

Abstract

The development of cold-chain independent reagents and assay systems for use in the field remain a priority for the military. The costs and logistical challenges of keeping a large inventory refrigerated from manufacture to point of use are incompatible with a smaller, more agile military. The Ruggedized Antibody Program (RAP) is an integrated portfolio of research and development efforts designed to enhance currently fielded antibody products through affinity maturation and stabilization with the goal of producing detection products which can tolerate temperature extremes while maintaining function. Under this program, antibody sequences from the Critical Reagents Program are provided to contracted performers who apply their technologies to improve the stability and specificity of the antibody. Edgewood Chemical Biological Center provides program support, standardized antigens, and uniform testing of the starting material and improved products from performers.

In addition to contracting antibody 'hardening' work, the RAP is researching means to use antibody fragments in detection products in lieu of full-length antibodies in order to capitalize on the greater speed and lower cost of synthetic antibody screening and production techniques. Finally, work is underway to both engineer existing antibodies, and to identify novel antibodies against target antigens, and graft these antibody fragments or complementarity-determining regions into known thermostable full-length scaffolds developed by collaborators (AnaptysBio, AxioMx Inc.) in cases where antibody fragments are ultimately unsuitable.

Antigen Production

Targets for the RAP program include a variety of bacterial toxins (ricin and SEB), as well as antigenic viral proteins, such as surface proteins for Ebola, Chikungunya, and Crimean-Congo hemorrhagic fever. Toxins are obtained through commercial sources, while non-toxic viral proteins are produced in-house via recombinant expression in the eukaryotic Expi293 cell line (Thermo Fisher) in order to retain native glycosylation patterns.

Ebola Virus Antigen Production

The Ebola virus glycoprotein (EBV GP) is a highly ranked target for antibody development in the RAP program. Purification of this His₆-tagged protein involved an initial separation from media components using immobilized-metal affinity chromatography (IMAC), followed by elution from two consecutive Q columns. Approximately 1 mg of purified EBV GP was obtained per 30 mL of eukaryotic transfection.

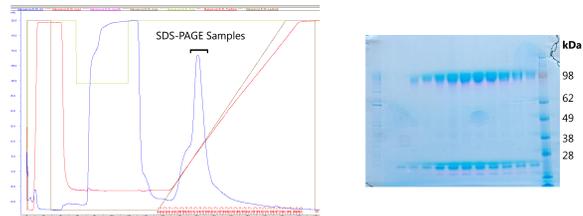


Figure 1. A representative chromatogram of EBV GP elution from consecutive Q columns (left) and an SDS-PAGE gel of the purified fractions (right). Due to its high level of glycosylation, purified EBV GP has an approximate molecular weight of 100 kDa, whereas the molecular weight is 73 kDa.

