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Supporting Information for:

Superantigens promote *Staphylococcus aureus* bloodstream infection by eliciting pathogenic interferon-gamma (IFNγ) production

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This PDF file includes:

Text S1 to S3
Figures S1 to S6
Tables S1 and S2

Supplementary Text

Text S1: Construction of MW2 Δ*sec* mutant

Markerless deletion of *sec* in MW2 was performed using the pKor1 allelic replacement system (1). Briefly, a 598 bp fragment upstream of *sec* was amplified with Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher) using the primers (Table S2) pKOR-sec-upstream-For and pKOR-sec-upstream-Rev along with a 576 bp region downstream of *sec* amplified by the primers pKOR-sec-downstream-For and pKOR-sec-downstream-Rev 2. These products contained a 12 bp overlap and were spliced together at a ratio of 1:1 using primers pKOR-sec-upstream-For and pKOR-sec-downstream-Rev, creating an insert of 1203 bp in total. This insert was integrated into empty pKOR1 using BP clonase (Thermo Fisher) according to the manufacture's instructions. The cloned plasmids were transformed into *E. coli* XL1-Blue and screened for plasmids containing the insert. The confirmed knockout construct was chemically transformed into *E. coli* SA30B (2) to methylate the plasmid for electrotransformation into *S. aureus* MW2 (3). The *sec* knockout was created as described previously (1) and candidate constructs were screened by PCR using primers SEC-screen-For and SEC-screen-Rev (Table S2).

Text S2: Construction of pCM29::*seb* and pCM29::*sec* complementation plasmids

SEB and SEC-complementation plasmids for *S. aureus* SAg null mutants were created as previously described, with modifications (4). Briefly, SAg coding sequences were cloned into a pCM29 vector containing the active promotor of the leukocidin LukMF' (5). To achieve this, pCM29::pLukM-sGFP was digested with KpnI and EcoRI to remove the sGFP coding sequence while retaining the *lukM* promotor sequence. SAg insert fragment forward primers were designed to contain endogenous RBS upstream of the start codon as this would be removed from the plasmid with the *sgfp* gene. Sequences of *seb* and *sec* were amplified respectively from COL and MW2 genomic DNA using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher) with primers listed in Table S2. PCR products were digested with

KpnI and EcoRI to prepare the SAg insert for ligation. Complementation inserts were ligated into pCM29 that had the *sgfp* removed with T4 ligase (NEB). Following ligation, plasmids were further digested with MluI to inactivate any contaminating pCM29 that still retained the *sgfp*. After this step, ligations were transformed into *E. coli* SA30B (2) for appropriate methylation before transformation, of sequence positive constructs, into electrocompetent *S. aureus* using a protocol previously described (3).

Text S3: Flow cytometry analysis of murine cells

After isolation from murine livers immune cell viability was first determined using Fixable Viability Dye eFluor™ 506 (Thermo Fisher) and then subsequently stained anti-CD4-PE-Cy5 (clone RM4-5, Thermo Fisher) anti-CD45r(B220)-V450 (clone RA3-6B2, BD), anti-F4/80-A647 (clone BM8, Biolegend), anti-Ly6G-A700 (clone RB6-8C5, Biolegend), anti-Ly6C-BV711 (clone RB6-8C5, Biolegend), and anti-CD11b-PE (clone M1/70, Biolegend). Cells were fixed overnight with 1% paraformaldehyde prior to analysis. Events were acquired and data analyzed as outlined above. Events were acquired using a LSR II (BD Biosciences), and data were analyzed using FlowJo v10.7.1 (TreeStar).

