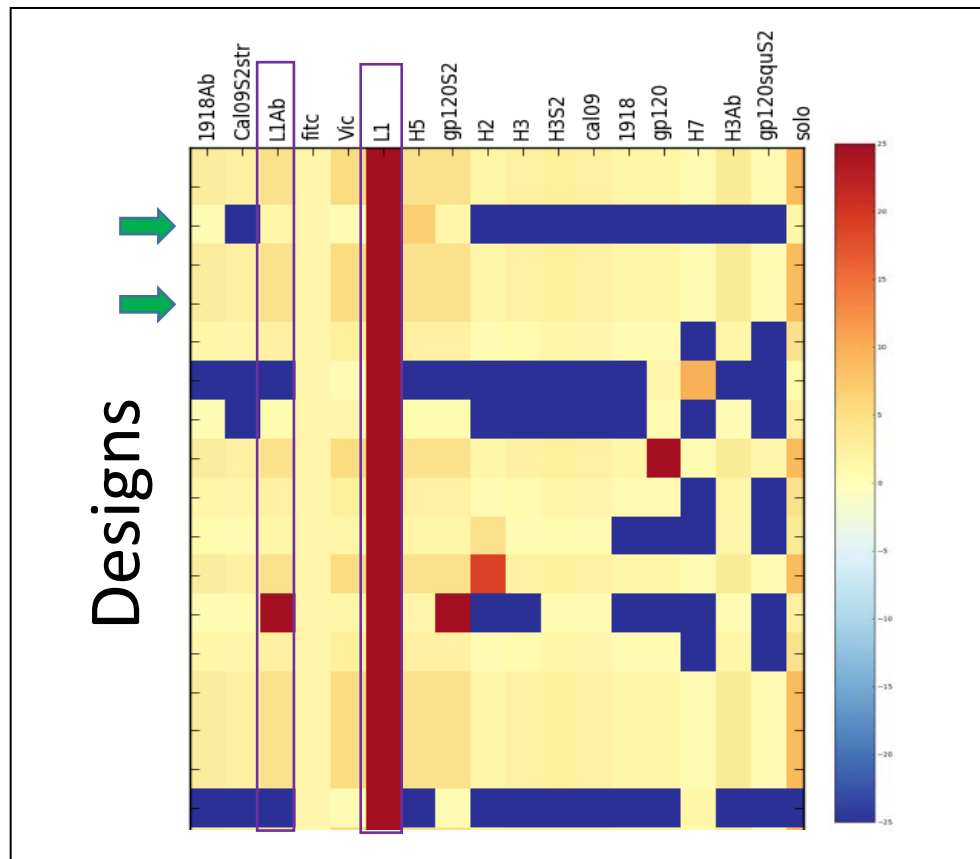


Figure 9. Heat map of enrichment values for Rosetta candidates that bind to L1R and negative-control proteins, which are listed across the top as bait. Enrichment values for candidates selected during screening are color-coded as follows: blue indicates that the candidate is not represented in a given pool, yellow indicates neutral or depleted levels, and red indicates enrichment. Any score above 25 is shown as 25. All of the constructs shown can bind L1R (purple rectangle). L1Ab is L1R that has been preincubated with antibody 7d11. Binding of a candidate to L1R alone but not to L1Ab, suggests that the candidate binds on the same face as the 7d11 antibody. All other recombinant bait molecules were used as negative controls. Green arrows indicate the two candidates that moved forward for affinity maturation.

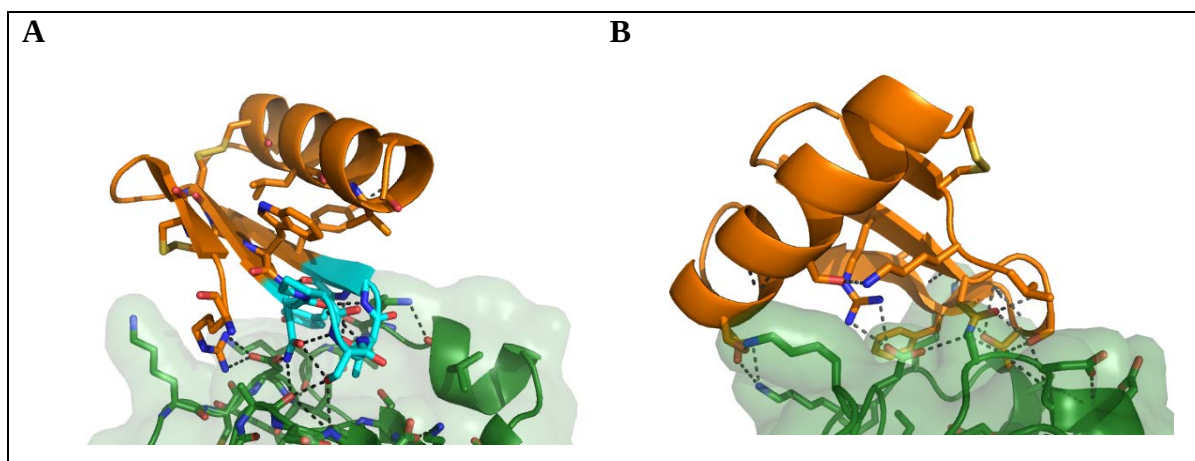


Figure 10. Structural models of the two Rosetta candidates that advanced to affinity maturation, which are indicated by arrows in Figure 9. The two designs (orange) are shown in their predicted binding mode with L1R (green). Panel A shows the 7d11 binding interface (cyan).

3. **TASK 2: CREATION OF A THERMOSTABLE IgG SCAFFOLD-BASED SINGLE-CHAIN VARIABLE FRAGMENT (scFv) LIBRARY**

A substantial number of biodetection technologies that are used in austere environments rely on antibodies to recognize threat targets. However, cold-chain requirements limit the utility and cost effectiveness of antibody-based assays. The development of a thermally stable antibody library and the subsequent use of this library would provide the Department of Defense with significant savings in the production of novel, thermostable antibodies that can operate without dependence on a cold-chain infrastructure. Therefore, in collaboration with AxioMx Inc., a phage-based library and the methods needed for isolation and production of thermostable antibodies were developed. These methods leveraged the DARPA-initiated Antibody Thermostability Program IgG framework as a scaffold of the antibody production pipeline. The library was then transferred to ECBC to enable in-house production of thermostable antibodies against targets of interest.

The AnaptysBio thermostable IgG backbone scaffold was originally built as part of the DARPA Ruggedized Antibody Program. AxioMx was subcontracted to adapt this IgG backbone as a universal acceptor for both heavy and light complementarity-determining region (CDR) grafts of scFv library candidates that were built for this purpose. This work had two phases: (1) build an IgG scaffold that is readily amenable to grafting of scFvs that are selected against future targets; and (2) develop an scFv library with a diversity of at least 1×10^{10} clones. The scFv library can be constructed by amplifying the parental scFv gene via error-prone PCR for example, which will limit mutagenesis to the variable regions. Emerging scFvs from the custom scFv library (Figure 11) would more readily be directly graftable into the IgG scaffold for future efforts and for custom, thermostable monoclonal antibody reagents.

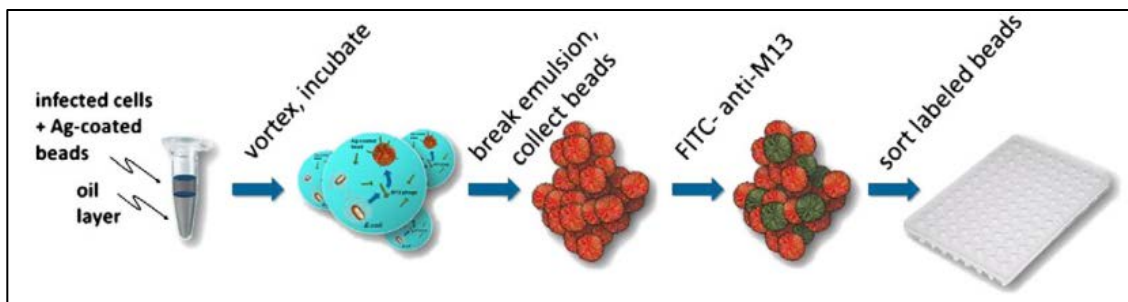


Figure 11. AxioMx affinity maturation of phage-displayed scFv sequences via emulsion screening.

Figure 11 shows that bacteria infected with a scFv phage library were added to antigen-coated beads in oil, mixed, and incubated to allow phage propagation and phage–scFv–antigen-bead binding. After emulsion disruption was performed, the beads were collected and probed with fluorescent antibody-recognizing phage to quantify and sort them based on the relative amount of bound scFv per bead. These candidates could then be used to seed another round of affinity maturation.

3.1 Adapt AnaptysBio Thermostable IgG Backbone to Build a scFv → IgG Splicing Pair

The original AnaptysBio Thermostable IgG was not designed to be a generic scaffold for scFv grafting. As such, AxioMx personnel adapted this DARPA framework for functionality in an *Escherichia coli*-based display system. The hypervariable domain from a known antibody sequence was grafted into a thermostable scFv framework, affinity-optimized, and then grafted into the DARPA-based IgG scaffold. Affinities were quantified using the PerkinElmer (Waltham, MA) AlphaScreen bead-based assay technology. Thermostability was assessed using the Life Technology (Grand Island, NY) Sypro orange protein gel stain that fluoresces upon binding to hydrophobic surfaces. The scFv binding increased ~8-fold after affinity maturation relative to the initial graft (Figure 12), with an ~52% increase in affinity for the lead scFv grafted into the IgG thermostable backbone compared with the original IgG (Figure 13). Analysis of thermostability using Sypro orange gel stain (Table 3) demonstrated that the >70 °C IgG melting temperature was achieved with this construct. The success of the scFv → IgG graft approach prompted the development of an scFv thermostable phage library (Section 3.2).

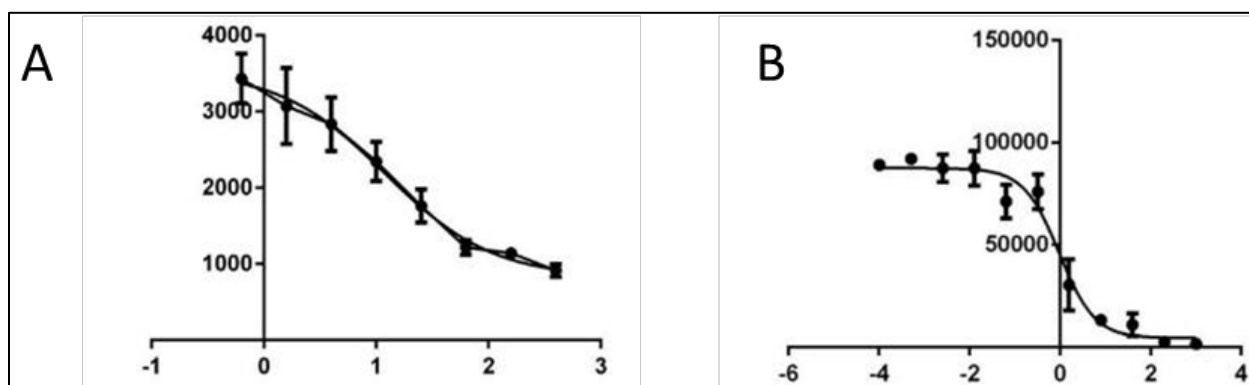


Figure 12. An scFv affinity enhancement from (A) the starting scFv with $K_D = 7.6$ nM to (B) the lead scFv after affinity maturation with $K_D = 0.96$ nM, as determined using the PerkinElmer AlphaScreen technology.

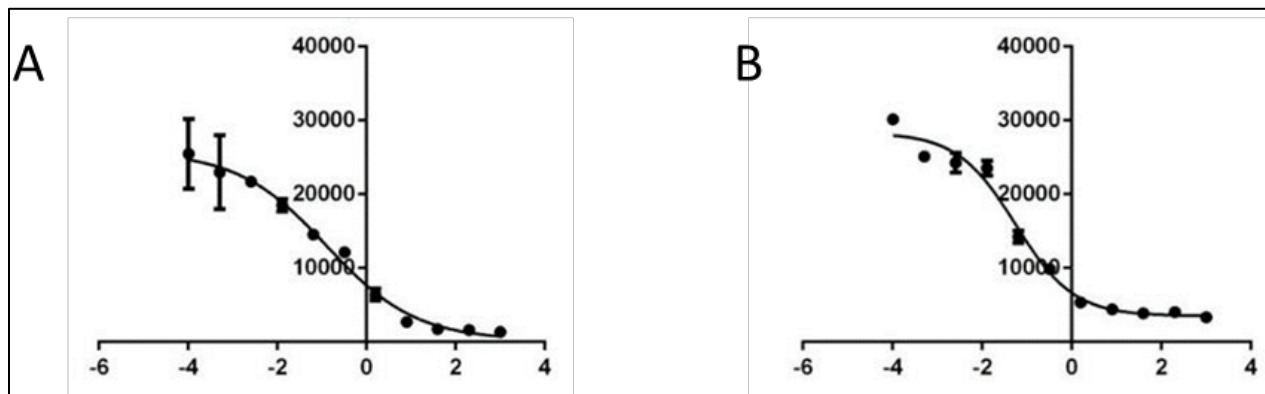


Figure 13. IgG affinity enhancement from (A) the original IgG with $K_D = 0.128$ nM to (B) the lead IgG after affinity maturation with $K_D = 0.076 - 0.092$ nM across several different lots of material, as determined using the PerkinElmer AlphaScreen technology.

Table 3. Affinity Reagent Thermostability

AxioMx Identification	Description	T_m (°C)	Genetic Change
Original scFv	mscFv	53	ECBC sequence
Starting graft	hscFv	63.5	AxioMx graft
Lead scFv	hscFv	68	Stability-matured graft
Final IgG	hIgG	77	Stable scFv spliced into DARPA IgG

T_m , melting temperature; mscFv, mouse single-chain variable fragment; hscFv, humanized single-chain variable fragment; hIgG, human immunoglobulin G.

Note: This information was determined using SYPRO orange fluorescence protein gel stain for the original scFv, for the initial AxioMx graft, for the design, for the affinity optimized “lead” scFv, and for the final IgG constructed by grafting the lead scFv into the DARPA-derived IgG thermostable backbone.

3.2 Develop an scFv Library with a Diversity of at Least 1×10^{10} Clones

With the development of a thermostable scaffolding amenable to grafting scFvs that were identified by phage display emulsion screening, attention was shifted to building an scFv library. Single-vector constructs were designed, which were composed of heavy (V_H) and light (V_L) variable domains connected by a peptide linker (Figure 14). During this process, only the CDRs were targeted for mutagenesis, and the focus was on positions with the highest variability, as observed in the Kabat database by Johnson et al. (5). (The database can be accessed at <http://immuno.bme.nwu.edu>.)

A library framework template (Figure 15A) was cloned into the pAPIII₆ vector. The resulting phagemid was transformed into CJ236 *E. coli* cells, which lack deoxyuridine-triphosphatase (dUTPase) and uracil-*N*-glycosylase activity. Cultures of CJ236 containing the pAPIII₆ phagemid were then grown to an optical density at 600 nm (OD_{600nm}) of 0.4–0.6 and inoculated with M13-K07 helper phage. A modified Kunkel mutagenesis strategy was used to generate the library of scFv clones (Figure 15B). After overnight incubation, phage particles were precipitated from supernatants that were clarified by centrifugation, and uracilated single-stranded DNA (ssDNA) was isolated by ethanol precipitation (Figure 15C).

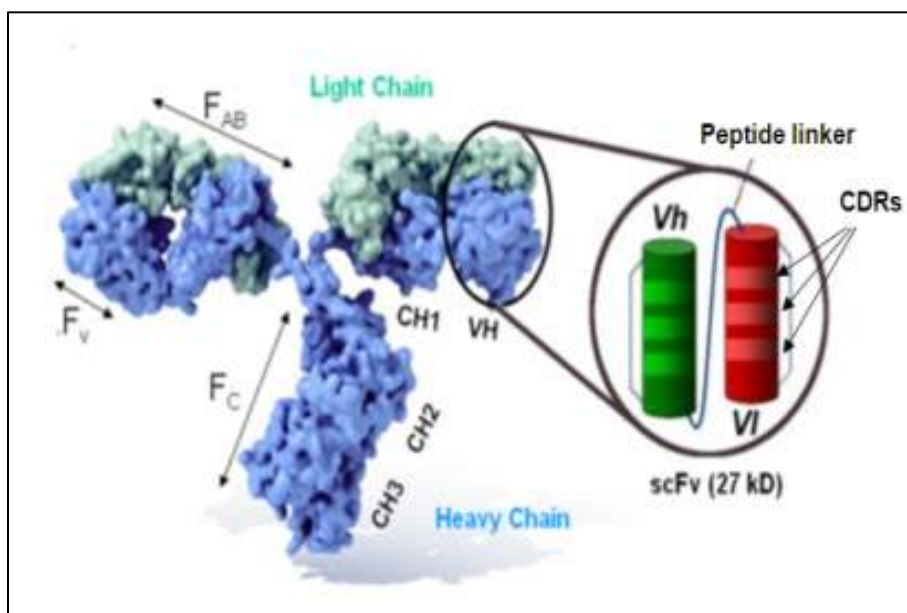


Figure 14. Library construction strategy. Combinatorial changes were limited to the heavy and light chain CDRs of the antibody scaffold, and focused on the most variable positions, as observed in the Kabat database of known antibody sequences.