CDR Grafts to Thermostable Host

Antigen	Light Chain Sequences			Heavy Chain Sequences		
	CDR 1	CDR 2	CDR 3	CDR 1	CDR 2	CDR 3
Bot A	RSSTGAVTSSNYDN	GTDNRPP	ALWYSHHWV	GVAFSSWWMN	RYPGDDGTNYNGKFKD	EGVYDYG
Bot A	RSSTGAVTSSNYAN	GTDNRPP	ALWYSHHWV	GFTFTYDIN	WIYPGNGRTEYSEKFK	GSHYDFD
Bot	KSSQSLNSRTRKNYLA	WASTRES	KQSYNLRT	GYTFSSFWIE	EILPSSGNTNCKEKFQ	PYQGLYVYAMDY
Ebola	KASQDVSTAVA	SASYRYT	QQHYSTPWT	GYTLTXGMX	WINTYTGATFADDFQK	GYGNVAGTWFPY
Ebola	KSSQSLNSRTRKNYLA	LVSRLDS	WQGTHTLPT	GYTFTDNDL	DIYPSGNTNYNCKFKG	AGFDY
Ebola	RSSQSLVHSGSTYHL	KVSIRFS	SQSTHVPT	GYTFSSWWMN	QIYLGDDGTNYNGKFKD	GNRYRDEGDC
Lassa Fever	TSSQSLVHSGNTYHL	LVSTRFS	SQSTHVPT	GFSLNYGVH	VIWRGGRTDYNAAFMS	NGFNAMDY
Lassa Fever	RASQSDVDTYGNFHF	RASNLES	QQSNEDPYT	GYSTIDYAWN	YITYSGSTNYNPSLKS	KYGGYVPMYD
Lassa Fever	KSSQSLVSSDQKNYLA	WASTRES	HQYLSWT	GFTFTNYWMT	RIDPYDSETHYQDFQK	RLGLVYAMDY
Lassa Fever	RASESVDSYGNFSFMH	RASNLES	QQSNADPYT	GYSTGYMWH	YISYNGKTSYNGKFKG	FHYDYRGLLGTAGKL
Rift Valley Fever Virus	KASQDVSTAVA	SASYRYT	QQHYSTPWT	GYTFSSWWMN	EILPSSGNTNYNCKFKG	RANWVVFY
Rift Valley Fever Virus	KASQSDVDTYGNFHF	AASNLES	QQSDDEPWT	GFTFSNYGMS	TISSGGTYTYPDSVKG	-
SEB	KASQVINNLS	RVSRLVD	LQYDFPWT	GFSSDYMYI	TISDGGYTYLDSVKG	DDAMDY
VEEV	KASQVINNLS	RVSRLVD	LQYDFPWT	GFNIKTYIH	EILPSSGNTNCKEKFQ	SEGYGNFPFAY
Vaccinia	RASQSDVDTYGNFHF	YSTRLHS	QQGNTLPT	GYTFSSWWMN	QIYLGDDGTNYNGKFKG	GGLLRGFTY
Vaccinia	RSSTGAVTSSNYAN	GTDNRPP	ALWYSHHWV	GVAFSSWWMN	QIYLGDDGTNYNGKFKG	GGLLRGFTY
West Nile Virus	KASQDVSTAVA	WTSTRHT	QQHYTMPLT	GVAFSSWWMN	RHPGDDGNTYNGEFA	EGNDYDFDV
West Nile Virus	KASQDVSTAVA	WASTRHT	QHMYATPRT	GFTFSYDMS	SIYHGGNTYYPDSVKG	ERYDGDFFDY
Anaptys MS2	RSSQSLVHSGNTYHL	KVSNRFS	SQSTHVPT	GFTFSYAMS	TISTGGTYTYPDSVKG	QGDFGDWYFDV
Vaccinia	RASQSISSYLN	AASSLQS	QQSYSTPWT	GYTFTRVWMMH	YINPSTGYTEYNQKFKD	TTVDGDFAY

Table 1. Complementarity determining regions (CDRs) that were identified via the Rosetta Antibody software (Gray Lab, JHU) or manual alignment based on sequence conservation. Sequences of the mRNAs expressed in various hybridoma lines supplied by the CRP were obtained by PCR-amplifying the antibody variable regions using primers designed by Essono et al. 2003, and then sequencing the PCR products.