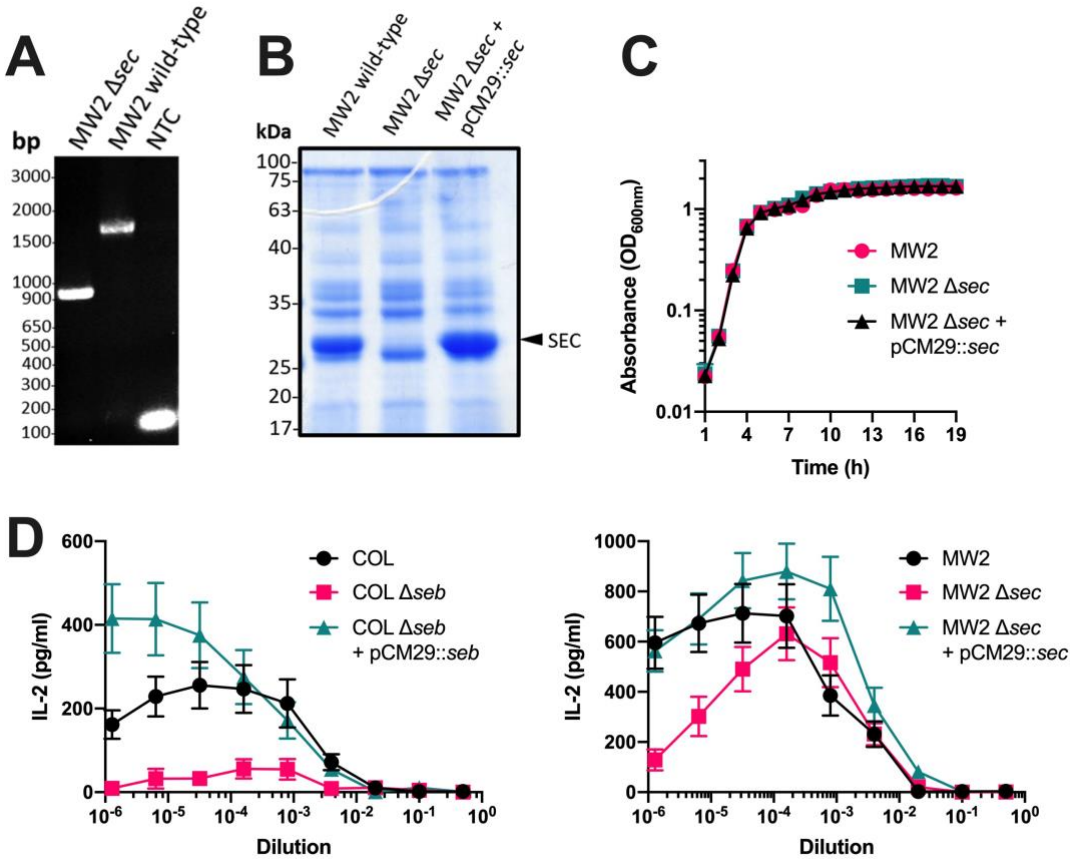

Figure S1. Validation of *S. aureus* COL and MW2 superantigen mutants. (**A**) Following allelic replacement, the deletion of *sec* gene was confirmed by PCR using primer SEC-screen-For and SEC-screen-Rev (Table S2). PCR products were run on a 1% gel and a PCR with genomic DNA from wild-type MW2 was included for comparison, along with a no template control (NTC). (**B**) *S. aureus* MW2 strains were grown in BHI broth for 8 h and the secreted profile was assessed by SDS PAGE analysis on 12% acrylamide gel stained with instant blue (Coomassie based stain). (**C**) To ensure that the *sec* deletion in MW2 had no impact on general viability, growth curve analysis was performed on the MW2 strains. Each clone was grown in TSB over the course of 19 h with measurements taken every hour. Data shown are mean ± SEM. (**D**) To confirm the ability of these constructs to be able to activate T cells, IL-2 production was determined. PBMC from human blood where isolated and stimulated with a titration of supernatant from *S. aureus* COL or MW2 strains. Supernatants were taken from cultures grown for 8 h in BHI prior to use in these assays. Data shown are mean ± SEM from 8 donors.