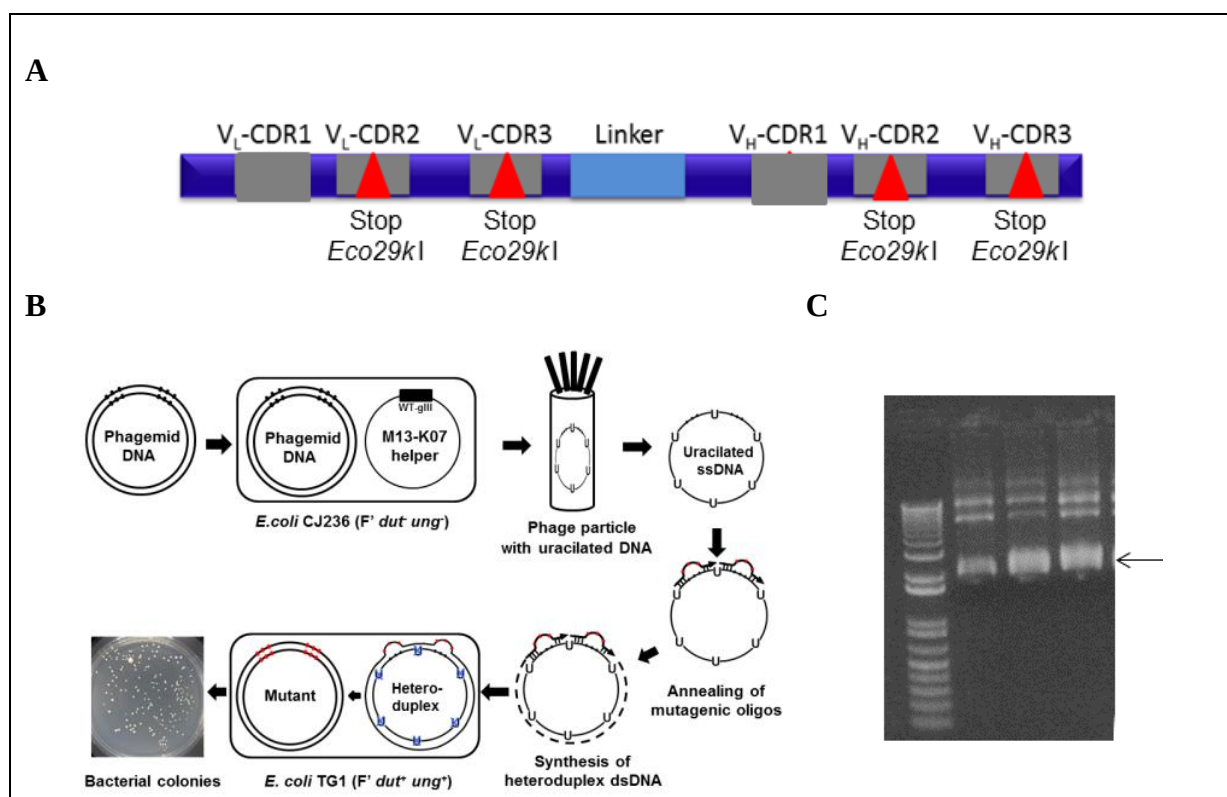


Figure 15. Library construction by modified Kunkel mutagenesis.

Figure 15A shows an scFv framework template containing stop codons and Eco29kI restriction sites (isoschizomeric with SacII) that was cloned into the pAPIII₆ vector to allow downstream *in vivo* elimination of unreacted templates from the pool of recombinant clones. Figure 15B shows the Kunkel mutagenesis (6) workflow, which allows 10⁷ colonies per transformation. Phagemid DNA (panel 15A) is electroporated into CJ236 *E. coli* that are then infected with M13-K07 helper virus to amplify those phage particles to yield uracilated ssDNA. The ssDNA is annealed to two phosphorylated mutagenic oligonucleotides that prime the synthesis of heteroduplex, double-stranded DNA (dsDNA) in the presence of T7 DNA polymerase, T4 ligase, and deoxynucleotides. The resulting dsDNA is purified and electroporated into TG1 cells, where the uracilated parental strand is degraded, and the mutant strand is preserved and converted into the replicative form of the phagemid DNA. Figure 15C shows the results when uracilated ssDNA was isolated from clarified supernatant by precipitating phage particles using the QIAprep M13 kit (Qiagen, Inc.; Hilden, Germany), the resuspension of the phage pellet with iodide buffer, and the ethanol precipitation of the ssDNA (indicated by arrow at right in Figure 15).

Isolated uracilated ssDNA was hybridized with phosphorylated oligonucleotides that pseudorandomly encoded mutations at positions that were previously identified as highly variable regions within the L2, L3, H2, and H3 CDRs. NNK codons, which encode all 20 amino acids and one stop codon (Figure 16A), were introduced at 13 positions in the L2, L3, and H2 CDRs. The H3 CDR length was varied between 8, 11, or 14 amino acids, which necessitated a reduction in the likelihood that a stop codon may be introduced. Thus, NNK codons were used at

four positions, whereas NMY codons (Figure 16B) were used at 6, 9, or 12 positions: NMY codons generate eight amino acids and no stop codons. In vitro DNA synthesis using T7 DNA polymerase, T4 ligase, and deoxynucleotides generates circular, heteroduplex (with and without uracil insertions) dsDNA. Cotransformation of the heteroduplex dsDNA, together with a vector encoding inducible Eco29kI restriction enzymatic activity, into an *E. coli* strain such as TG1 enriches the diversity of replicative phagemid genomic ssDNA as follows: First, the parental uracilated strand is degraded by the intact dUTPase and uracil-*N*-glycosylase activity that is found in the cell. Second, the inducible restriction digestion activity eliminates >90% of the unreacted framework template (Figure 15A) that may be remaining. Finally, cells were allowed to grow to an OD_{600nm} of 0.4 before the KM13 helper phage was added to generate the phage library. The cells were grown to an OD_{600nm} of 1.0 before glycerol stocks were created (Figure 17).

		Second letter					
		U	C	A	G		
First letter	U	UUU } Phe UUC } UUA } Leu UUG }	UCU } Ser UCC } UCA } UCG }	UAU } Tyr UAC } UAA } Stop UAG }	UGU } Cys UGC } UGA } Stop UGG }	U C A G	Third letter
	C	CUU } CUC } Leu CUA } CUG }	CCU } CCC } CCA } Pro CCG }	CAU } His CAC } CAA } Gln CAG }	CGU } CGC } Arg CGA } CGG }	U C A G	
	A	AUU } Ile AUC } AUA } AUG } Met	ACU } ACC } ACA } Thr ACG }	AAU } Asn AAC } AAA } Lys AAG }	AGU } Ser AGC } AGA } Arg AGG }	U C A G	
	G	GUU } GUC } Val GUA } GUG }	GCU } GCC } Ala GCA } GCG }	GAU } Asp GAC } GAA } Glu GAG }	GGU } GGC } Gly GGA } GGG }	U C A G	

		Second letter					
		U	C	A	G		
First letter	U	UUU } Phe UUC } UUA } Leu UUG }	UCU } Ser UCC } UCA } UCG }	UAU } Tyr UAC } UAA } Stop UAG }	UGU } Cys UGC } UGA } Stop UGG }	U C A G	Third letter
	C	CUU } CUC } Leu CUA } CUG }	CCU } CCC } CCA } Pro CCG }	CAU } His CAC } CAA } Gln CAG }	CGU } CGC } Arg CGA } CGG }	U C A G	
	A	AUU } Ile AUC } AUA } AUG } Met	ACU } ACC } ACA } Thr ACG }	AAU } Asn AAC } AAA } Lys AAG }	AGU } Ser AGC } AGA } Arg AGG }	U C A G	
	G	GUU } GUC } Val GUA } GUG }	GCU } GCC } Ala GCA } GCG }	GAU } Asp GAC } GAA } Glu GAG }	GGU } GGC } Gly GGA } GGG }	U C A G	

Figure 16. scFv mutagenesis oligonucleotides. (A) NNK codons with sequence NN(G/T) encode all 20 amino acids (yellow highlights) and one stop codon (red highlight). (B) NMY codons with sequence N(C/A)(C/T) generate eight amino acids (yellow highlights).

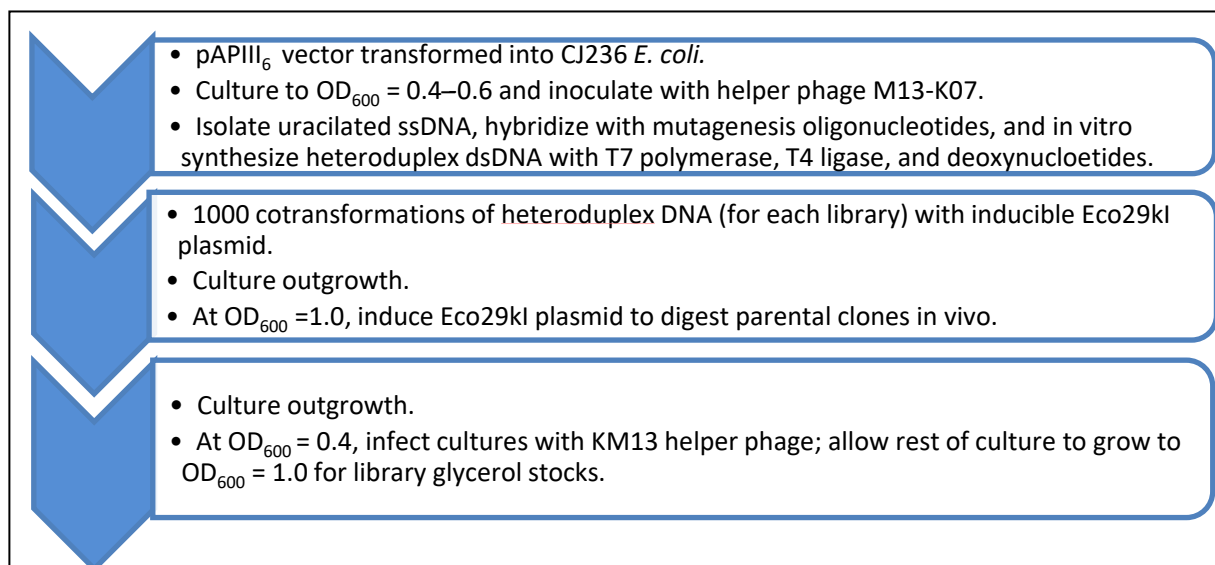


Figure 17. Example of library manufacturing workflow.

Phage isolated from supernatants were sequenced to validate library composition. Overall, the amino acid distributions were as predicted (Figure 18A,B). Sequencing showed an approximately 70:30% split between 8 and 11 amino acid H3 lengths, and <2% had 14 amino acids (Figure 18C). The library met all of the following desired specifications: diversity ($>10^{10}$ clones), amino acid distribution, and compatibility for grafting into a thermostable IgG backbone. These specifications were by design and were demonstrated via successful construction of a Rivax-binding thermostable IgG (funded by and reported to the Defense Threat Reduction Agency [DTRA] program CB3636.) Together, 100 tubes, each containing 0.1 mL with 4×10^{13} phage/mL were produced (Figure 19) for transition to ECBC, which completed the CB3646 Task 2 milestone.

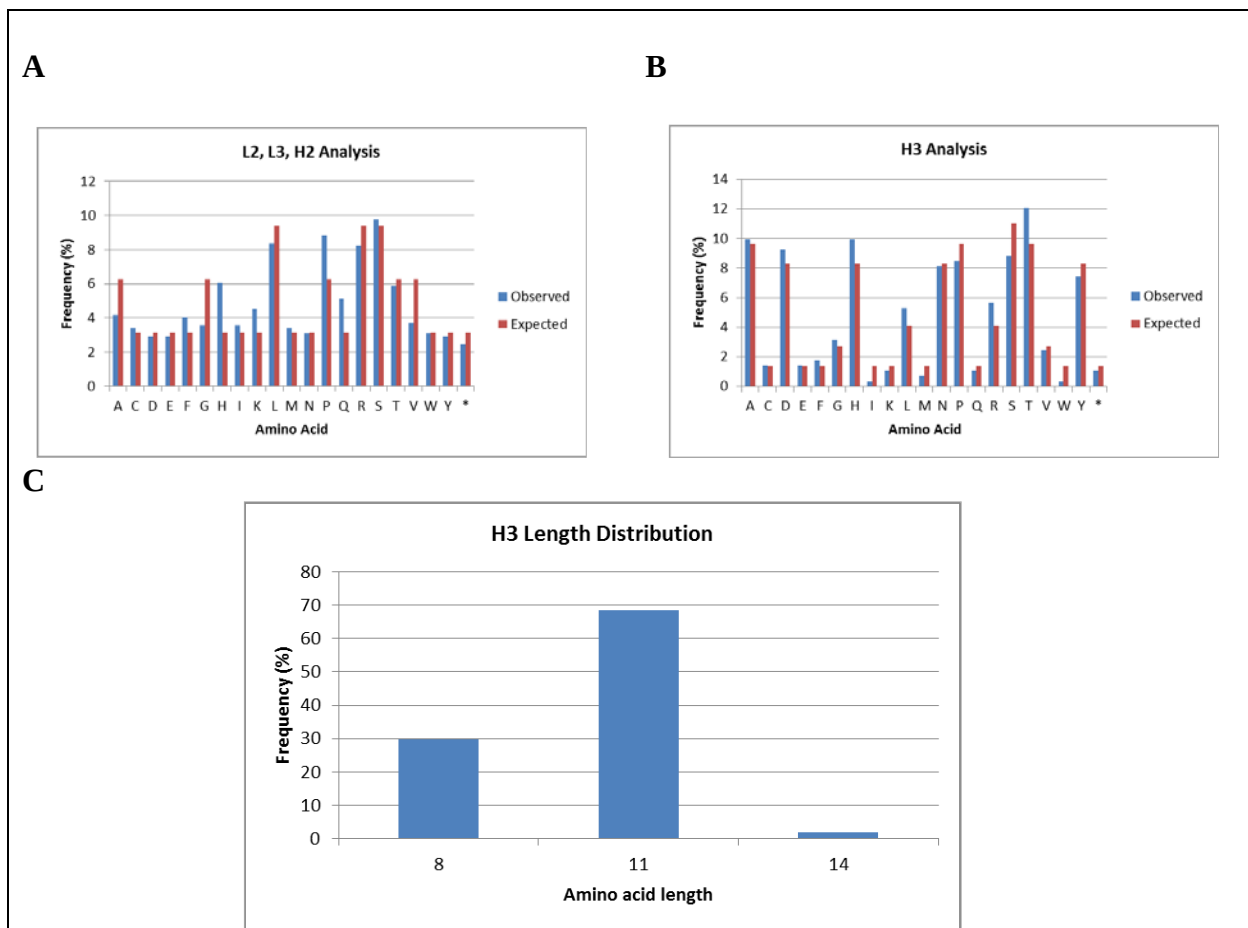


Figure 18. Library validation. Sequence validation of a subset of phage particles met design objectives. Observed amino acid composition was well-correlated to prediction for CDRs (A) L2, L3, and H2, as well as (B) CDR H3, which had been designed to vary in overall amino acid length. (C) Length distribution of CDR H3, as observed in a subset of phage particle constructs.

Library size determination:

$$\begin{aligned}
 & (\text{number of cells}) \times (\text{dilution factor}) \times (\text{volume plated}) \times \\
 & (\% \text{ fully incorporated oligonucleotides}) \times (\text{number of transformations}) \\
 & 10 \times (10^6) \times 5 \times 0.9 \times 1000 = 4.5 \times 10^{10} \text{ unique clones}
 \end{aligned}$$

Phage precipitated from liquid cell culture growth:

$$\begin{aligned}
 \text{Phage titer} &= (\text{number of cells}) \times (\text{dilution factor}) \times (1000) = \\
 & (4) \times (10^{10}) \times (1000) = 4 \times 10^{13} \text{ phage/mL}
 \end{aligned}$$

Figure 19. Calculated phage library diversity.

4. TASK 3: CRYSTALLOGRAPHY DATA COMPILATION

Fabs alone or in complex with their cognate antigen were crystallized for structure determination. Three structures were solved: the anti-ricin AxioMx IgX 2691 bound to recombinant RiVax at 2.7 Å, the anti-*Vaccinia* AnaptysBio 7d11 graft Fab alone at 2.64 Å, and the anti-*Vaccinia* AnaptysBio 7d11 graft bound to recombinant L1R at 2.15 Å.

Immunoassay development and optimization is greatly facilitated by the availability of antigen epitope information. Although primary sequence epitopes can be elucidated through peptide library screening efforts, nonsequenced adjacent tertiary structural epitopes are more readily visualized using structural studies. Similarly, structure-based protein engineering efforts benefit strongly from the availability of experimentally derived structural data. X-ray crystallography is the gold standard technique for determining the three-dimensional structures of proteins, and it has become a commodity technique. Many contract laboratories and core facilities offer automated crystallization, diffraction, and molecular-replacement structure solution as fee-for-service tasks. Originally, it was proposed that three to five affinity reagents (recombinant or antibody targets) would be prepared and submitted for crystallization and structural characterization with no recovery plans (i.e., no further attempts would be made if diffracting crystals were not obtained). These three to five samples could include co-crystallization efforts with recombinant targets, where available. Targets included the thermostable AnaptysBio scaffold graft of the anti-*Vaccinia* antibody 7d11 alone and in complex with its recombinant antigen, L1R. The anti-L1R 7d11 graft antibody was particularly of interest to support molecular modeling being performed as part of DTRA project CB3636. Protein-processing and crystallography efforts were performed through a subcontract with Cedars-Sinai Medical Center (Los Angeles, CA; POC: Dr. Paul Hubbard).

4.1 Crystal Structure of RiVax Complex with AxioMx IgX 2691 at 2.7 Å

4.1.1 Summary of RiVax–IgX 2691 Fab Structure

The crystal structure of the Fab of AxioMx IgX 2691 was solved in complex with its antigen, RiVax, to 2.7 Å resolution (Figure 20A). There are two copies of the complex in the asymmetric unit that share similar quaternary structures. Contacts between the hypervariable loops of the Fabs and RiVax are shown to be almost identical (Figure 20B). Comparison to a related ricin-A–Fab complex with a protein data bank (PDB) ID of 4KUC (7), shows that although both types of Fabs bind to the same face of the antigen (Figure 21A), there are significant differences in the epitope, as highlighted in Figure 21B. Comparison of both Fab hypervariable loops shows that CDR-H1 and CDR-H3 are structurally similar, which is reflected in their sequence similarity, whereas all three loops of the light chain and CDR-H3 show large topological differences. The latter accounts for the significant difference in the molecular surface area of the antigen that lies within 4 Å of the Fab: 658 Å² for the Fab–RiVax complex and 531 Å² for 4KUC. Figure 22 shows refined electron density surrounding the six CDRs, which highlights the map quality and the high degree of confidence in the accuracy of the refined model.