Heavy Chain	Light Chain
<p>CDR 1</p> <p>..GSLRLSCAAS GFTFSSYAMS WVRQAPGKGL..</p> <p>..GSLRLSCAAS GFTFSSYAMS WVRQAPGKGL..</p> <p>..GSLRLSCAAS GYTFTRVWMMH WVRQAPGKGL..</p> <p>..GSLRLSCAAS GFTFSSYAMS WVRQAPGKGL..</p> <p>CDR 2</p> <p>..KGLEWVC TISTGGTYTYPDSVK GRFTCS..</p> <p>..KGLEWVC SISDYGSITYSADSVK GRFTCS..</p> <p>..KGLEWVC YINPSTGYTEYNQKFKD GRFTCS..</p> <p>..KGLEWVC SISTGGDTHYQDSVK GRFTCS..</p> <p>CDR 3</p> <p>..EDTAVYYCAR QGDFGDWYFDV WGAGTITVTV..</p> <p>..EDTAVYYCAR GGGTFDI WGAGTITVTV..</p> <p>..EDTAVYYCAR TTVDGDFAY WGAGTITVTV..</p> <p>..EDTAVYYCAT NRGWYFDY WGAGTITVTV..</p>	<p>CDR 1</p> <p>..PASISC RSSQSLVHSGNTYHL WYQQR..</p> <p>..PASISC KSSQTLNLSRTRKNYLA WYQQR..</p> <p>..PASISC RASQSISSYLN WYQQR..</p> <p>..PASISC SARSSVSYMY WYQQR..</p> <p>CDR 2</p> <p>..RPGQSPRLLIY KVSNRFS GVPDRFSGSGS..</p> <p>..RPGQSPRLLIY WASTRES GVPDRFSGSGS..</p> <p>..RPGQSPRLLIY AASSLQS GVPDRFSGSGS..</p> <p>..RPGQSPRLLIY RTSNLAS GVPDRFSGSGS..</p> <p>CDR 3</p> <p>..EAEDVGVYFC SQSTHVPT FGGGTRKLEIK..</p> <p>..EAEDVGVYFC KQSNLMT FGGGTRKLEIK..</p> <p>..EAEDVGVYFC QQSISYPT FGGGTRKLEIK..</p> <p>..EAEDVGVYFC QQHVSYPPT FGGGTRKLEIK..</p>

- Anaptys anti-MS2 CDR region
- anti-Coxiella (oCom1) CDR graft
- anti-Vaccinia (7D11) CDR graft
- anti-B.anthraxis (oBclA) CDR graft
- Thermostable Anaptys IgG backbone

Table 2. We are grafting the complementarity-determining regions (CDRs) of antibodies with desirable antigen affinities and specificities into thermostable/robust IgG molecules. Predicted CDRs of target-binding antibodies were converted into coding DNA and incorporated into a single-stranded megaprimers (~500bp). New CDRs were PCR amplified into heavy and light chain plasmids coding for the thermostable Anaptys or AxioMx IgG backbone, and were subsequently sequence verified by the Synthesis & Sequencing Facility at the Johns Hopkins University School of Medicine.

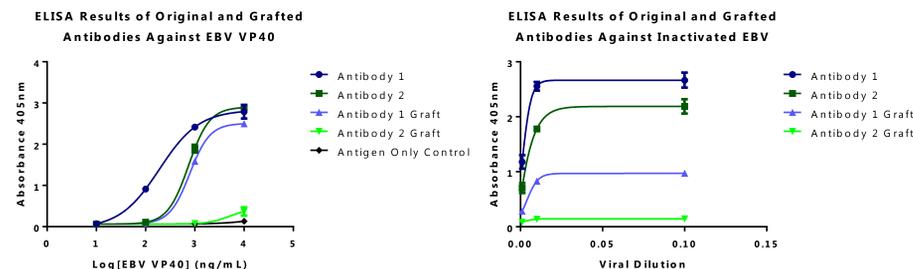


Figure 2. ELISA data showing EBV VP40 binding of antibodies derived from existing CRP hybridomas lines (Antibody 1 and 2) and corresponding grafted antibodies containing CDRs from these antibodies grafted into a thermostable IgG scaffold (Antibody Graft 1 and 2). Binding was assessed against purified EBV VP40 protein (left) and inactivated Ebola virus (right). The antigen only control (left, black) shows the 405 nm background absorbance in the absence of antibody. Antibody 1 retained most of its EBV VP40 protein binding affinity upon grafting into the thermostable scaffold, whereas Antibody 2 lost its ability to bind to this antigen. The grafted antibodies both displayed weaker binding to the inactivated virus when compared to the unmodified antibodies.