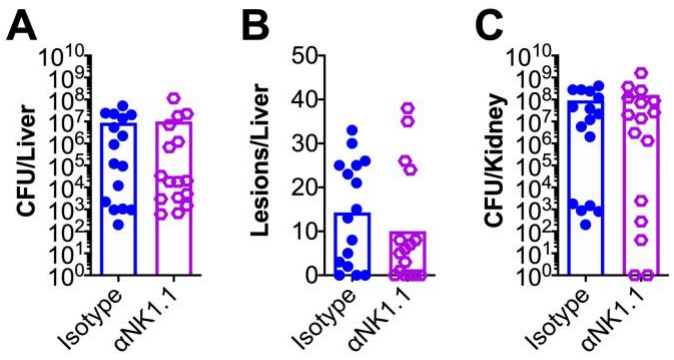

Figure S2. Depletion of NK1.1+ cells does not alter *S. aureus* burden or pathology during bloodstream infections. *In vivo* depletions in DR4-B6 mice were performed with monoclonal antibodies to deplete NK1.1 cells prior to intravenous infection of *S. aureus* COL. *In vivo* liver bacterial burden (**A**), liver pathology (**B**), and kidney bacterial burden (**C**) was assessed 96 h post i.v. challenge. Each data point represents an individual mouse, and the bar indicates the geometric mean for CFUs/organ, and the median for lesions/organ. No significant differences were detected.

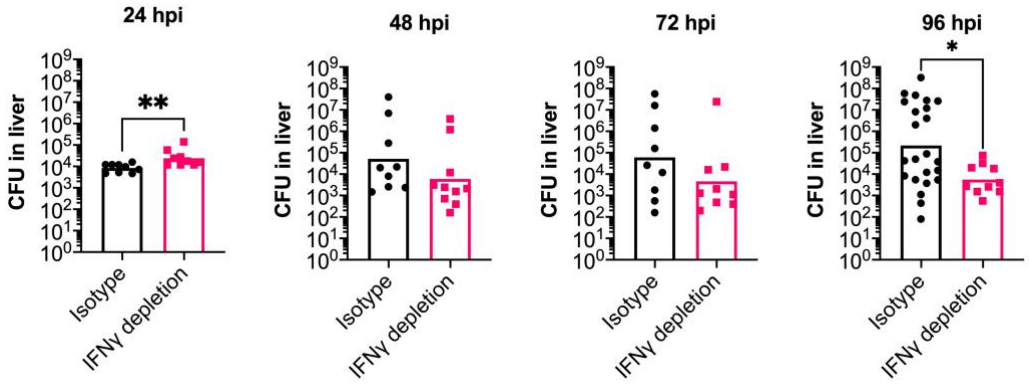

Figure S3. Daily Time-course of *S. aureus* liver burden during bloodstream infection. Animals were treated with the isotype control or IFNγ-depleting antibody 18 h prior to infection with *S. aureus* COL. Following infection, animals were sacrificed from each group at the 4 timepoints show, and livers were harvested from each animal and bacterial burdens determined. Each dot represents an individual mouse, and the bar indicates the geometric mean. Significant differences were determined using the Mann-Whitney test (* $p < 0.05$, ** $p < 0.01$,). For comparison, data shown in the 96 hpi timepoint are the same data included in Fig 3b (lacking the IL-17A depletion and isotype controls).