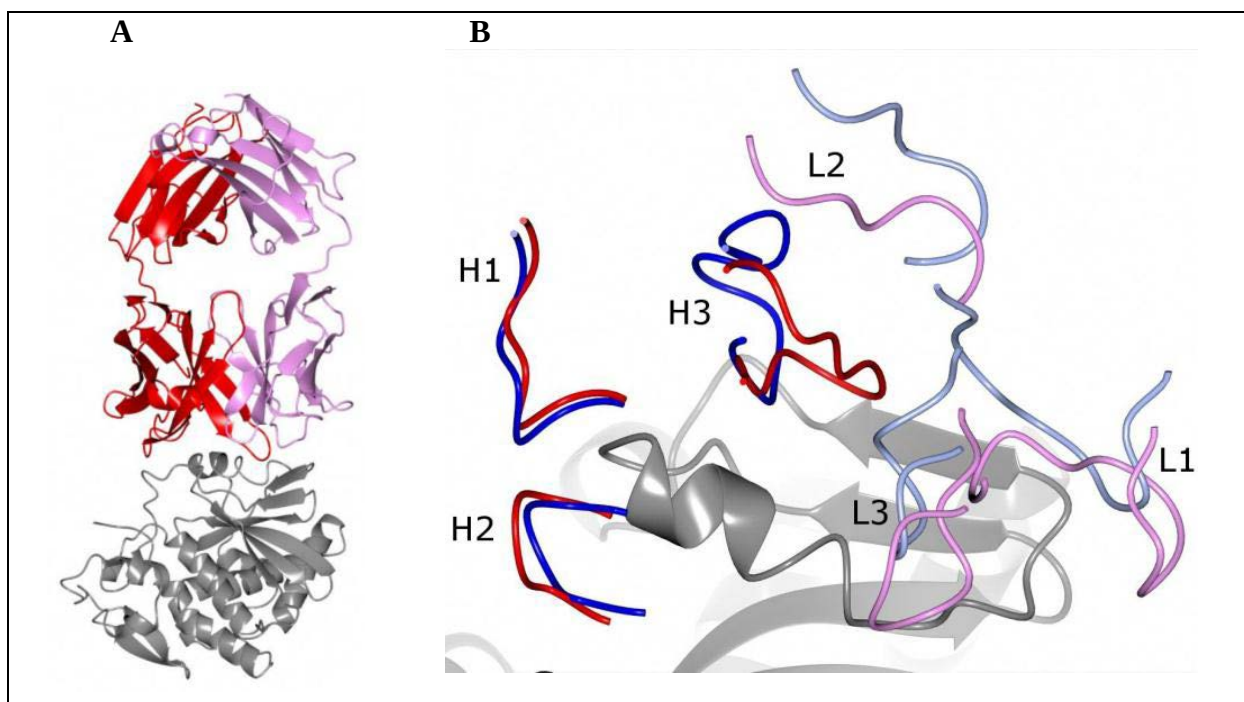


Figure 20. Ribbon diagram of the Fab–RiVax complex. (A) Heavy chain of Fab (red), light chain (pink), and RiVax (gray). (B) Superimposition of the Fab–RiVax complex and PDB ID: 4KUC with the antigen and hypervariable loops illustrated. (Coloring scheme for RiVax and AxioMx IgX 2691 Fab as shown in panel A. The 6C2 Fab (4KUC) heavy and light chain hypervariable loops are colored dark and light blue, respectively. The structurally conserved H1 and H2 loops are to the left of the figure.

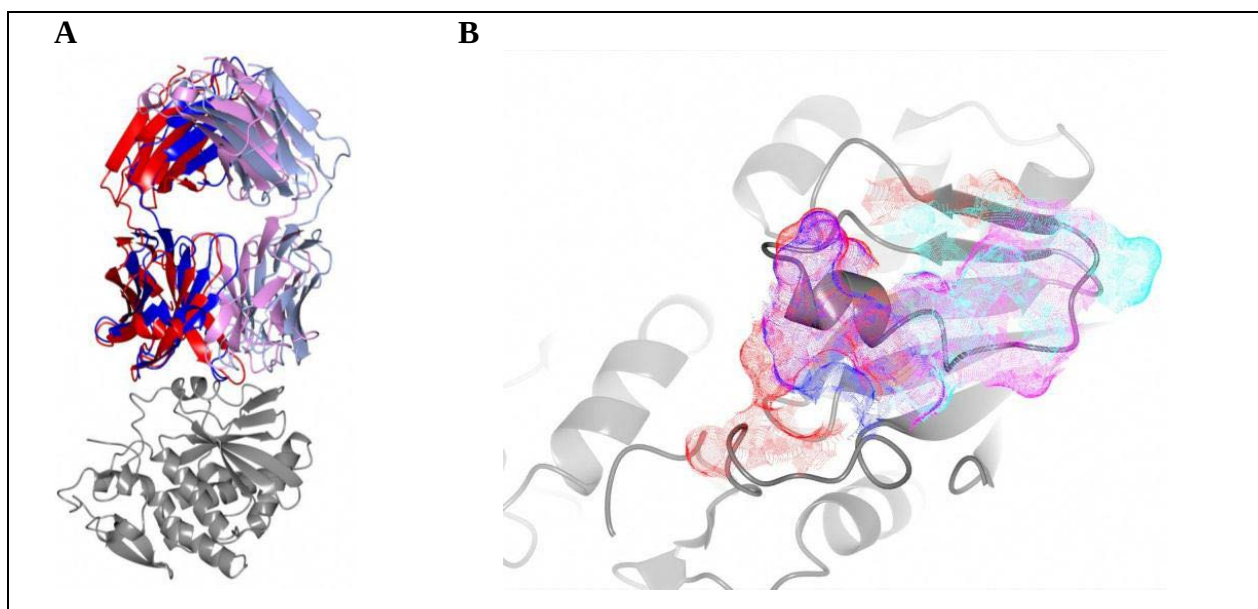


Figure 21. Superimposition of the Fab–RiVax complex and PDB ID: 4KUC showing both Fabs (A) binding the same region of the antigen (coloring scheme as shown in panel B). (B) Molecular surface representation that highlights the differences in the epitope recognized by each Fab. Regions contacting the heavy chains are in red and blue, and those contacting the light chains are in pink and light blue for AxioMx IgX 2691 Fab and 6C2 Fab (4KUC), respectively.

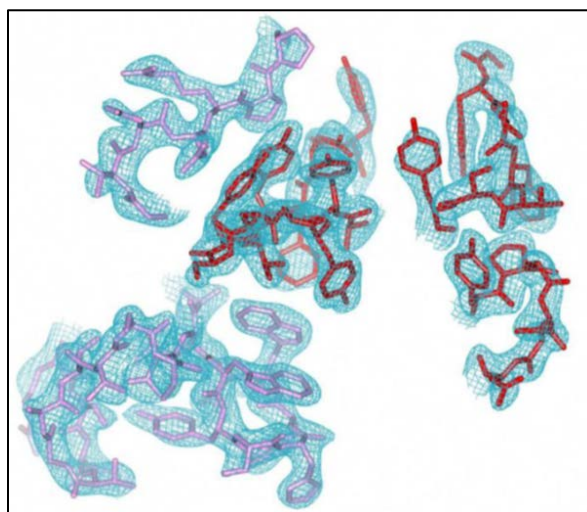


Figure 22. Refined $|2F_o - F_c|$ electron density of RiVax–IgX 2691 Fab. The surrounding six CDRs are colored in blue and contoured at 1.0 σ . (Left) light chain loops (pink cylinders) and (right) heavy chain loops (red).

4.1.2 IgX 2691 Fab Preparation

Anti-RiVax antibody was frozen at approximately 8 mg/mL in PBS and 15% glycerol and sent to Cedars-Sinai. An aliquot of this solution was subsequently thawed and digested using the Pierce Fab preparation kit (catalog no. 44985; Thermo Fisher Scientific, Inc.; Waltham, MA), which uses immobilized papain for antibody cleavage. For this procedure, 0.5 mL of antibody was exchanged into digestion buffer, incubated with immobilized papain overnight at room temperature, and then the Fab was separated and purified using a protein A column.

4.1.3 RiVax–IgX 2691 Fab Size-Exclusion Chromatography (SEC)

Fab was concentrated to 12.6 mg/mL in PBS using a Millipore centrifugal filter unit with a 10 kDa molecular weight cutoff (MilliporeSigma; Burlington, MA). This was then mixed in an equimolar ratio with RiVax at 0.92 mg/mL concentration in 20 mM sodium phosphate and 20% glycerol before being passed over a HiLoad 16/600 Superdex 200 size-exclusion column (GE Healthcare Life Sciences; Pittsburgh, PA) using 150 mM sodium chloride and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.5 as an elution buffer. Peak fractions were pooled and concentrated to 9 mg/mL (assuming a 280 nm extinction coefficient of 1.112) using a Millipore centrifugal filter unit with a 10 kDa molecular weight cutoff (Figure 23).

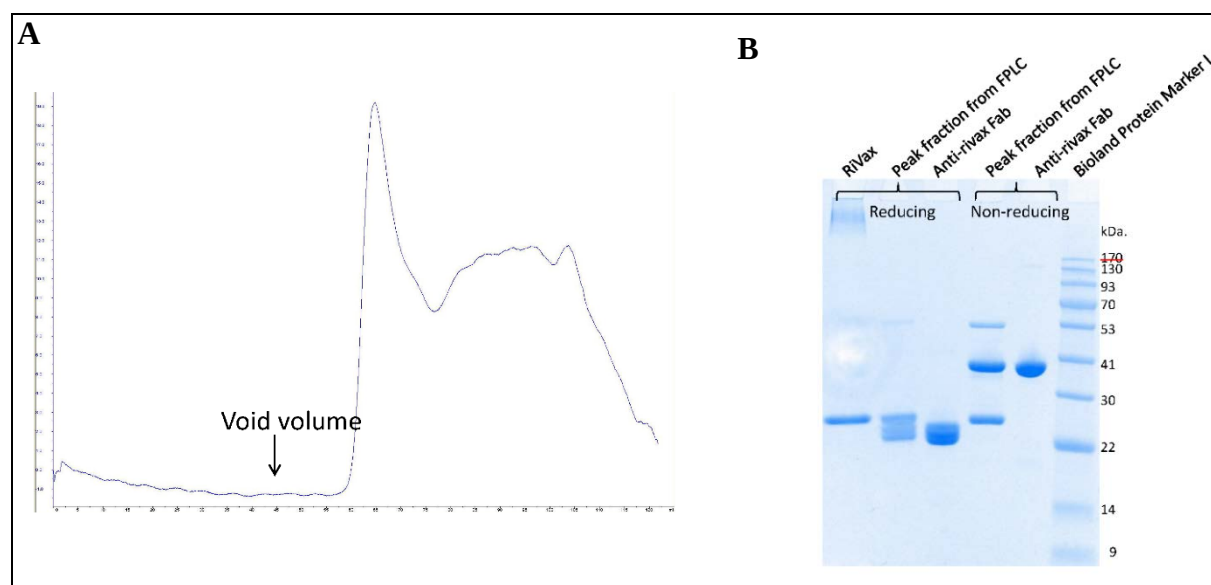


Figure 23. AxioMx IgX 2691 and anti-RiVax Fab preparation.

Figure 23 shows the RiVax–IgX 2691 Fab complexes that were formed by mixing 851 μ g of RiVax in 20 mM sodium phosphate and 20% glycerol with 945 μ g of IgX 2691 Fab in PBS from the Pierce Fab preparation kit. Figure 23A shows peak fractions of the complex as they were pooled and concentrated after elution from a HiLoad 16/600 Superdex 200 SEC column using 20 mM HEPES at pH 7.5 and 150 mM sodium chloride buffer that was run at a

1 mL/min flow rate and 1 mL fractions. Figure 23B shows the peak fractions that contain both the RiVax and IgX 2691 Fab, as shown in a 12% SDS-PAGE (lanes 2 and 4). The band at ~53 kDa in the fast protein liquid chromatography peak fraction was unexpected, although the absence of the band in both the RiVax and Fab-only fractions suggests that it might be a hyperstable complex that is not denatured under gel conditions. Note that the 53 kDa band is more intense in nonreducing conditions relative to reducing conditions, as might be expected given the disulfide stabilization in IgX 2691.

4.1.4 RiVax–IgX 2691 Fab Crystallization

Crystal screening and optimization was performed with protein at 9 mg/mL and at 18 °C. A number of positive results were found with the Hampton Research polyethylene glycol (PEG)/Ion condition E12 screening kit (Hampton Research; Aliso Viejo, CA), which produced crystals that diffracted to approximately 3.5 Å when tested using the in-house X-ray source. The crystals were optimized to produce hotdog-like crystals in 18% PEG 3350 and 10% tacsimate at pH 5.0. These hotdog-like crystals diffracted at 2.7 Å at the synchrotron X-ray source (Figure 24).

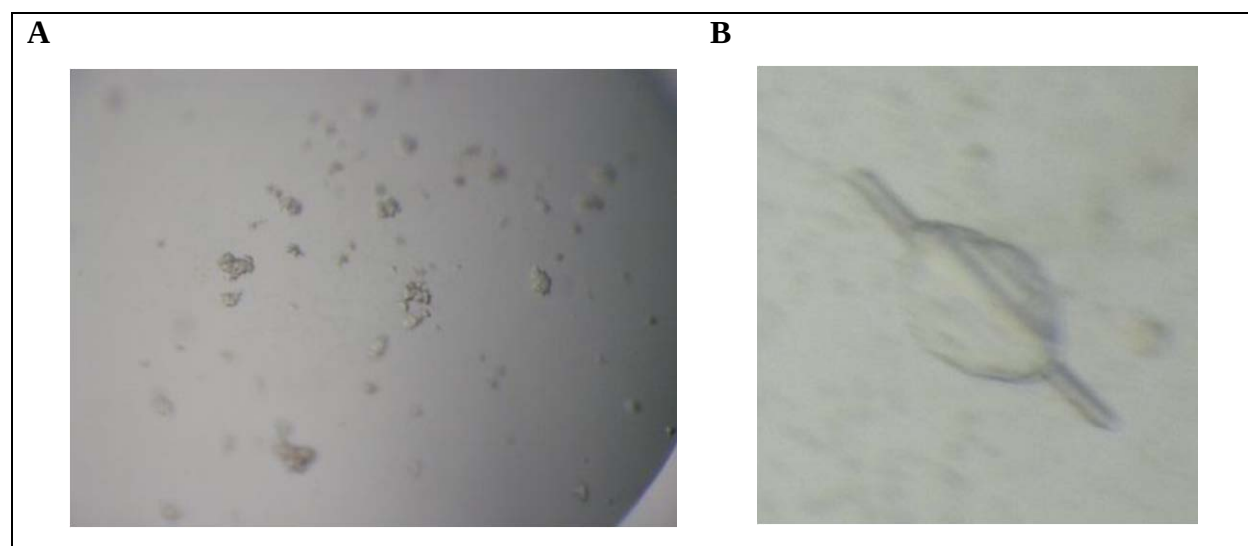


Figure 24. RiVax–IgX 2691 Fab co-crystals. (A) Image of initial positive result from condition E12 of Hampton Research PEG/Ion screen, and (B) image of optimized crystal.

4.1.5 RiVax–IgX 2691 Fab Data Collection and Processing

Crystals were harvested in nylon cryo-loops and plunged into liquid nitrogen without the need for cryo-protectant. Data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL; Stanford University; Menlo Park, CA) using beam-line BL7-1 with the beam positioned at the “sausage” end of the hotdog-like crystal. Slight ice rings were observed in the diffraction images, but these were sufficiently weak and did not affect the overall quality of the data. A summary of data collection details and statistics is provided in Table 4.

Table 4. RiVax–IgX 2691 Fab Crystal Data Collection Details

Parameter	Value
Radiation source	SSRL/BL7-1
Detector name (distance to crystal)	ADSC Q315R (300 mm)
Wavelength	1.1271 Å
Oscillation angle per image	0.5°
Exposure time per image	60 s
Number of images collected	360

Integration of diffraction data was performed using iMosflm software (Medical Research Council Laboratory of Molecular Biology; Cambridge, England) (8), and scaled to 2.7 Å using the Scala programming language (9). Five percent of the reflections were set aside for calculation of R_{free} , which is a statistical measurement that assesses the quality of a model made using X-ray crystallographic data. Results are summarized in Table 5.

Table 5. RiVax–IgX 2691 Fab Crystal Data Collection Statistics

Parameter	Value
Resolution range (highest resolution shell)	70.85–2.70 Å (2.85–2.70 Å)
R_{merge}	13.5% (61.2%)
Total number of observed reflections	132082 (19696)
Total number of unique reflections/multiplicity	41874 (6091)/3.2 (3.2)
Mean I/σ	7.9 (2.2)
Completeness	99.5% (93.8%)
Average mosaicity	0.64
Space group	P21
Unit cell dimensions	$104.4 \times 70.9 \times 116.3$ Å $90.0 \times 116.7 \times 90.0^\circ$

R_{merge} describes the agreement between multiple measurements of a data set; mean I/σ is the mean signal-to-noise ratio; and numbers in parentheses indicate the standard deviations.

4.1.6 RiVax–IgX 2691 Fab Structure Phasing, Model Building, and Refinement

Initial phases were calculated using the molecular-replacement technique as implemented in the PHASER crystallographic software (University of Cambridge; Cambridge, England) (10). The published crystal structure of RiVax, in complex with another neutralizing Fab, was used as the search model (PDB ID: 4KUC) (7) with the antigen and variable domains of the Fab split into separate search ensembles. Two copies of the complex were found in the asymmetrical unit, giving a final translation function Z-score of 19.8. The resulting map allowed placement of the constant domains of the Fab using the MOLREP molecular replacement software (A.A. Vagin; CCP4 Research Complex at Harwell; Oxon, U.K.) (11). The model was then automatically rebuilt with the correct Fab sequence using the BUCCANEER protein model-building software (The University of York; York, England) (12). Alternating rounds of model

building were performed using the crystallographic object-oriented toolkit (COOT) software (Medical Research Council Laboratory of Molecular Biology) (13). Refinement was accomplished using the macromolecular crystallographic refinement program, REFMAC5, (University of York) (14). These processes were repeated until all possible residues had been correctly fitted including all residues of the six CDRs. Waters were subsequently included, and the whole model was refined until R_{free} convergence was achieved. The translation–liberation–screw (TLS) vibrational motion parameters were included and refined during the last round of refinement.

