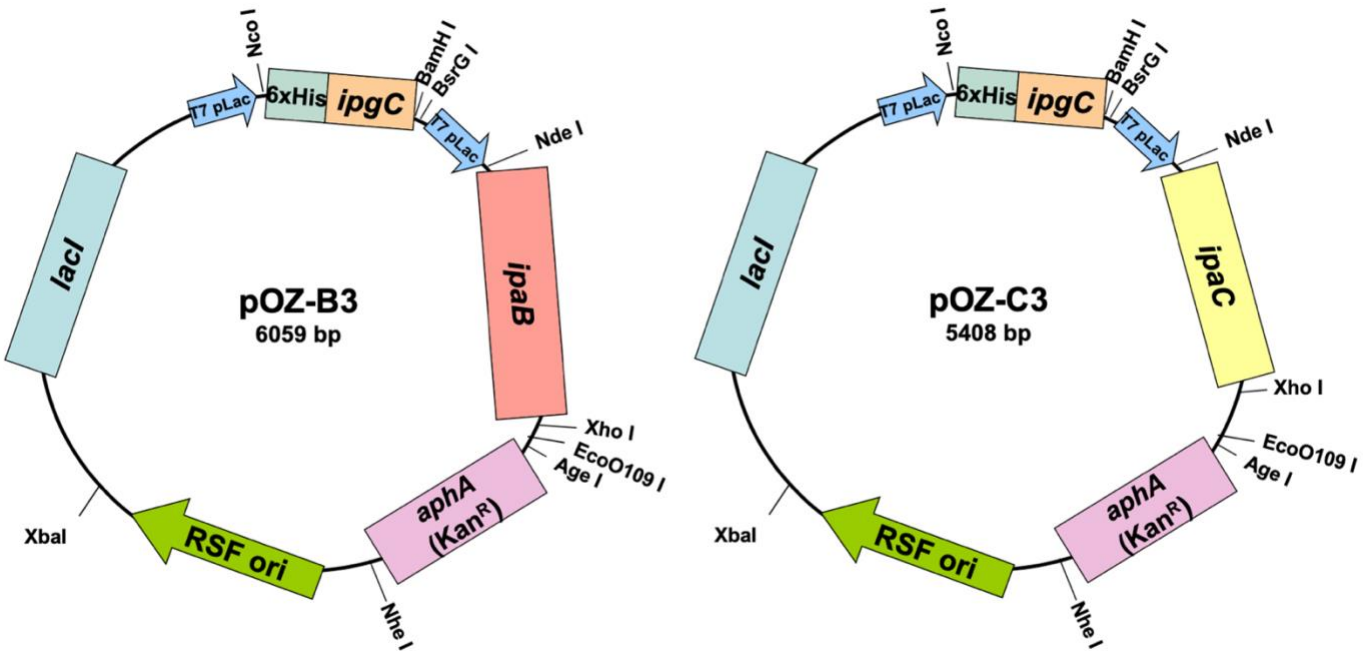
Supplemental Material for

Development of the *Shigella flexneri* 2a, 3a, 6 and *S. sonnei* Artificial Invaplex ($Invaplex_{AR}$) vaccines

K. Ross Turbyfill[1*], Kristen A. Clarkson[1], Edwin V. Oaks[2], Daniel V. Zurawski[3], Anthony R. Vortherms[1], and Robert W. Kaminski[1†]

[1] Department of Diarrheal Disease Research, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, MD, USA
[2] Patuxent Research and Consulting Group, Gambrills, MD, USA
[3] Wound Infections Department, Bacterial Diseases Branch, Walter Reed Army Institute of Research, Silver Spring, MD, USA

***Corresponding author:** K. Ross Turbyfill, M.S.
Walter Reed Army Institute of Research
503 Robert Grant Avenue
Silver Spring, MD 20910
Phone: 301-319-9943
E-mail: kevin.r.turbyfill.civ@health.mil



Supplemental Figure S1. Construction of pOZ-B3 and pOZ-C3 recombinant expression plasmids for IpaB and IpaC. Previous IpaB and IpaC constructs used for Invaplex$_{AR}$ were designed and constructed by the Picking laboratory (1) and these previous pCAYCDuet-IpaB/pET15b-HTIpgC and pCAYCDuet-IpaC/pET15b-HTIpgC plasmids served as the source of the *ipaB*, *ipaC*, and *ipgC* genes, all derived from *S. flexneri* 2a. The *aad*A gene (streptomycin/spectinomycin resistance gene; Str^R/Spec^R) was cut from the pCDFDuet-1 (Novagen) and ligated the IpaB-pACYCDuet expression vector and the resulting construct called pIpaB-*aad*A. The 6xHis-tagged *ipgC* insert was cloned into the NcoI/BamHI sites of pIpaB-*aadA* resulting in IpaB/HTIpgC Duet vector and transformed into BLR (DE3) protein expression strain (pOZ-B1). The p15A ori was replaced with the pCDF ori to create the pOZ-B2 clone. The pRSF1030 ori and the *aph*-1 (kanamycin resistance cassette) were cut from the pRSFDuet™-1 (Novagen) and cloned into XbaI/AgeI sites of the pOZ-B2 expression vector. The new construct, pOZ-B3, was transformed was transformed into the BLR

(DE3) protein expression strain. The cloning for *ipaC* was the same as above for the *ipaB* construct.