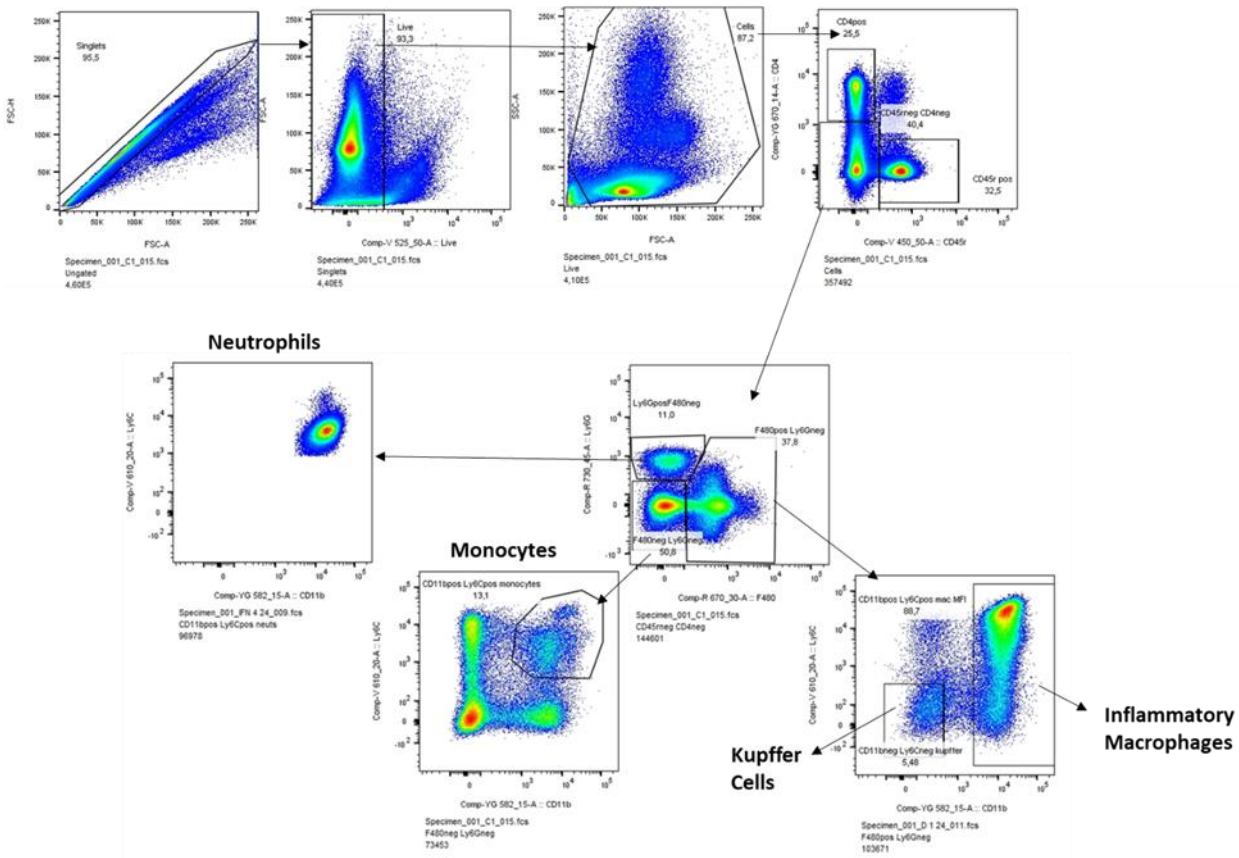

Figure S4. Flow cytometry gating strategy for liver analysis. Following isolation from murine livers, immune cells were analyzed by flow cytometry. Prior to cytometer analysis cells were stained with antibodies against F4/80, CD11b, Ly6C, Ly6B, CD45r and CD4. Also included was a live/dead stain to determine cell viability. Schematic of cell phenotyping is shown with arrows denoting the workflow for gating. Cells were gated first on whether they were liver or dead and the live population was subsequently gated for the singlet population. Single liver cells were checked for the expression of CD4 and CD45r markers and the double negative population (i.e. majority myeloid derived cells) were gated for further analysis. Cells were then checked for their expression of F4/80 and Ly6G. Cells that were subsequently high for Ly6G but negative for F4/80 were checked for CD11b and Ly6C expression and classed as neutrophils. Ly6C and F4/80 double negative cells were gated and checked for CD11b and Ly6C expression, with double positive cells classed as monocytes. F4/80 positive and ly6G negative cells were checked for CD11b and Ly6C expression. Cells that were negative for both were considered resident macrophages i.e., Kupffer cells. Cells remaining cells in this group that expressed high levels of CD11b were considered inflammatory macrophages.