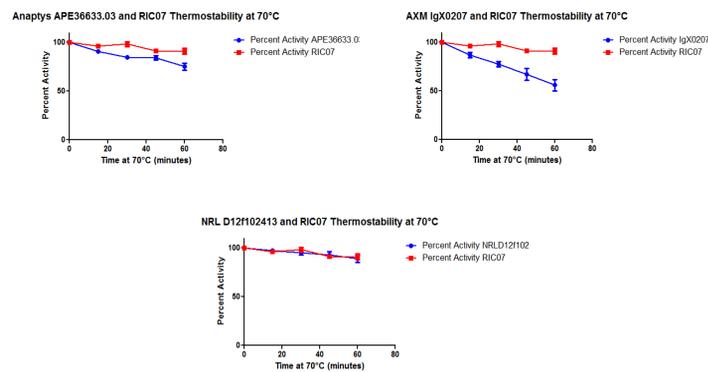


Figure 3. 5800 RU of Rivax was covalently tethered to a Biacore Series S CM5 Sensor Chip using standard amine coupling chemistry. A linear curve was created using unheated sample diluted in PBS. Samples were heated to 70°C for 0, 15, 30, 45, and 60 minutes. Samples were diluted using two different dilution factors and injected in triplicate for thirty seconds at 10µl/min. All antibody grafts maintained binding activity upon heating to 70°C, demonstrating their thermal stability.

Phage Display for Antibody Discovery

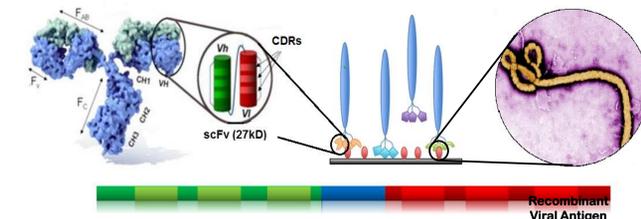
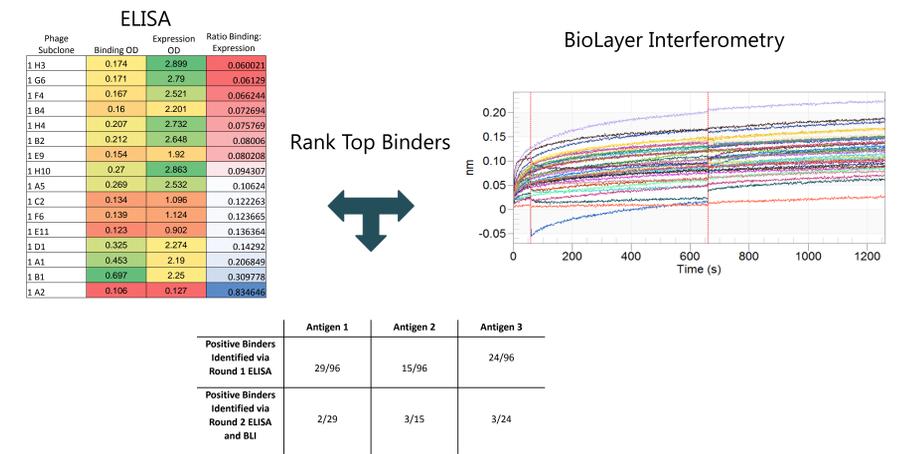


Figure 4. Phage display strategy to optimize CDR recognition. An scFv library expressed on the surface of phage was used to identify scFvs binders which are derived from a newly designed thermostable IgG scaffold. CDR regions from the binding scFvs can be directly incorporated (grafted) back into the full-length thermostable IgG with little to no distortion of CDR conformation.



Graft CDRs into Thermostable Scaffold

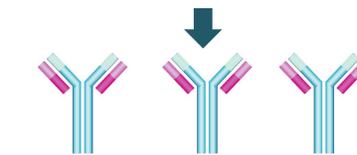


Figure 5. Phage- scFv binders were identified via an initial ELISA screening. A second, more stringent ELISA, in conjunction with BioLayer Interferometry, was used to rank the candidate scFvs. CDRs of the top binders were chosen for grafting into a thermostable, full length IgG scaffold.

Conclusions

The Ruggedized Antibody Program has created novel antibodies against a variety of viral antigens and bacterial toxins by grafting the CDRs of existing antibodies into a thermostable scaffold, as well as through the discovery of novel scFv binders through the use of a phage display library. These reagents can be incorporated into cold-chain independent assay systems to identify biological agents in the field.

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