The final PDB is based upon the Kabat numbering convention as follows: RiVax chains are labeled A and B, heavy chains are H and I, light chains are L and M, and water molecules are chain W (Table 6).

Table 6. RiVax–IgX 2691 Fab Structure Refinement and Model Statistics

Parameter	Value
Resolution range	103.94–2.70 Å
$R_{\text{work}}/R_{\text{free}}$	22.5/29.2%
rmsd bond lengths/angles	0.005 Å/0.682 °
Number of residues modeled	1375 (6 chains)
Number of water molecules modeled	89
Average B-factor of all residues	35.8 Å ²
Average B-factor of all waters	19.2 Å ²
Ramachandran plot (RAMPAGE software; University of Cambridge) (15):	
Favored	1319 (97.2%)
Allowed	36 (2.7%)
Outliers	2 (0.1%)

rmsd, root mean square deviation; R_{work} , residual factor or reliability factor.

4.2 Crystal Structure of the Fab of Anaptys 7d11 at 2.64 Å

4.2.1 Summary of AnaptysBio 7d11 Fab Structure

The crystal structure of the Fab from AnaptysBio 7d11 was solved to 2.64 Å resolution (Figure 25A). Two copies of the fragment in the asymmetric unit are related by 2-fold, noncrystallographic symmetry, and they share a near-identical tertiary structure with a $C\alpha$ rmsd of 0.5 Å over 430 residues of both heavy and light chains. It should be noted that as the Fab was crystallized in the absence of its antigen, crystal packing forces could have played a role in influencing the observed loop topologies.

Structural comparison with two published anti-L1R Fabs, in complex with the antigen L1R, shows that sequence similarity within the CDRs is reflected by conservation in topology. Fab 7d11 of PDB ID: 2I9L (4) shares identical CDR residues with AnaptysBio 7d11, whereas Fab M12B9 of PDB ID: 4U6H (16) only shares identical or near-identical residues for CDRs H1 and H2; structural similarities are illustrated in Figure 25B. Close inspection shows

that although the H3 loops of AnaptysBio 7d11 and Fab 7d11 of PDB ID: 2I9L are composed of identical amino acids, there is a variation in backbone structure surrounding residues Asp98 and Gly99 (Kabat numbering)—the more disordered loop of the former Fab structure is likely a result of being crystallized in the absence of L1R, and therefore, lacked interactions with its epitope (Figure 26).

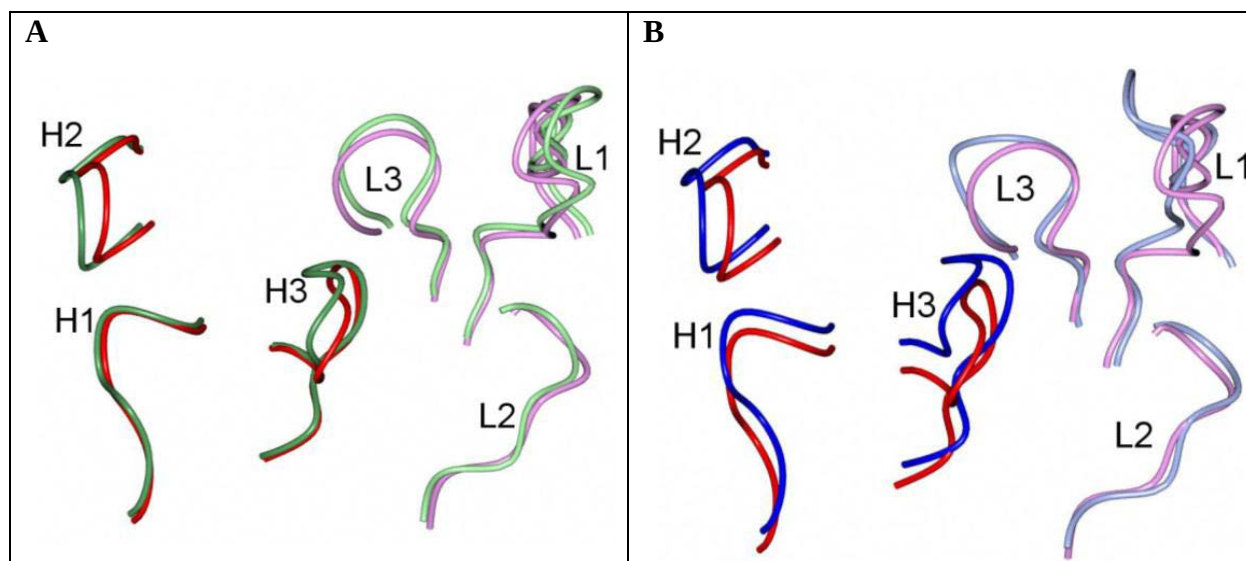


Figure 25. CDR superimposition of Fab 7d11 (PDB ID: 2I9L) versus AnaptysBio 7d11. (A) Heavy chains (green) and light chains (light green) onto the corresponding loops of AnaptysBio 7 d11 (red and pink). (B) Superimposition of the CDRs of Fab M12B9 (PDB ID: 4U6H) with heavy chains (blue) and light chains (light blue) onto the corresponding loops of AnaptysBio 7d11.

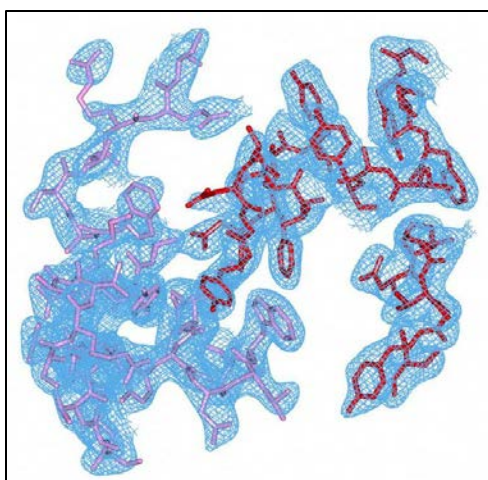


Figure 26. Refined $|2F_o - F_c|$ electron density of Anaptys 7d11 Fab surrounding the six CDRs (blue), which are contoured at 1.0 σ . (Left) light chain loops (pink cylinders) and (right) heavy chain loops (red).

4.2.2 AnaptysBio 7d11 Fab Preparation

The AnaptysBio 7d11 antibody was frozen at approximately 3.8 mg/mL in PBS and 15% glycerol and sent to Cedars-Sinai. An aliquot of this solution was subsequently thawed and digested using the Pierce Fab preparation kit (catalog no. 44985), which uses immobilized papain for antibody cleavage. For this procedure, 0.5 mL of antibody was exchanged into digestion buffer, incubated with immobilized papain overnight at room temperature, and then the Fab was separated and purified using a protein A column.

4.2.3 AnaptysBio 7d11 SEC

The Fab eluates from the protein A were combined and then passed over a HiLoad 16/600 Superdex 200 size-exclusion column using 150 mM sodium chloride and 20 mM HEPES at pH 7.5 as the elution buffer. Peak fractions were pooled and concentrated to 9.3 mg/mL (assuming a 280 nm extinction coefficient of 1.4) using a Millipore centrifugal filter unit with a 10 kDa molecular weight cutoff (Figure 27).

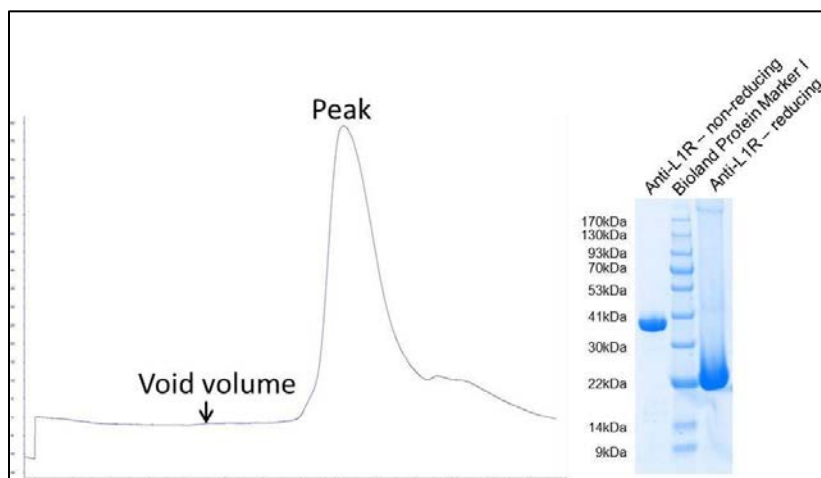


Figure 27. AnaptysBio 7d11 Fab size-exclusion chromatogram showing (left) peak fractions of the 7d11 Fabs that were pooled and concentrated, and (right) SDS-PAGE gel confirming that the fractions contain high-purity Fab.

4.2.4 AnaptysBio 7d11 Fab Crystallization

Crystal screening and optimization were performed with protein at 8.3 mg/mL and 18 °C. Many positive results were found, but all of these were very thin, multilayered crystals that diffracted poorly. Crystals from Hampton Research Crystal Screen condition G2 appeared to have the thickest plates and diffracted in-house to approximately 10 Å. The crystals were optimized using the microseeding technique in 0.2 M ammonium sulfate, 24% 5000 monomethyl ether (MME), and 0.5 M 2-(*N*-morpholino)ethanesulfonic acid (MES) at pH 6.5 (Figure 28).



Figure 28. Image of optimized AnaptysBio 7d11 Fab crystal.

4.2.5 AnaptysBio 7d11 Fab Data Collection and Processing

Crystals were harvested in nylon cryo-loops and plunged into liquid nitrogen without the need for cryo-protectant. Data were collected using the in-house X-ray source. A summary of the data collection details and statistics is provided in Table 7.

Table 7. AnaptysBio 7d11 Fab Data Collection Details

Parameter	Description
Radiation source	Rigaku MicroMax-007 HF
Detector (distance to crystal)	Rigaku R-Axis IV++ (220 mm)
Wavelength	1.5418 Å
Oscillation angle per image	0.5°
Exposure time per image	420 s
Number of images collected	171 and 360

Rigaku Oxford Diffraction; The Woodlands, TX.

Due to technical issues with the generator, two datasets were collected and merged during data processing. The xia2 data processing system (xia2 HQ; Chilton, U.K.) (17) was used for automated data processing, and X-ray detector software (XDS; Max Planck Institute for Medical Research; Heidelberg, Germany) (18) was used to integrate, merge, and scale the data. Five percent of the reflections were set aside for the calculation of R_{free} . The results are summarized in Table 8.

Table 8. AnaptysBio 7d11 Fab Data Collection Statistics

Parameter	Value
Resolution range (highest resolution shell)	42.68–2.64 Å (2.71–2.64 Å)
R_{merge}	12.8% (60.0%)
Total number of observed reflections	140200 (7162)
Total number of unique reflections/multiplicity	26809 (1940)/5.2 (3.7)
Mean I/σ	9.8 (1.9)
Completeness	99.9% (99.9%)
Average mosaicity	0.22
Space group	P21
Unit cell dimensions	$88.5 \times 55.3 \times 95.0$ Å $90.0 \times 101.4 \times 90.0^\circ$

Numbers in parentheses indicate the standard deviations.

4.2.6 AnaptysBio 7d11 Fab Phasing, Model Building, and Refinement

Initial phases were calculated using the molecular-replacement technique as implemented in PHASER (10). This step used the crystal structures of two sequence-related Fabs as search models, PDB ID: 4J1U (19) for the light chain and PDB ID: 4LLW (20) for the heavy chain, with the antigen and variable domains of the Fabs split into separate search ensembles. Two copies of the complex were found in the asymmetric unit, which resulted in a final translation function Z-score of 22.8. The model was then automatically rebuilt with the correct Fab sequence using the python-based hierarchical environment for integrated xtallography (PHENIX) software (Lawrence Berkeley Laboratory; Berkeley, CA) (21). Alternating rounds of model building using COOT software (13) and refinement using the PHENIX software were performed until all possible residues had been correctly fitted, including all residues of the six CDRs. Waters were subsequently included and the whole model was refined until R_{free} convergence was achieved, which included refining the TLS parameters during the last round of refinement (Table 9).

Amino acids of the final PDB are based on the Kabat numbering convention as follows: heavy chains are H and I, light chains are L and M, and water molecules are chain W.

Table 9. AnaptysBio 7d11 Fab Refinement and Model Statistics

Parameter	Value
Resolution range	40.02–2.64 Å
$R_{\text{work}}/R_{\text{free}}$	22.6/27.9 %
rmsd bond lengths/angles	0.003 Å/0.712 °
Number of residues modeled	864 (4 chains)
Number of water molecules modeled	122
Average B-factor of all residues	47.6 Å ²
Average B-factor of all waters	36.0 Å ²
Ramachandran plot (RAMPAGE software) (15):	
Favored	804 (94.6%)
Allowed	37 (4.4%)
Outliers	(1.1%)

4.3 Crystal Structure of the AnaptysBio 7d11 Graft Fab in Complex with L1R at 2.15 Å

4.3.1 Summary of L1R–AnaptysBio 7d11 Fab Structure

The crystal structure of the Fab from AnaptysBio 7d11 was solved in complex with its antigen, L1R, to 2.15 Å resolution (Figure 29A). Comparison with a related L1R/Fab complex, PDB ID: 2I9L (4), shows that the Fab–epitope interface is highly conserved and contains only CDRs from the heavy chain, which form significant contacts with the antigen (Figure 29B). This observation is expected because the hypervariable loops of both the heavy and light chains are identical between the two Fabs. However, superimposition of L1R from both structures shows a slight relative rotation between the Fab and antigen (overall $\text{C}\alpha$ rmsd of 1.8 Å for 85% of residues from the complex). This topological difference is most likely related to differences in crystal packing rather than differences in amino acid composition of the Fab (Figure 30).

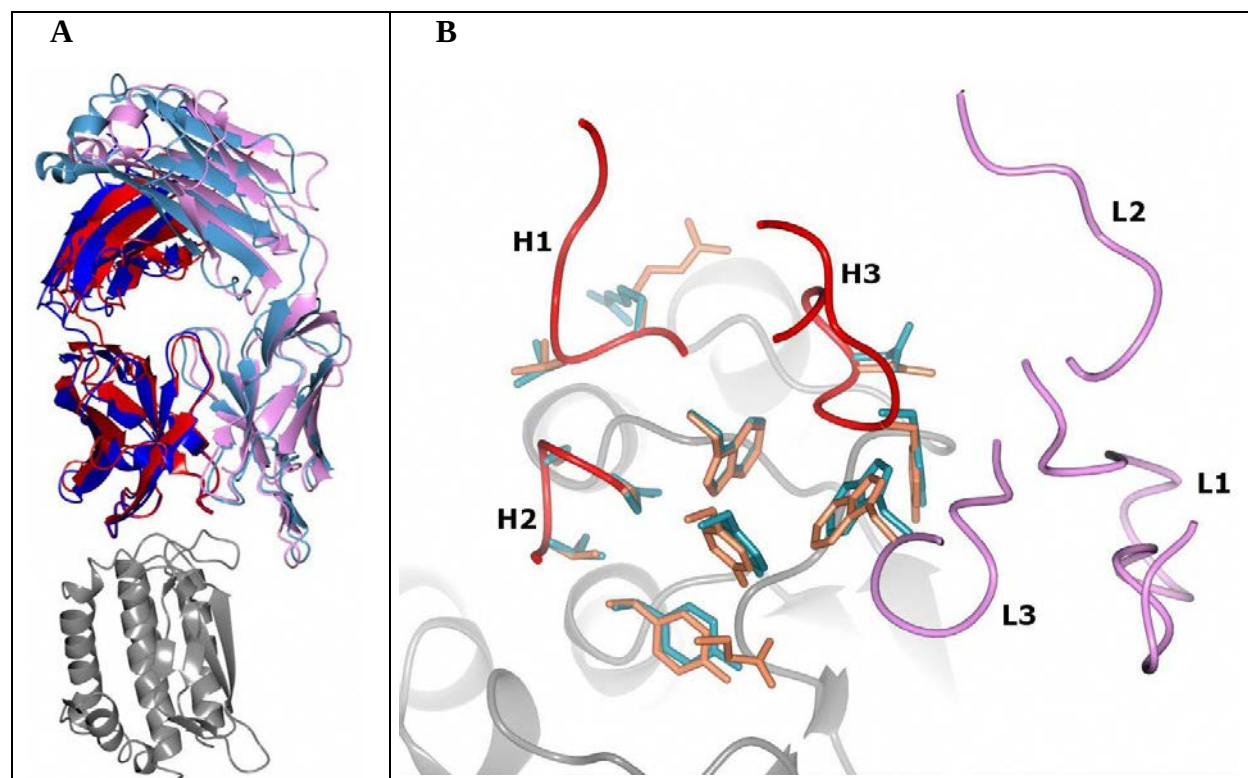


Figure 29. Ribbon diagram showing superimposition of the AnaptysBio 7d11–L1R complex and PDB ID: 2I9L. (A) L1R (gray); the heavy and light chains of AnaptysBio 7d11 (red and pink, respectively); and the heavy and light chains of 7D11 (dark blue and light blue, respectively). (B) Diagram highlighting conservation of side chains (AnaptysBio 7d11 [orange] and 7D11 [teal]) from the hypervariable loops of the heavy and light chains that lie within 4 Å of L1R (coloring scheme as shown in panel A).

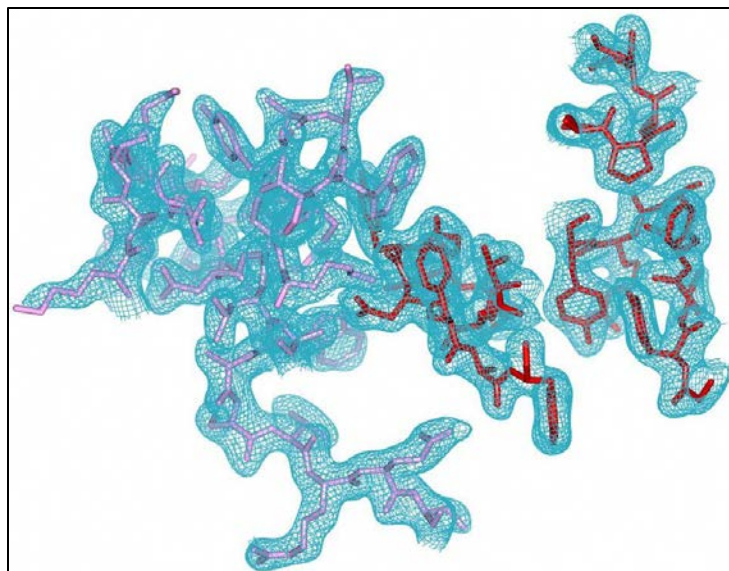


Figure 30. Refined $2|F_o - F_c|$ electron density of L1R–AnaptysBio 7d11 Fab structure. The surrounding six CDRs (blue) are contoured at 1.0σ . (Left) light chain loops (pink cylinders) and (right) heavy chain loops (red).