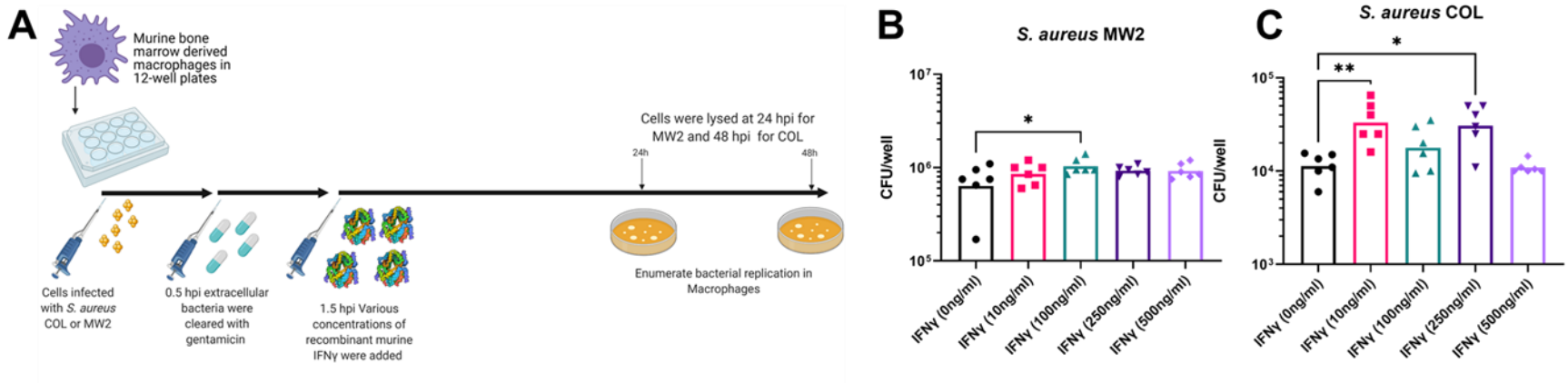

Fig S5: Murine Bone marrow derived macrophages are not permissive to *S. aureus* replication and this is not clearly reversed by IFNγ dosing. Schematic outlining the procedure for intracellular *S. aureus* replication in murine bone marrow derived macrophage (BMDM) after dosing with recombinant murine IFNγ. *S. aureus* recovered from murine macrophages after incubation at 24 h for strain MW2 (D) and 48 h for strain COL (E) with varying concentrations of recombinant IFNγ. Each dot represents macrophages from an individual mouse and the bar represents the geometric mean for CFUs/well. Significant differences between 0 ng/ml of IFNγ and other concentrations were determined using the Kruskal-Wallis test with uncorrected Dunn's test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$).