Notably, at the selection steps following transformation, cells were placed at 30 °C (rather than 37 °C) and in the presence of glucose to help restrict any leaky translation of the protein products until the final construct was made. Without these implementations, positive colonies containing the plasmid were not attained. Therefore, it is thought these steps resulted in a more stable, less toxic plasmid throughout the cloning process, which helped to then generate the final plasmid construct. This cloning strategy, in addition to the chaperone being driven by the same promoter, are thought to be the reasons behind the 100-fold increase in product yields.

Supplemental Table S1. Results of Visual Inspection of cGMP *Shigella* IpaB at different storage temperatures.

Storage Temperature	48 Hrs	72 Hrs	7 Days	30 Days	3 Months	6 Months	9 Months	12 Months	18 Months	24 Months	36 Months
-80°C	0	0	0	0	0	0	0	0	0	0	No Sample
4°C	0	0	0	0	0	0					
25°C	0	0	0	0							
40°C	0	0									

Supplemental Table 1. From the total cGMP purified IpaB bulk material, a 35 mL aliquot was subdivided into smaller aliquots of 1.5 mL into sterile 1.5 mL-Eppendorf polypropylene centrifuge tubes. While the bulk of the tubes were stored at -70 ± 10°C, the same storage temperature as the bulk cGMP *Shigella* IpaB protein, additional tubes were placed at 4°C, 25°C, and 40°C as suggested by FDA Guidance for Industry Q1A(R2) Stability Testing of New Drug Substances and Products, November 2003, Revision 2. Parameters of relative humidity and exposure to light were not controlled or assessed. Visual inspection was used as a qualitative indicator of significant precipitation and aggregation and potential contamination with visible particles observed unaided by the naked eye. 0 = No precipitation; + = Subjective amount of precipitation with + being visible precipitation, ++ light precipitation, +++ moderate precipitation, ++++ heavy precipitation. No precipitation was noted for any storage temperature at any time point post-manufacture for the cGMP *Shigella* IpaB lot. The bulk purified cGMP *Shigella* IpaB protein was stored frozen at -70 ± 10°C.

Supplemental Table S2. Results of Visual Inspection of cGMP *Shigella* IpaC at different storage temperatures.

Storage Temperature	48 Hrs	72 Hrs	7 Days	30 Days	3 Months	6 Months	9 Months	15 Months	18 Months	24 Months	37 Months
-80°C	0	0	0	0	0	0	0	0	0	0	0
4°C	0	0	0	0	0	++					
25°C	+	+	++	+++							
40°C	+++	++++									

Supplemental Table 2. From the total cGMP purified IpaC bulk material, a 35 mL aliquot was subdivided and stored at the various temperatures described above for IpaB. Visual inspection was used as a qualitative indicator of significant precipitation and aggregation and potential contamination with visible particles observed un-aided by the naked eye. 0 = No precipitation; + = Subjective amount of precipitation with + being visible precipitation, ++ light precipitation, +++ moderate precipitation, ++++ heavy precipitation. Precipitation was recorded and all tubes were then centrifuged at 14,500 x *g* for 5 mins in an Eppendorf centrifuge. The supernatant was removed and placed in a second, appropriately labeled 1.5 mL centrifuge tube. If no precipitation or pellet is observed after centrifugation, then the tube was treated as if it were a supernatant tube. Storage at cold temperature, specifically frozen at -70 ± 10°C, is best suited for long-term storage of cGMP *Shigella* IpaC Lot 1771 drug substance. The bulk purified cGMP *Shigella* IpaC protein was stored frozen at -70 ± 10°C.

Supplemental Table S3.
Quantities of Antigen delivered to guinea pigs in a 25 µg dose

Shigella species	LPS Multiplier	Total Protein Concentration (mg/mL)	IpaC:IpaB Ratio (Gel Densitometry)	LPS (10^6 EU) delivered in 25 µg dose for gpigs	IpaC (µg) delivered in 25µg dose	IpaB (µg) delivered in 25µg dose
Shigella flexneri 2a	1X	1.2	3.8:1	0.31	19.8	5.2
	2X	1.7	6.9:1	0.43	21.8	3.2
	4X	2	7.8:1	0.79	22.2	2.8
	8X	0.76	9.4:1	2.01	22.6	2.4
Shigella flexneri 3a	1X	0.8	2.5:1	0.23	17.9	7.1
	2X	1.1	3.8:1	0.36	19.8	5.2
	4X	1.03	5.8:1	1.33	21.3	3.7
	8X	1	5.8:1	1.95	21.3	3.7
Shigella flexneri 6	1X	1.5	2.1:1	0.27	16.9	8.1
	2X	1.4	4.1:1	0.63	20.1	4.9
	4X	1.5	4.0:1	1.03	20.0	5.0
	8X	1.5	3.9:1	3.67	19.9	5.1
Shigella sonnei	1X	0.58	2.6:1	0.25	18.1	6.9
	2X	0.78	5.6:1	0.35	21.2	3.8
	4X	0.92	9.4:1	0.87	22.6	2.4
	8X	0.93	7.2:1	2.39	22.0	3.0

Supplemental Table 3. The densitometric ratio for each Invaplex$_{AR}$ formulation and the total protein concentration of the formulation were used to calculate the concentration of IpaC in the total product and this amount was then subtracted from the total protein concentration to get the concentration of IpaB in the formulation. From these resulting values, the amount of IpaC and IpaB per 25 µg Guinea pig dose was calculated.

REFERENCES

1. Birket SE, Harrington AT, Espina M, Smith ND, Terry CM, Darboe N, Markham AP, Middaugh CR, Picking WL, Picking WD. 2007. Preparation and characterization of translocator/chaperone complexes and their component proteins from Shigella flexneri. Biochemistry 46:8128-37.