4.3.2 AnaptysBio 7d11 Fab Preparation

The client supplied the anti-L1R antibody frozen at approximately 2 mg/mL in PBS. An aliquot of this solution was subsequently thawed and digested using the Pierce Fab preparation kit (catalog no. 44985), which uses immobilized papain for antibody cleavage. For this procedure, 0.5 mL of antibody was exchanged into digestion buffer, incubated with immobilized papain overnight at room temperature, and then the Fab was separated and purified using a protein A column.

4.3.3 L1R–AnaptysBio 7d11 Fab Complex Formation and SEC

The Fab eluates from the protein A were combined and then passed over a HiLoad 16/600 Superdex 200 size-exclusion column using 150 mM sodium chloride and 20 mM HEPES at pH 7.5 as the elution buffer. Peak fractions were pooled and concentrated to 9.3 mg/mL (assuming a 280 nm extinction coefficient of 1.4) using a Millipore centrifugal filter unit with a 10 kDa molecular weight cutoff (Figure 31).

The Fab was concentrated to 10 mg/mL in PBS using a Millipore centrifugal filter unit with a 10 kDa molecular weight cutoff. This was then mixed in a 4–5 excess molar ratio with client-supplied L1R at an 8 mg/mL concentration in PBS before being passed over a HiLoad 16/600 Superdex 75 size-exclusion column using 150 mM sodium chloride and 20 mM HEPES at pH 7.5 as the elution buffer and running at 1 mL/min. Peak fractions were pooled and concentrated to 10 mg/mL (assuming a 280 nm extinction coefficient of 1.311) using a Millipore centrifugal filter unit with a 10 kDa molecular weight cutoff.

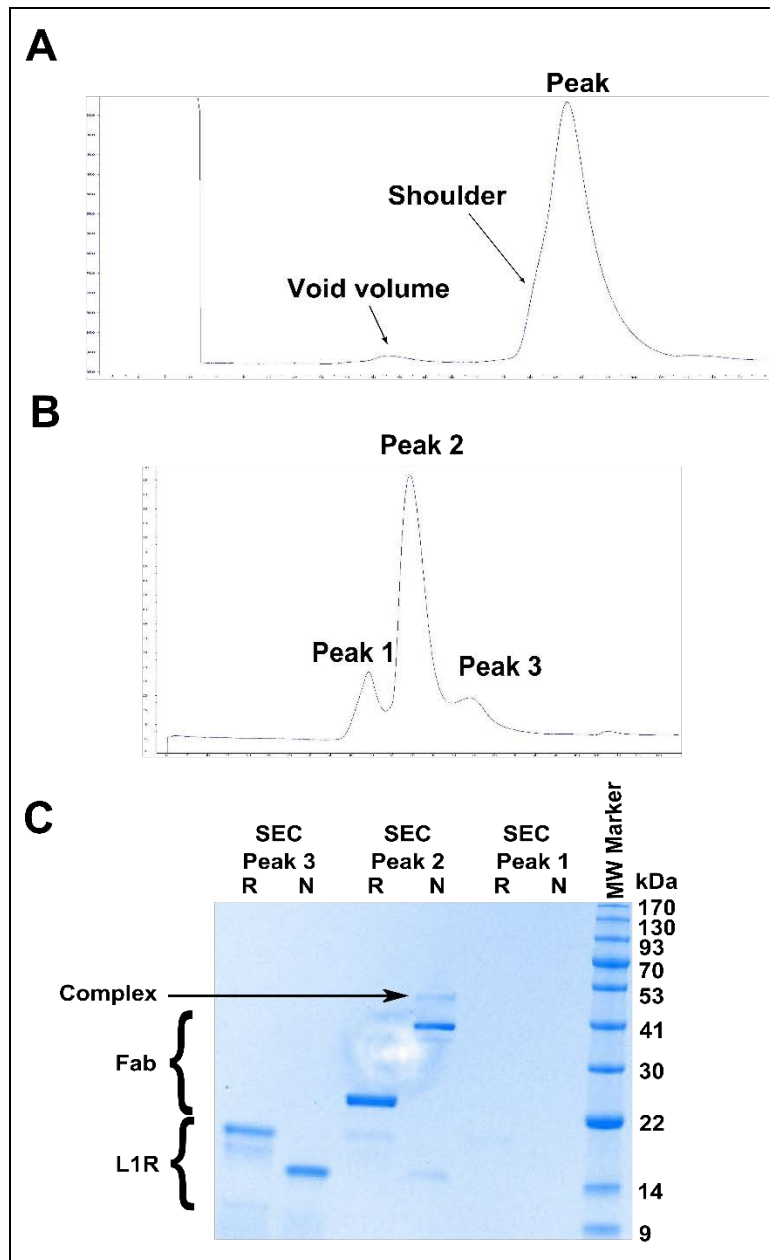


Figure 31. Purification of the L1R-anti-L1R Fab complex.

L1R-anti-L1R Fab complexes were made by equimolar mixing of 1.2 mg anti-L1R Fab with 0.72 mg L1R and diluted with 4 mL of 20 mM HEPES at pH 7.5, 150 mM sodium chloride. Figure 31A shows the results from the L1R-anti-L1R Fab mixture that was loaded onto an S200 SEC column to collect 1 mL fractions at 1 mL/min with a running buffer of 20 mM HEPES at pH 7.5, 150 mM sodium chloride. Figure 31B shows the peak fraction recovered from the S200 column, which was concentrated to 10 mg/mL; mixed with an additional 3.2 mg L1R (4- to 5-fold molar excess); diluted with 4 mL of 20 mM HEPES at pH 7.5, 150 mM sodium chloride; and run on an S75 SEC column as stated herein. Figure 31C shows the results from a

polyacrylamide gel (12%) with reduced (R) and nonreduced (N) samples, which indicates that Peak 2 exhibited the complex mixed with Fab and L1R alone, and Peak 3 was of L1R alone.

4.3.4 L1R–AnaptysBio 7d11 Fab Crystallization

Crystal screening was performed with 10 mg/mL of protein at 18 °C. A number of positive results were found, and the Hampton Research Index condition G6 produced a crystal that diffracted to approximately 3.5 Å using the in-house X-ray source (Figure 32). For screening, the crystal was mounted on a nylon cryo-loop and plunged into liquid nitrogen without cryo-protectant.



Figure 32. Image of initial positive result for L1R–AnaptysBio 7d11 Fab complex from condition G6 of the Hampton Research Index screen.

4.3.5 L1R–AnaptysBio 7d11 Fab Data Collection and Processing

After an initial diffraction trial of the crystal that was obtained with the Hampton Research Index condition G6, the crystal was placed back in the drop, and the plate was resealed.

Approximately 3 weeks later, the crystal was re-examined for diffraction using the same mounting and cryo-cooling procedure as before. The crystal showed good diffraction to better than 2.2 Å; however, weak diffraction from additional lattices was also observed, but this was subsequently shown not to adversely affect data quality. A summary of data collection details and statistics is given in Table 10.

Table 10. L1R–AnaptysBio 7d11 Fab Data Collection Details

Parameter	Value
Radiation source	Rigaku MicroMax-007 HF
Detector (and distance to crystal)	Rigaku R-Axis IV++ (175 mm)
Wavelength	1.5418 Å
Oscillation angle per image	0.5°
Exposure time per image	420 s
Number of images collected	546

Integration of diffraction data were performed using XDS (18) and scaled to 2.15 Å using the AIMLESS software (Medical Research Council Laboratory of Molecular Biology) (22), and 5% of the reflections were set aside for the calculation of R_{free} . Results are summarized in Table 11.

Table 11. L1R–AnaptysBio 7d11 Fab Data Collection Statistics

Parameter	Value
Resolution range (highest resolution shell)	29.00–2.15 Å (2.22–2.15 Å)
R_{merge}	8.3% (40.4%)
Total number of observed reflections	172471 (10166)
Total number of unique reflections/multiplicity	29772 (1955)/5.8 (5.2)
Mean I/σ	11.8 (3.2)
Completeness	94.7% (72.3%)
Average mosaicity	0.24
Space group	$P2_1$
Unit cell dimensions	$65.2 \times 56.7 \times 83.7$ Å $90.0 \times 109.7 \times 90.0^\circ$

Numbers in parentheses indicate the standard deviations.

4.3.6 L1R–AnaptysBio 7d11 Fab Structure Phasing, Model Building, and Refinement

Initial phases were calculated using the molecular-replacement technique, as implemented in PHASER (10). For this technique, the previously solved crystal structure of Fab AnaptysBio 7d11 alone and L1R from PDB ID: 2I9L were used as search models, and the heavy and light chains of the Fab were split into separate search ensembles. One copy of the complex was found in the asymmetric unit, and it gave a final translation function Z-score of 43.3. Then the model was automatically rebuilt with the correct Fab sequence using the PHENIX software (21). Alternating rounds of model-building using the COOT software (13) and refinement using the REFMAC5 program (14) were performed until all possible residues had been correctly fitted, including all residues of the six CDRs. Waters were subsequently included, and the whole model was refined until R_{free} convergence was achieved.

The final PDB is based on the Kabat numbering convention as follows: The L1R chain is labeled A; the heavy and light chains are labeled H and L, respectively; and water molecules are chain W (Table 12).

Table 12. L1R–AnaptysBio 7d11 Fab Structure Refinement and Model Statistics

Parameter	Value
Resolution range	78.80–2.15 Å
R _{work} /R _{free}	18.2/24.7%
rmsd bond lengths/angles	0.002 Å/0.993°
Number of residues modeled	604 (3 chains)
Number of water molecules modeled	256
Average B-factor of all residues	37.2 Å ²
Average B-factor of all waters	39.3 Å ²
Ramachandran plot (RAMPAGE software) (15):	
Favored	569 (96.0%)
Allowed	22 (3.7%)
Outliers	2 (0.3%)

5. TASK 4: USING UCNPS TO CREATE MORE SENSITIVE AND MULTIPLEX-CAPABLE LFIS.

Using conventional gold nanoparticles or UCNP reporters and otherwise identical antibodies and construction, LFIs were manufactured to detect the ricin A chain toxin surrogate. Estimated LODs were quantified relative to a threshold that was determined based on replicate measurements of analyte-free buffer runs for both gold- and UCNP-based LFIs. UCNP LFIs demonstrated a 4- to 10-fold increase in sensitivity to analyte relative to analogous gold LFI detection limits, which illustrated that UCNPs offer comparable, if not better, sensitivity for a common target. Because UCNPs are amenable to the same coupling chemistries and manufacturing handling processes that are already in widespread use for the production of gold LFIs, the use of UCNPs can offer a low-cost improvement of immunoassay performance that can be easily integrated into existing production streams.

Very small UCNPs are now commercially available to perform highly tunable two-photon upconversion through anti-Stokes emission, for example, to emit strong green light when illuminated with near-infrared light. It is important to note that the multiphoton absorption-phosphorescence upconversion process is completely free of the autofluorescence issues that are associated with ultraviolet excitation of biomolecules, which is a common problem in fluorescence-based methods (23). Increasingly, UCNPs are being employed in whole-animal imaging (24, 25) and in lateral flow-based applications (26–28). Proteins such as antibodies can be conjugated to these materials to offer a potentially significant enhancement to LFIs by enabling a lower LOD compared with the conventional OD-based detection of gold particles and by allowing genuine multiplexing by using nanoparticles that are tuned to emit different wavelengths of light (Figure 33). Here, we provide a direct comparison of UCNP-based LFIs to industry-standard gold nanoparticle-based LFIs (Figure 34).

Initial studies, described in Section 5.1, used the anti-MS2 coat protein (anti-MS2CP) AnaptysBio thermostable IgG to optimize protocols and conditions. This was chosen for its well-characterized behavior and for the abundance of antibody and antigen material that was available in-house.

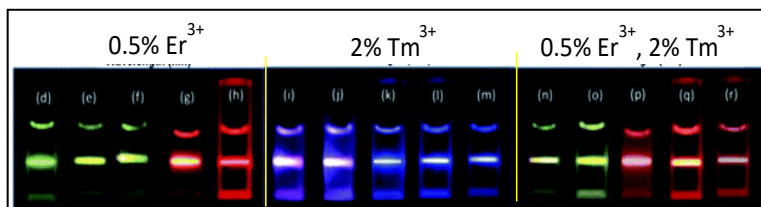


Figure 33. The visible emissions of YF_3 nanocrystals, which were stimulated by infrared excitation at ~ 980 nm, are tuned by doping with erbium (Er), thulium (Tm), or both as the fraction of Yb^{3+} increases from 10–90% (2).

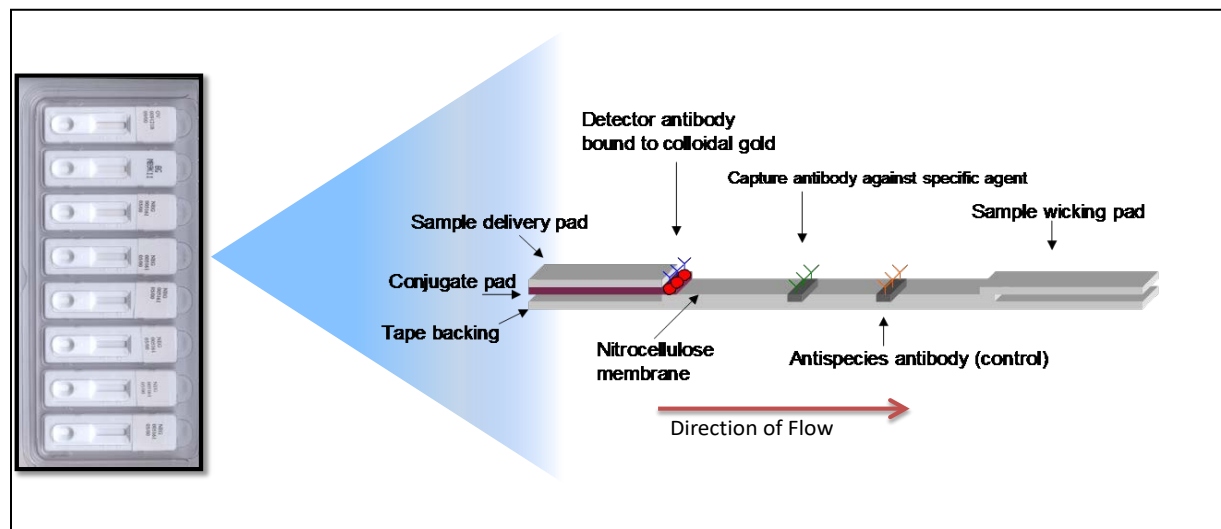


Figure 34. LFI process.

Figure 34 shows the LFI process, in which a sample containing an analyte is placed on a sample pad. The fluid then migrates laterally from the sample pad, and the analyte binds to an antibody that is conjugated to colloidal gold. The analyte that is bound to the antibody–nanoparticle conjugate migrates along the membrane where it binds to a second capture antibody (specific to the target) that forms the test line. Unbound antibody–gold conjugate then binds to the control line (antispecies antibody), which indicates a successful assay.

5.1 Immobilize Antibodies on UCNPs and Assay Affinity

Antibodies were covalently coupled UCNPs that were acquired from the manufacturer Intelligent Material Solutions, Inc. (Princeton, NJ), or they were gold nanoparticles that were developed using conventional 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) chemistry. Using available recombinant MS2CP and anti-MS2CP antibodies, ELISA studies confirmed that antibody–UCNP conjugates could still bind antigen and that nontarget interactions, such as interactions with bovine serum albumin (BSA), were within acceptable limits (Figure 35). Free anti-MS2CP and UCNP-coupled antibodies were able to recognize plated recombinant MS2CP, but UCNPs alone could not do this. Similarly, no binding to BSA was observed for the anti-MS2 antibodies, free UCNPs, or UCNP-coupled antibodies. In these initial experiments, the relative active antibody concentration on the UCNP surface was not quantified.

A second approach to demonstrate antibody–MS2CP binding was specific as follows: the total amount of free antibody that was conjugated to the UCNPs was varied in anticipation that higher coupling densities of antibodies to the UCNP surface should show greater reactivity to the protein target. Increasing the coupling densities from 50 to 100 to 250 μL of coupled antibodies showed an increase in signal intensity at a high dilution of UCNP–antibody probe (Figure 36). True quantitative comparison was not pursued because the fraction of active antibody on individual UCNP–antibody preparations, and the relative concentrations of surface-accessible epitopes on the plate surfaces were not defined. However, global nonlinear least-squares fitting to the semi-quantitative Hill equation indicated that an approximately one-to-one binding model adequately described the data. Increasing the coupling densities yielded UCNP–antibody probes with comparable responses to free antibody alone. Together, these data demonstrated effective antibody–UCNP coupling strategies.

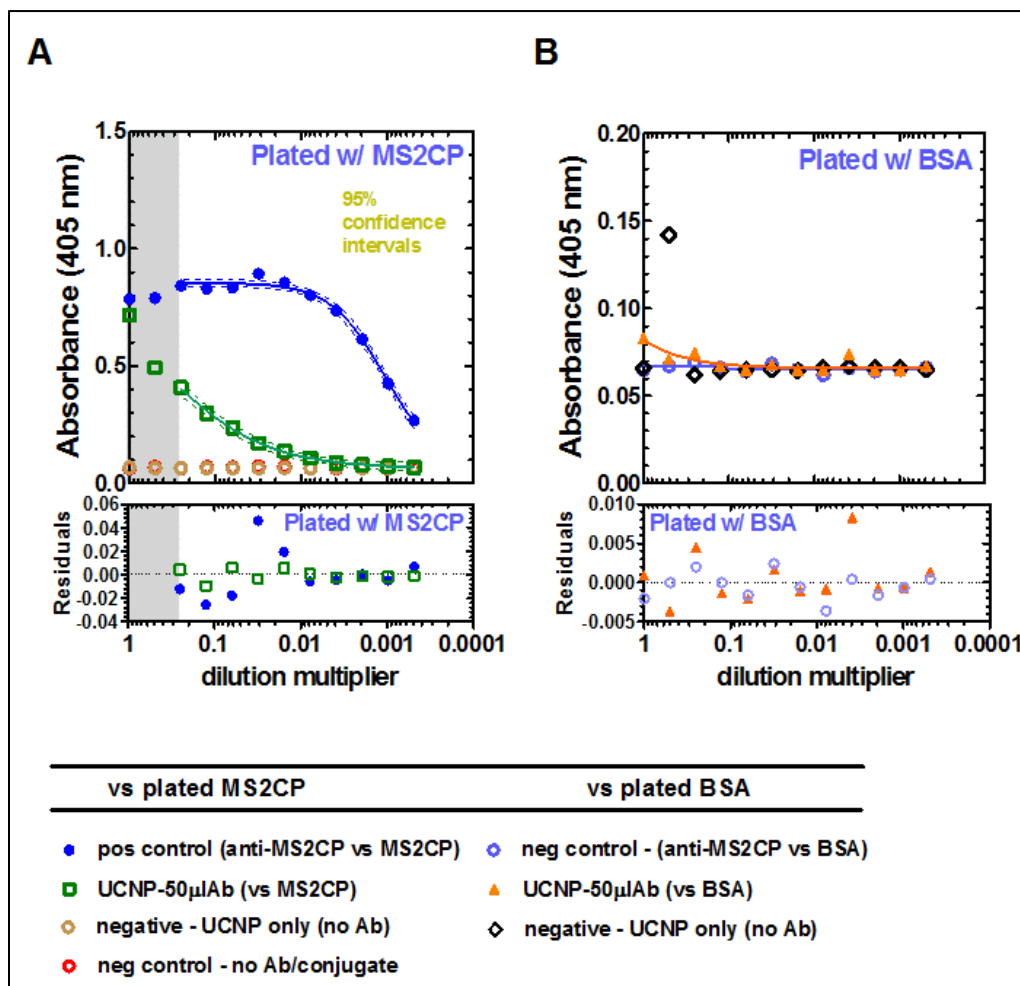


Figure 35. Affinity for UCNP-coupled antibodies for antigen or nonspecific target. (A) Results from plates that were coated with 200 ng/well of MS2CP and were probed with (1) unreacted UCNPs (open gold circles), (2) 40 μ g/mL of free anti-MS2CP antibodies (solid blue circles), (3) UCNPs coupled with 50 μ L of anti-MS2CP antibody (open green squares), or (4) milk diluent and blocking solution (Kirkegaard and Perry Laboratory, Inc. [KPL]; Gaithersburg, MD) (open red circles). These were then diluted 2-fold across the plate. Data, except that shaded with a gray background, were fitted to a Hill equation to obtain a semi-quantitative measure of affinity. (B) Results from plates that were coated with 200 ng/well of BSA and were probed with (1) unreacted UCNPs (black open diamonds), (2) 40 μ g/mL of free anti-MS2CP antibody, or (3) UCNPs coupled with 50 μ L of anti-MS2CP antibody (open cyan circles). Curves were fitted to a Hill equation for illustrative purposes. The lower graphs in panels A and B report the residuals of the fits.

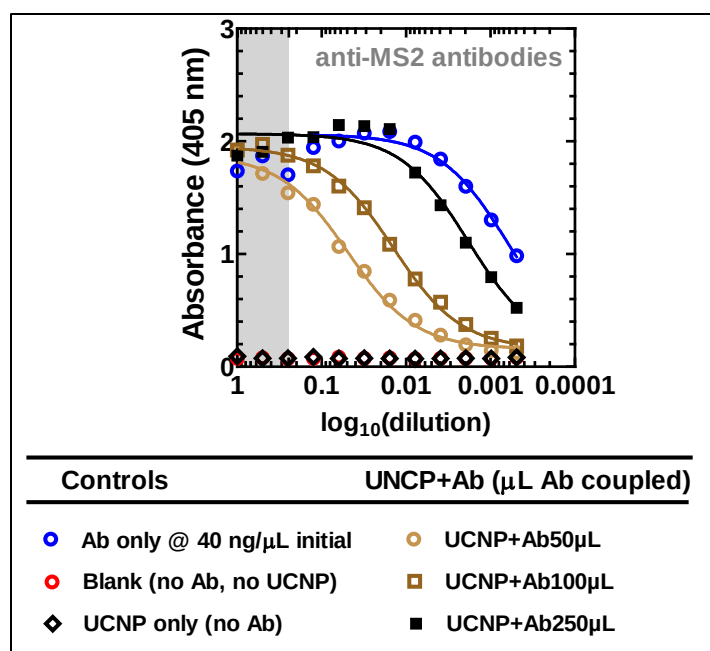


Figure 36. Comparison of affinity for titrating UCNP–antibody-coupling ratios.

Plates that were coated with 200 ng/well of MS2CP were probed with (1) unreacted UCNPs (open black diamonds); (2) 40 μg/mL of free anti-MS2CP antibodies (open blue circles); and (3) UCNPs coupled with increasing anti-MS2CP antibody-coupling ratios as follows: 50 μL of Ab (open gold circles), 100 μL of Ab (open brown squares), 250 μL of Ab (filled black squares), or milk diluent and blocking solution (KPL) (open red circles). These were diluted 2-fold across the plate. Data, excluding the free antibody with the shaded gray background, were fitted to a Hill equation to obtain a semi-quantitative measure of affinity.

5.2 Estimate LOD for IgG–Nanoparticle Adducts in LFI-like Sandwich Assays

5.2.1 UCNP-Only LOD on LFI Strips

Before considering the limit of antigen detection for UCNP-coupled antibodies, the LOD of the UCNPs that were sprayed directly onto an LFI strip was first determined through a collaboration with Intelligent Material Solutions, Inc. and the Centers for Disease Control and Prevention (CDC; Atlanta, GA; POC: Dr. Sukwan Handali). Nitrocellulose LFI strips were sprayed with UCNPs and quantified by emission lifetime measurements that were obtained using a customized Hewlett-Packard Fluorocount BF10000 system (Palo Alto, CA) with an avalanche photodiode detector and a 980 nm excitation source (Figure 37).

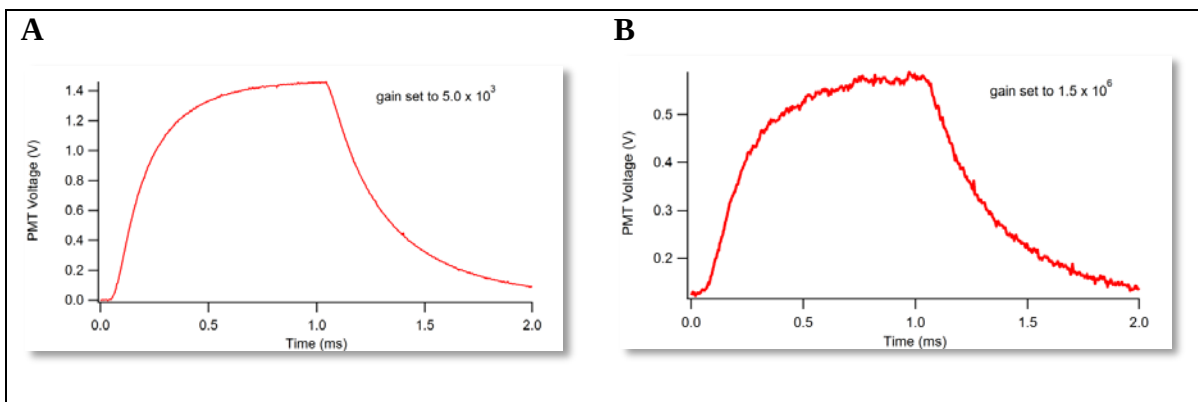


Figure 37. Time-dependent emission from UCNPs at (A) 625 ng/μL and (B) 40 pg/μL.

The photomultiplier tube (PMT) gain was initially set to 0.4V control voltage (5×10^3) but was subsequently adjusted upward for the low range to 0.9V control voltage (1.5×10^6). The electronic gain was 10^5 . No light-tight enclosure was used, and thus a low level of ambient light was present. The excitation source was a 2W 980 nm laser, and the emission was filtered with a 700 nm short-pass filter. The laser was turned on and off to allow for gated detection. The time-dependent emission from the 625 ng/μL strip was fit to a double-exponential with the following parameters:

$$A_1 = 0.534 \pm 0.092 \text{ V}$$

$$\tau_1 = 0.158 \pm 0.11 \text{ ms}$$

$$A_2 = 0.805 \pm 0.84 \text{ V}$$

$$\tau_2 = 0.398 \pm 0.033 \text{ ms}$$

where A_1 and A_2 are the first and second amplitude changes in response to the first and second exponentials, respectively, and τ_1 and τ_2 are the first and second time constants, respectively.

The location of the UCNP stripe on the LFI was readily detectable over 4 orders of magnitude and down to 40 pg/mL (Figure 38). This method was much more sensitive than, for example, the 1 μg/mL LOD that was observed for biotin–streptavidin-coupled gold nanoparticles in a dot blot format (28). Additional studies demonstrated that detection was relatively insensitive to the focusing depth of the UCNP stripe (Figure 39A) and was well-defined across the transverse width of the UCNP band (Figure 39B). After this proof-of-principle demonstration that UCNPs are detectable on an LFI strip, efforts were shifted to using UCNP emission to detect antibody recognition of analyte (Section 5.2.2).

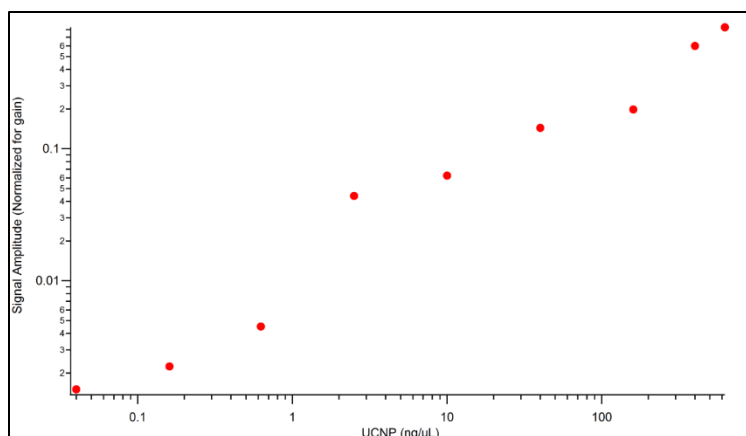


Figure 38. UCNP titration curve from LFI strips after normalizing for additional gain on the low end (lowest three data points).

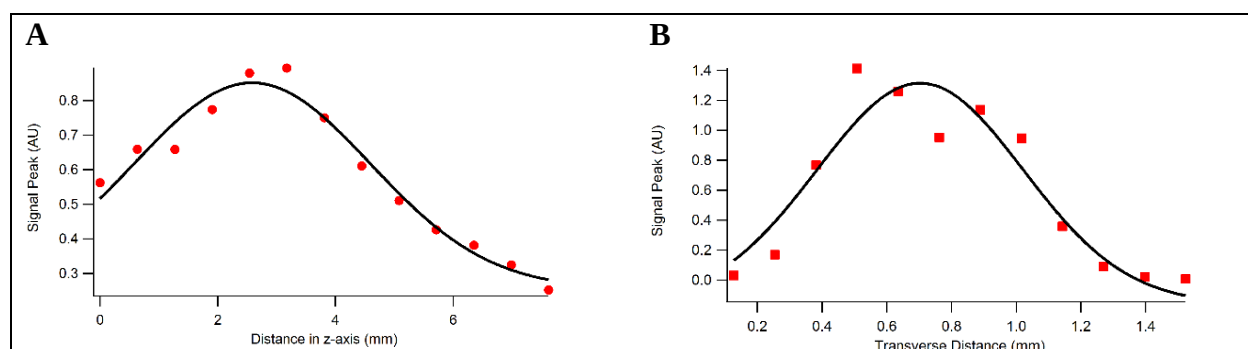


Figure 39. UCNP distribution on LFI membrane coated with 625 ng/μL UCNP per stripe. (A) Z-axis focus was adjusted in increments of 0.025 in. and then converted to millimeters. The resulting signal was plotted and fit to a Gaussian. The plot shows relatively large insensitivity to changes in the z-axis detection threshold. (B) The UCNP signal in the transverse direction (the direction of flow in an operational assay) was measured at optimal z-axis position in increments of 0.005 in., converted to millimeters, fitted to a Gaussian, and plotted.

5.2.2 ELISA Using UCNP Emission Detection in 96-Well Format

It was concluded that UCNP-conjugated antibodies were able to detect their target using standard horseradish peroxidase (HRP)-conjugated antibodies as a reporter in ELISA studies (Section 5.1). In this study, the objective was to test for upconversion emission of UCNPs coupled to antibodies and captured on a surface that was coated by antigen. ELISA plates were coated with recombinant MS2CP, blocked with milk diluent, washed, probed with UCNP-coupled antibodies, and washed again. The presence of antibodies was evaluated using a Leica SP5 confocal/multiphoton microscope (Leica Microsystems; Wetzlar, Germany) to visualize green UCNP emission after near-IR excitation. Figure 40A shows the bright-field distribution of UCNP–antibody conjugates on the plate surface, whereas Figure 40B shows UCNP emission within a subfield of the bright-field image. Hardware limitations precluded the visualization of the entire microtiter well area. Full-signal integration to quantify an LOD, and a serial dilution

series demonstrated the qualitative reduction in signal with decreasing antibody concentration. Previous ELISA studies (cf. Figures 35 and 36) demonstrated that neither UCNP alone nor UCNP–antibody conjugates bind nonspecifically to the ELISA plates under the assay conditions used. The bright green emission of the UCNP was evident with very low background signal. With these promising results in hand, the construction of a continuous-wave excitation detection breadboard device was initiated using commercial off-the-shelf components.

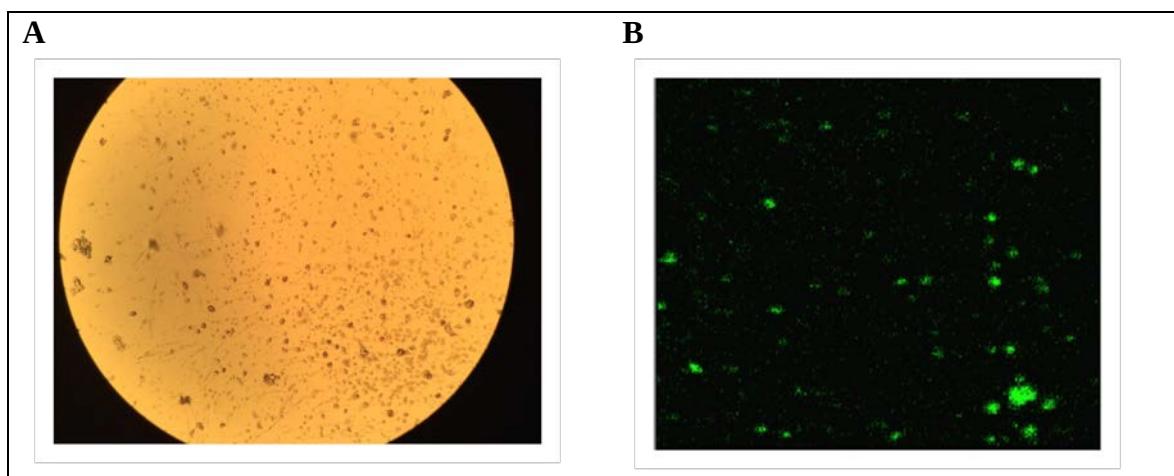


Figure 40. Visualization of antibody-coupled UCNPs. (A) Bright-field view of UCNP–antibody at 10× magnification and 2500 × 2500 μm field of view. (B) UCNP–antibody emission under 980 nm excitation, with a 369 × 369 μm field of view; a subset of the bright-field image in panel A.

5.2.3 Detection of Antibody Conjugates in LFI Format

Before visualizing UCNP–antibody on LFI strips by near-IR upconversion, the antibody detection of MS2CP was first confirmed. LFI strips that were striped with recombinant MS2CP (provided by Dr. Sukwan of the CDC) were soaked in a PBS (10 mM sodium phosphate at pH 7.4, 137 mM sodium chloride, and 2.7 mM potassium chloride) solution of gold nanoparticle-coupled, anti-MS2CP antibodies for 10 min (Figure 41). Figure 41A illustrates a typical LFI configuration that was used here for development and testing efforts. Bio-layer interferometry studies showed that both gold nanoparticle-coupled and UCNP-coupled anti-MS2CP antibodies can bind recombinant protein (data not shown), and these should be successful in a sandwich ELISA format.

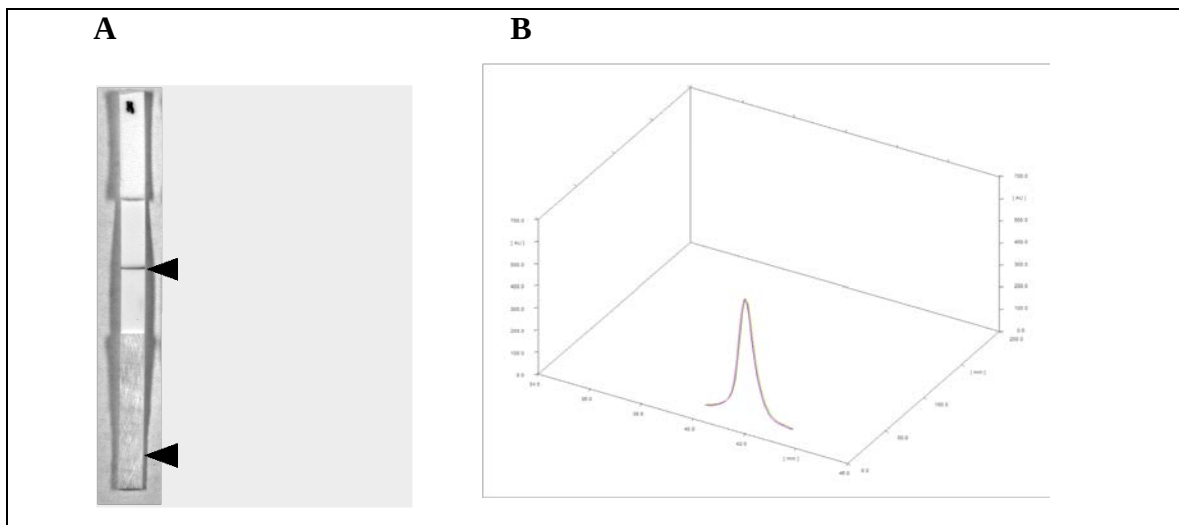


Figure 41. Validation of LFI production process. (A) A nitrocellulose LFI strip that was striped with recombinant MS2CP at 0.66 mg/mL and probed with 1 μ L of 30 μ g/ μ L gold-conjugated AnaptyBio anti-MS2CP antibody that was run in PBS buffer for 10 min. The black spot at the top of the LFI strip is an identification mark. (B) Triplicate scan of an optical dispersion of the LFI strip in panel A was performed at 536 nm using a thin-layer chromatography (TLC) Scanner 4 (CAMAG) and analyzed using winCATS software (CAMAG Chemie-Erzeugnisse und Adsorptionstechnik AG; Muttentz, Germany).

5.3 Construction of an LFI UCNP Detection System

With the demonstration that antigen can be detected by antibodies that are coupled to UCNPs with low cross-reactivity to unrelated proteins and that UCNPs in an LFI format have a signal response that is proportional to UCNP concentration, we began construction of a detection system (Figure 42) with support from Intelligent Material Solutions, Inc. and Mount Mitchell Optics (Asheville, NC). The instrument was built around the Thorlabs, Inc. (Newton, NJ) modular cage mount system. Light from a variable power (0–450 mW), 970 nm laser is passed through a collimating lens before being directed into a 45° dichroic “cold mirror”. The cold mirror reflects <7% of infrared light into an avalanche photodiode for power monitoring and permits the remainder to pass through to the sample. Visible green light from infrared-excited UCNPs is reflected by the cold mirror into a PMT, which transforms the incident light emission into a voltage change. This voltage change is quantified by an analog-to-digital converter (ADC) that is interfaced via a universal serial bus (USB) to a laptop computer. The green emission from the LFI strips that were coated with 200 μ g/mm of UCNPs demonstrated a linear relative response between the PMT that reported the sample emission compared with the silicon detector that monitored the light through the system as the laser power was increased (Figure 43).

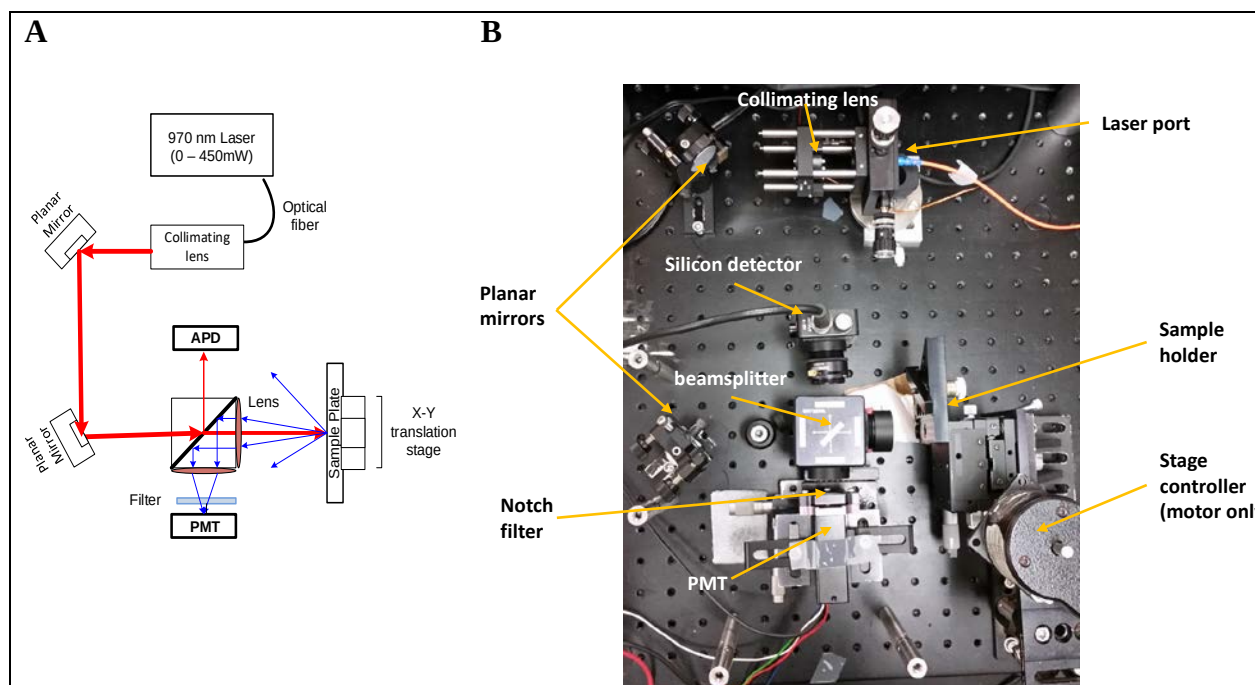


Figure 42. Optical (A) schematic and (B) breadboard implementation of the UCNP detection system that highlights the primary components.

The UCNP detection system is described as follows: Light from a variable power, 0–450 mW continuous-wave 970 nm (infrared) laser (not pictured in Figure 42B) passes through an optical fiber (orange fiber, top right of Figure 42B) and through a collimating lens. The light is then reflected by two planar mirrors into a cold dichroic mirror filter cube (beamsplitter, center of Figure 42B). A small portion (7%) of the laser beam is split into an avalanche photodiode detector (silicon detector, top center of Figure 42B) to monitor the incident beam intensity. The remainder of the beam is directed at the LFI cassette (not pictured) that is held in place on a stage (sample holder, middle right of Figure 42B). The sample holder position can be mechanically scanned in the flow direction and manually adjusted in the remaining two directions. Photons emitted by the nanocrystals are reflected by the dichroic mirror into a PMT (lower middle, Figure 42A and B) and through a notch filter that protects the PMT against undesired laser background detection. When the laser is at full power, the system transfers infrared light to the sample plane with >73% efficiency. The PMT and silicon detector signals are processed by an ADC (not pictured) and transferred to a computer via a USB connection.

The prototype system (Figure 42) delivers 180–320 mW laser power to the sample plane as the laser-setting knob is varied between 0.77 and 1.0 as shown in Figure 43. The silicon detector (Si) output voltage is directly proportional to laser power and serves as a convenient readout of the incident laser intensity. The PMT is set at 0.2 V gain (Figure 43), which is the lowest manufacturer-recommended setting. PMT sensitivity is linear with a gain voltage up to 0.9 V, which offers a broad dynamic range for significantly lower UCNP concentrations.

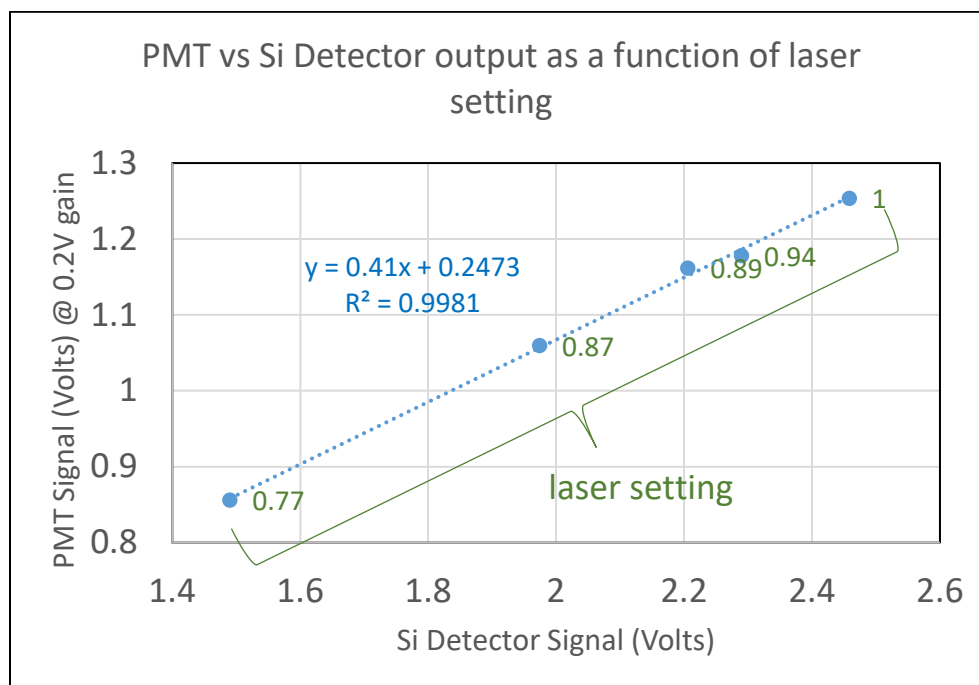


Figure 43. Proportionality of UCNP emission to laser intensity for UCNPs striped onto an LFI membrane at 200 $\mu\text{g}/\text{mm}$.

5.4 Construction of Anti-Ricin LFIs Using Gold or UCNP Reporters

Anti-ricin LFIs were manufactured with either conventional gold nanoparticles (AuNPs) or UCNPs and materials that are otherwise identical. The manufacturing was accomplished through collaboration with a commercial LFI production company (Maxim Biomedical Inc.; Rockville, MD). The LFIs were optimized and manufactured under industry standard conditions to facilitate a performance comparison using LFIs that were produced under the best-case conditions.

5.5 Quantification of Anti-Ricin Gold or UCNP LFI Response

LFIs were dosed with varying concentrations of purified recombinant ricin A chain protein in replicate studies that were performed on different days (cf., Figure 44). AuNPs were read using the commercially-available CAMAG LFI scanner, whereas the UCNP-based LFIs were quantified using the detector prototype described in Section 5.3 (Figure 42). Results from LOD studies spanned 0.1–1000 ng/mL for the gold LFIs and 0.05–500 ng/mL for the UCNP LFIs. Note that the gold and UCNP control line intensities were markedly depleted for analyte concentrations of 500 ng/mL and higher, relative to intensities observed at lower analyte concentrations. The LOD was estimated as the analyte concentration whose signal was at or above a minimum detection threshold defined as the mean $\pm$ 2 standard deviations for triplicate blank (buffer only) runs (Figure 44). For comparison, a semi-quantitative LOD was estimated by calculating the concentration at which a semi-log extrapolated fit crosses the aforementioned detection threshold. The UCNP-based LFIs exhibited an LOD of 10 pg recombinant ricin A

chain, whereas the AuNP LFIs were at least four times less sensitive with an estimated LOD of 25–100 pg. Figure 45 shows the response of an UCNP LFI exposed to 20 pg of ricin A chain overlaid with the intensity (background) of an UCNP LFI dosed with buffer alone. Table 13 provides the results obtained in the LOD studies.

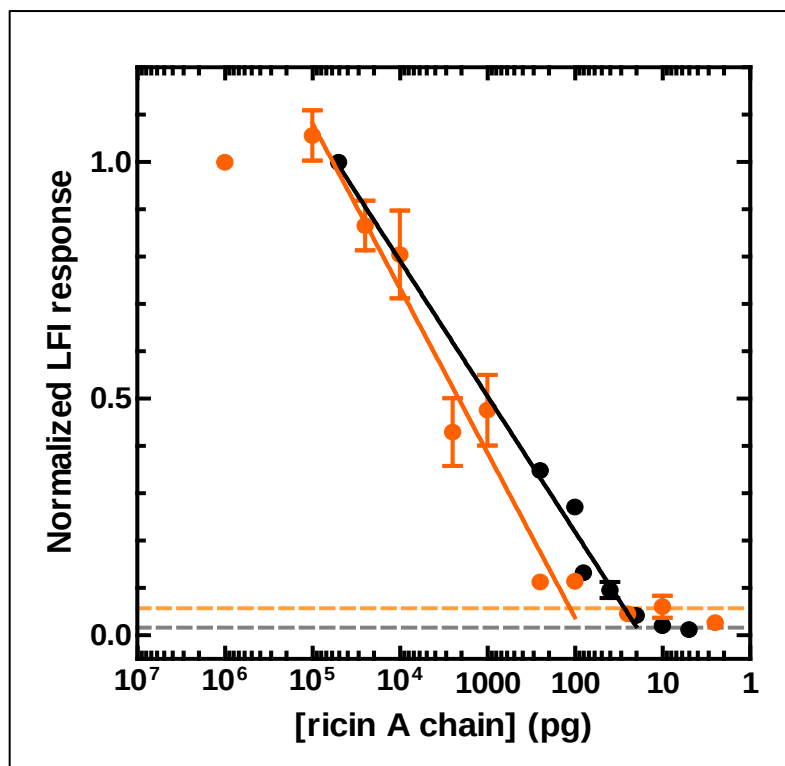


Figure 44. Performance of nanocrystals relative to gold anti-ricin LFIs (black and orange filled circles, respectively) that were constructed with identical reagents and materials, and tested with recombinant ricin A chain protein. The estimated LOD for the gold LFIs was 30–100 pg versus that of the nanocrystal LFIs at 10 pg. The detection limit threshold was calculated as the mean plus twice the standard deviation of a blank LFI that was run with no analyte (orange horizontal dashed line for gold LFIs; gray dashed line for UCNPs).

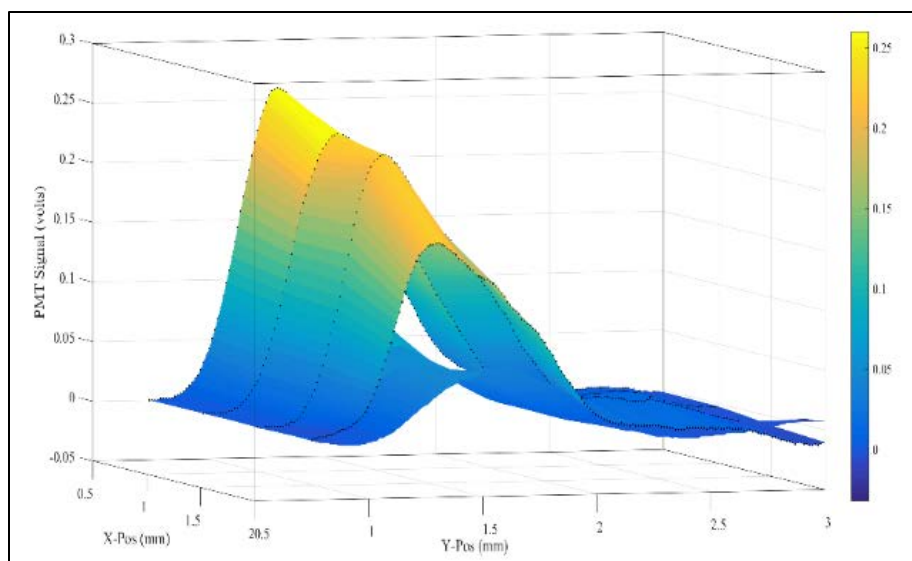


Figure 45. Signal intensity of UCNP LFI detection of 20 pg analyte relative to background observed for buffer alone. The x position is along the LFI width, and the y position traces the length of the LFI along the flow direction.

Table 13. Integrated Peak Areas of Test Line Intensity for Gold and UCNP-based LFIs

[Ricin A chain] (pg)	Gold LFI Peak Area (mean $\pm$ SEM)	Gold LFI Replicates	UCNP LFI Peak Area (mean $\pm$ SEM)	UCNP LFI Replicates
1000000	6367*	1	—	—
100000	6724 $\pm$ 339*	2	—	—
50000	—	—	6.71*	1
25000	5512 $\pm$ 333	3	—	—
10000	5125 $\pm$ 591	4	—	—
2500	3459 $\pm$ 328	2	3.61	1
1000	3030 $\pm$ 475	3	—	—
250	719 $\pm$ 97	4	2.95	1
100	728 $\pm$ 28	2	1.75	1
80	—	—	1.30 $\pm$ 0.46	3
40	—	—	0.62 $\pm$ 0.11	3
25	290 $\pm$ 53	3	—	—
20	—	—	0.27 $\pm$ 0.05	3
10	385 $\pm$ 147	2	0.132 $\pm$ 0.023	3
5	—	—	0.079	1
2.5	168	1	—	—
0 (blank)	236 $\pm$ 54	3	0.051 $\pm$ 0.015	3

* Test line area is >5% greater than control line area.

Notes: LFIs were dosed with recombinant ricin A chain analyte. Table reports the mean and standard error of the mean (SEM) for the number of replicates indicated.

— indicates no test was performed.

6. METHODS

6.1 Reagents

EDCI and *N*-hydroxysulfosuccinimide (sNHS) were purchased from Chem-Impex International, Inc. (Wood Dale, IL). Dimethylsulfoxide (DMSO) and MES were obtained from Sigma-Aldrich (St. Louis, MO). UCNPs (15 × 35 nm rods), comprising a NaY₄ lattice doped with 20% ytterbium and 2% erbium coated in a polyacrylic acid shell, were provided by Intelligent Material Solutions, Inc. Purified recombinant ricin A chain protein was obtained from Vector Laboratories, Inc. (Burlingame, CA) as a 1 mg/mL solution in 10 mM phosphate, 150 mM sodium chloride at pH 7.5, 0.08% sodium azide, 50 mM 2-mercaptoethanol, and 0.1% polyethylene glycol (MW 3300). The AnaptysBio APE 987.03 anti-MS2CP antibody, AnaptysBio anti-ricin antibody APE 36633.03, and the AxioMx, Inc. IgX 2691 anti-ricin antibody are thermostable antibodies that were produced as part of the DARPA-funded Antibody Technology Program. Bovine serum albumin was purchased from Thermo Fisher Scientific, Inc.

6.2 Antibody-Reporter Conjugation

Similar to the protocol described by Tjon Kon Fat et al. (29), a solution of EDCI (1 μL, 0.4 μmol, 250 mM); sNHS (1 μL, 0.4 μmol, 250 mM); MES (2.6 mg, 13 μmol); and 150 μL of DMSO was added to a UCNPs suspension in (1.5 mg in 250 μL of 50:50 water–DMSO) or 40 nm gold chloride nanoparticles (Sigma-Aldrich) at room temperature with a final pH of 6. The solution was stirred at room temperature for 1 h, and the resulting sNHS-activated UCNPs were isolated by centrifugation (7000 × *g* for 10 min). After decanting the supernatant, AnaptysBio anti-MS2CP antibody (50, 100, or 250 μL of 2.35 mg/mL) or IgX 2691 anti-ricin antibody was then added and the UCNPs were resuspended in this solution by pipetting with an additional volume of MES buffer to bring the total reaction volume to 250 μL. The resulting solution was stirred at room temperature for 90 min, and then the antibody-conjugated UCNPs were isolated by centrifugation (7000 × *g* for 10 min), resuspended in buffered water (20% DMSO, 50 mM MES), centrifuged again (7000 × *g* at 10 min), decanted, and resuspended to provide the antibody-conjugated UCNPs as a solution (20% DMSO, 50 mM MES).

6.3 ELISA Studies

Standard ELISA protocols (30) were used to probe antibody–antigen recognition using an HRP-coupled secondary antibody to amplify the signal through the enzymatic action on 2,2'-azino-*bis*(3-ethylbenzthiazoline-6-sulphonic acid). Using this protocol, antigen (e.g., 2 μg of purified recombinant MS2CP) was coated onto Nunc polystyrene immuno-plates (Sigma-Aldrich) for 16 h. Plates were then incubated with 200 μL milk diluent (KPL) for 1 h, washed three times in a AquaMax 2000 plate washer (Molecular Devices LLC; Sunnyvale, CA), probed with the indicated nanocrystal-coupled antibodies for 1 h, washed again three times as previously stated, and incubated with a goat anti-human HRP-conjugated antibody (KPL) for 1 h. The plates were washed again before the HRP substrate was added. Optical absorbance at 405 nm was quantified using a BioTek Instruments, Inc. (Winooski, VT) Synergy H4 plate reader after 2 min.

6.4 LFI Construction

LFIs were manufactured following literature methods described by Tjon Kon Fat et al. (29). For this procedure, IgX 2691 anti-ricin antibodies were dialyzed and conjugated to gold nanoparticles or UCNPs, as described in Section 6.2, and dried onto a polyester sample pad (Whatman–Pall product; Pall Corporation; Port Washington, NY). The sample pad, nitrocellulose membranes (cellulose nitrate with polyester clear backing (Whatman–Pall), and the absorbent pad (compressed paper fibers; Whatman–Pall) were assembled on a vinyl adhesive backing card (Arista Biologicals Inc., Allentown, PA). The target-specific test line was striped with 0.5 $\mu\text{g}/4\text{ mm}$ of thermostable anti-ricin antibody APE 36633.03, and a control line was striped with 0.5 $\mu\text{g}/4\text{ mm}$ of anti-human goat antibody (SouthernBiotech; Birmingham, AL) using an Isoflow striper (Arista Biologicals, Inc.; Allentown, PA). The assembled lateral flow cards were then cut into 4 mm strips and installed in plastic LFI cassettes (Forsite Diagnostics Ltd; York, U.K.). The buffer used for this procedure was optimized for proper flow and binding, adequate pH control, and high concentration of BSA. It was made up of 50 mM Tris at pH 8.1, 150 mM sodium chloride, 10 mg/mL of BSA, and 0.1% ProClin 950 (Sigma-Aldrich).

6.5 Gold LFI Scans

The LFIs were then scanned with a CAMAG TLC scanner 4 using a 1 mm cross-section (three lanes, spaced 0.1 mm apart) at 536 nm to quantify peak areas for the test and control lines using the manufacturer-provided WinCATS software package (CAMAG Chemie-Erzeugnisse und Adsorptionstechnik AG; Muttenz, Germany).

6.6 Prototype Construction

The instrument was built around the modular Cage Mount System, and related accessories were all purchased from ThorLabs, Inc. (Newton, NJ), unless specified otherwise. A variable power (0–450 mW) 970 nm laser from Edmund Optics, Inc. (Barrington, NJ) was coupled via a fiber optic cable through a collimating lens (Edmund Optics, 25.0 mm diameter, 35.0 mm focal length, visible–near-infrared [VIS–NIR] coated Plano-Convex lens) and reflected by two planar mirrors into a filter cube that was equipped with a 45° dichroic cold mirror. The cold mirror reflected <7% of infrared light into a model DET100A avalanche photodiode for power monitoring. The cold mirror also permitted the remainder of infrared light to be focused through a 25.0 mm diameter, 35.0 mm focal length, VIS–NIR coated PlanoConvex lens (Edmund Optics) onto the sample plane. Visible green light from infrared-excited UCNPs was reflected by the cold mirror through a 25.0 mm diameter, 50.0 mm focal length, VIS–NIR coated PlanoConvex lens (ThorLabs, Inc.) into a Hamamatsu H5874-03 PMT (Hamamatsu Photonics, Hamamatsu City, Japan), which transforms incident light emission into a voltage change. This voltage change was quantified by a DATAQ Instruments, Inc. (Akron, OH) DI-245 ADC that was interfaced via USB to a laptop computer running the DATAQ WinDaq serial acquisition software (ver. 3.94). The LFI was translated using a Unidex 11 two-axis motion controller (Aerotech, Inc.; Pittsburgh, PA).

6.7 Nanocrystal LFI Scans

UCNP-based LFIs were dosed with recombinant ricin A chain that was diluted in the sample buffer (cf., LFI construction in Section 6.4). Because water absorbs the 980 nm infrared light that is used to excite the nanocrystals, the LFIs were placed in a passive desiccating chamber for over 4 h before the scans were performed. LFIs were mounted with a custom bracket onto the prototype and scanned along the y axis (i.e., flow direction) using a motorized stage at each of four x positions across the width of the LFI ticket. The PMT voltage was monitored as a function of time while scanning in the y direction at a constant rate of 0.025 or 0.05 mm/min through the test line position and stopping at the shoulder of the control line to prevent PMT burnout. Total peak area for the LFI test line was quantified by mathematically converting the PMT voltage time dependence to a position measurement using the scan rate and integrating across the four x-position scans using Mathworks (Natick, MA) MATLAB software, version 2016a 9.0.0.341360.

Blank

LITERATURE REFERENCES

1. Whitehead, T.A.; Chevalier, A.; Song, Y.; Dreyfus, C.; Fleishman, S.J.; De Mattos, C.; Myers, C.A.; Kamisetty, H.; Blair, P.; Wilson, I.A.; Baker, D. Optimization of Affinity, Specificity and Function of Designed Influenza Inhibitors Using Deep Sequencing. *Nat. Biotech.* **2012**, *30* (6), 543–548.
2. Chen, G.; Qiu, H.; Fan, R.; Hao, S.; Tan, S.; Yang, C.; Han, G. Lanthanide-doped Ultrasmall Yttrium Fluoride Nanoparticles with Enhanced Multicolor Upconversion Photoluminescence. *J. Mater. Chem.* **2012**, *22* (38), 20190–20196.
3. Koga, N.; Tatsumi-Koga, R.; Liu, G.; Xiao, R.; Acton, T.B.; Montelione, G.T.; Baker, D. Principles for Designing Ideal Protein Structures. *Nature* **2012**, *491* (7423), 222–227.
4. Su, H.-P.; Golden, J.W.; Gittis, A.G.; Hooper, J.W.; Garboczi, D.N. Structural Basis for the Binding of the Neutralizing Antibody, 7D11, to the Poxvirus L1 Protein. *Virology* **2007**, *368* (2), 331–341.
5. Johnson, G.; Wu, T.T. Kabat Database and Its Applications: 30 Years after the First Variability Plot. *Nucleic Acids Res.* **2000**, *28* (1), 214–218.
6. Huang, R.; Fang, P.; Kay, B.K. Improvements to the Kunkel Mutagenesis Protocol for Constructing Primary and Secondary Phage-Display Libraries. *Methods* **2012**, *58* (1), 10–17.
7. Zhu, Y.; Dai, J.; Zhang, T.; Li, X.; Fang, P.; Wang, H.; Jiang, Y.; Yu, X.; Xia, T.; Niu, L.; Guo, Y.; Teng, M. Structural Insights into the Neutralization Mechanism of Monoclonal Antibody 6C2 against Ricin. *J. Biol. Chem.* **2013**, *288* (39), 25165–25172.
8. Battye, T.G.; Kontogiannis, L.; Johnson, O.; Powell, H.R.; Leslie, A.G. iMOSFLM: A New Graphical Interface for Diffraction-Image Processing with MOSFLM. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2011**, *67* (4), 271–281.
9. Evans, P.; Scaling and Assessment of Data Quality. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2006**, *62* (1), 72–82.
10. McCoy, A.J.; Grosse-Kunstleve, R.W.; Adams, P.D.; Winn, M.D.; Storoni, L.C.; Read, R.J. Phaser Crystallographic Software. *J. Appl. Crystallogr.* **2007**, *40* (4), 658–674.
11. Vagin, A.; Teplyakov, A. Molecular Replacement with MOLREP. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66* (1), 22–25.
12. Cowtan, K. The Buccaneer Software for Automated Model Building. 1. Tracing Protein Chains. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2006**, *62* (9), 1002–1011.

13. Emsley, P.; Lohkamp, B.; Scott, W.G.; Cowtan, K. Features and Development of Coot. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66* (4), 486–501.
14. Murshudov, G.N.; Skubak, P.; Lebedev, A.A; Pannu, N.S.; Steiner, R.A.; Nicholls, R.A.; Winn, M.D.; Long, F.; Vagin A.A. REFMAC5 for the Refinement of Macromolecular Crystal Structures. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2011**, *67* (4), 355–367.
15. Lovell, S.C.; Davis, I.W.; Arendall, W.B. III; de Bakker, P.I.; Word, J.M.; Prisant, M.G.; Richardson, J.S.; Richardson, D.C. Structure Validation by C α Geometry: ϕ , ψ and C β Deviation. *Proteins: Struct., Funct., Genet.* **2003**, *50* (3), 437–450.
16. Kaever, T.; Meng, X.; Matho, M.H.; Schlossman, A.; Li, S.; Sela-Culang, I.; Ofra, Y.; Buller, M.; Crump, R.W.; Parker, S.; Frazier, A.; Crotty, S.; Zajonc, D.M.; Peters, B.; Xiang, Y. Potent Neutralization of *Vaccinia* Virus by Divergent Murine Antibodies Targeting a Common Site of Vulnerability in L1 Protein. *J. Virol.* **2014**, *88* (19), 11339–11355.
17. Winter, G.; Lobley, C.M.C.; Prince, S.M. Decision Making in xia2. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2013**, *69* (7), 1260–1273.
18. Kabsch, W. XDS. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66* (2), 125–132.
19. Wang, F.; Sen, S.; Zhang, Y.; Ahmad, I.; Zhu, X.; Wilson, I.A.; Smider, V.V.; Magliery, T.J.; Schultz, P.G. Somatic Hypermutation Maintains Antibody Thermodynamic Stability during Affinity Maturation. *P. Natl. Acad. Sci. U.S.A.* **2013**, *110* (11), 4261–4266.
20. Lewis, S.M.; Wu, X.; Pustilnik, A.; Sereno, A.; Huang, F.; Rick, H.L.; Guntas, G.; Leaver-Fay, A.; Smith, E.M.; Ho, C.; Hansen-Estruch, C.; Chamberlain, A.K.; Truhlar, S.M.; Conner, E.M.; Atwell, S.; Kuhlman, B.; Demarest, S.J. Generation of Bispecific IgG Antibodies by Structure-Based Design of an Orthogonal Fab Interface. *Nat. Biotechnol.* **2014**, *32* (2), 191–198.
21. Adams, P.D.; Afonine, P.V.; Bunkóczi, G.; Chen, V.B.; Davis, I.W.; Echols, N.; Headd, J.J.; Hung, L.-W.; Kapral, G.J.; Grosse-Kunstleve, R.W.; McCoy, A.J.; Moriarty, N.W.; Oeffner, R.; Read, R.J.; Richardson, D.C.; Richardson, J.S.; Terwilliger, T.C.; Zwart, P.H. PHENIX: A Comprehensive Python-Based System for Macromolecular Structure Solution. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *66* (2), 213–221.
22. Evans, P.R.; Murshudov, G.N. How Good Are My Data and What Is the Resolution? *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2013**, *69* (7), 1204–1214.
23. Mader, H.S.; Kele, P.; Saleh, S.M.; Wolfbeis, O.S. Upconverting Luminescent Nanoparticles for Use In Bioconjugation and Bioimaging. *Curr. Opin. Chem. Biol.* **2010**, *14* (5), 582–596.

24. Xiong, L.-Q.; Chen, Z.-G.; Yu, M.-X.; Li, F.-Y.; Liu, C.; Huang, C.-H. Synthesis, Characterization, and in Vivo Targeted Imaging of Amine-Functionalized Rare-Earth Up-Converting Nanophosphors. *Biomaterials* **2009**, *30* (29), 5592–5600.
25. Naccache, R.; Rodríguez, E.M.; Bogdan, N.; Sanz-Rodríguez, F.; Iglesias de la Cruz, M.d.C.; de la Fuente, A.J.; Vetrone, F.; Jaque, D.; García Solé, J.; Capobianco J.A. High Resolution Fluorescence Imaging of Cancers using Lanthanide Ion-Doped Upconverting Nanocrystals. *Cancers* **2012**, *4* (4), 1067–1105.
26. Zhao, Y.; Wang, H.; Zhang, P.; Sun, C.; Wang, X.; Wang, X.; Yang, R.; Wang, C.; Zhou, L. Rapid Multiplex Detection of 10 Foodborne Pathogens with an Up-Converting Phosphor Technology-Based 10-Channel Lateral Flow Assay. *Sci. Rep.* **2016**, *6*, 21342.
27. Corstjens, P.L.A.M.; de Dood, C.J.; Priest, J.W.; Tanke, H.J.; Handali, S.; the Cysticercosis Working Group in Peru. Feasibility of a Lateral Flow Test for Neurocysticercosis using Novel Up-Converting Nanomaterials and a Lightweight Strip Analyzer. *PLoS. Negl. Trop. Dis.* **2014**, *8* (7), e2944.
28. Linares, E.M.; Kubota, L.T.; Michaelis, J.; Thalhammer, S.; Enhancement of the Detection Limit for Lateral Flow Immunoassays: Evaluation and Comparison of Bioconjugates. *J. Immunol. Meth.* **2012**, *375* (1-2), 264–270.
29. Tjon Kon Fat, E.M.; Abrams, W.R.; Niedbala, R.S.; Corstjens, P.L.A.M. Chapter 11 - Lateral Flow Sandwich Assay Utilizing Upconverting Phosphor (UCP) Reporters. In *Methods in Cell Biology*; Conn, P.M., Ed; Elsevier B.V.: Amsterdam, Netherlands, 2012; pp 203–234.
30. Hornbeck, P.; Winston, S.E.; Fuller, S.A. Enzyme-Linked Immunosorbent Assays (ELISA). In *Current Protocols in Molecular Biology*; John Wiley and Sons, Inc.: Hoboken, NJ, 2001; pp 11.2.1–11.2.22.

Blank

ACRONYMS AND ABBREVIATIONS

ADC	analog-to-digital converter
AuNP	gold nanoparticle
BSA	bovine serum albumin
CDC	Centers for Disease Control and Prevention
CDR	complementarity-determining region
COOT	crystallographic object-oriented toolkit
DARPA	Defense Advanced Research Projects Agency
DLS	dynamic light scattering
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
dsDNA	double-stranded DNA
DTRA	Defense Threat Reduction Agency
dUTPase	deoxyuridine-triphosphatase
ECBC	U.S. Army Edgewood Chemical Biological Center
EDCI	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
ELISA	enzyme-linked immunosorbent assay
Fab	antibody fragment
FITC	fluorescein isothiocyanate
FY	fiscal year
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulfonic acid
HRP	horseradish peroxidase
hscFv	humanized single-chain variable fragment
hIgG	human immunoglobulin G
IgG	immunoglobulin G
K_D	dissociation constant
KPL	Kirkegaard and Perry Laboratory, Inc.
LFI	lateral flow immunoassay
LOD	limit of detection
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MME	monomethyl ether
MOLREP	molecular replacement software
MS2CP	recombinant MS2 coat protein
mscFv	mouse single-chain variable fragment
MW	molecular weight
MW-R	predicted molecular weight assuming a Raleigh spherical model
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PEG	polyethylene glycol
PHENIX	python-based hierarchical environment for integrated xtallography
pI	isoelectric point

PMT	photomultiplier tube
Q	quarter
REFMAC5	macromolecular crystallographic refinement program
R _{free}	a statistical measurement that assesses the quality of a model made using X-ray crystallographic data
RiVax	ricin vaccine
R _{merge}	agreement between multiple measurements of a data set
rmsd	root mean square deviation
R _{work}	residual factor or reliability factor
SAPE	streptavidin–phycoerythrin
scFv	single-chain variable fragment
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
SEM	standard error of the mean
sNHS	<i>N</i> -hydroxysulfosuccinimide
ssDNA	single-stranded DNA
SSRL	Stanford Synchrotron Radiation Lightsource
TLC	thin-layer chromatography
TLS	translation–liberation–screw (vibrational motion)
T _m	melting temperature
UCNP	upconverting nanoparticle
USB	universal serial bus
XDS	X-ray detector software
V _H	heavy variable domain
VIS–NIR	visible–near infrared
V _L	light variable domain

DISTRIBUTION LIST

The following individuals and organizations were provided with one Adobe portable document format (pdf) electronic version of this report:

U.S. Army Edgewood Chemical
Biological Center (ECBC)
RDCB-DRB-M
ATTN: Broomall, S.
Rosenzweig, C.

ECBC Technical Library
RDCB-DRB-BL
ATTN: Foppiano, S.
Stein, J.

Defense Threat Reduction Agency, R&D
J9-CBS
ATTN: Cronce, D.

Office of the Chief Counsel
AMSRD-CC
ATTN: Upchurch, V.

Department of Homeland Security
RDCB-PI-CSAC
ATTN: Mearns, H.

Defense Technical Information Center
ATTN: DTIC OA

G-3 History Office
U.S. Army RDECOM
ATTN: Smart, J.

ECBC Rock Island
RDCB-DES
ATTN: Lee, K.
RDCB-DEM
ATTN: Grodecki, J.