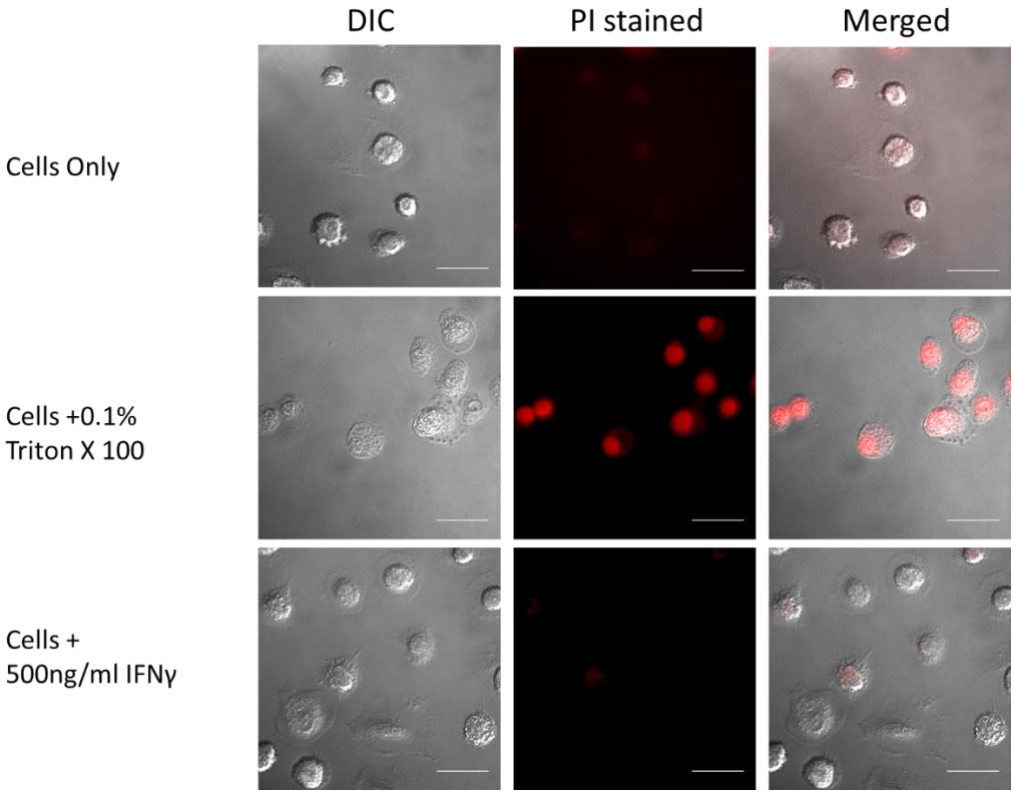

Fig S6. High doses of IFNγ have minimal impact on macrophage viability. Primary human macrophages were differentiated from blood. To ensure high dose IFNγ had little impact on cell viability cells were stained with propidium iodine (PI) to check for cell death. Differential interference contrast (DIC) microscopy was used to observe general cell morphology with red fluorescence used to determine the level of PI staining. Cells treated with IFNγ were observed after 24 h incubation and compared with cell in media only and a control positive for cell death, that had been treated with 0.1% triton X 100 10mins prior to microscopy.

Table S1. Bacteria and plasmids used in this study

Strain/plasmid	Description	Reference/Supplier
S. aureus		
COL	MRSA strain isolated in the 1960s (Clonal complex 8)	(6)
COL Δ*seb*	Null mutant of *seb* in COL background	(7)
COL Δ*seb* + pCM29::*seb*	COL Δ*seb* containing complementation plasmid expressing SEB	This Study
MW2	MRSA strain isolated in 1998 (Clonal complex 1)	(8)
MW2 Δ*sec*	Null mutant of *sec* in MW2 background	This Study
MW2 Δ*sec* + pCM29::*sec*	MW2 Δ*sec* containing complementation plasmid expressing SEC	This Study
E. coli		
XL1-Blue	General cloning strain	Stratagene
SA30B	DNA methylation strain	(2)
BL21 (DE3)	Protein expression strain	New England Biolabs
Plasmids		
pKOR1	Temperature-sensitive integration vector with inducible counter selection; Cmr	(1)
pKOR1::*sec*_del	pKOR1 with *sec* flanking regions inserted, Cmr	This study
pCM29::*pLukM*-sGFP	pCM29 vector containing the active promoter of the leukocidin LukMF'	(5)
pCM29::*pLukM-seb*	pCM29 vector containing the seb gene	This study
pCM29::*pLukM-sec*	pCM29 vector containing the sec gene	This study
pET28a	Protein expression plasmid, Kmr	Invitrogen
pET28a::*seb*	SEB protein expression plasmid, Kmr	(9)
pET28q::sebN23A	SEB_{N23A} protein expression plasmid, Kmr	(10)

[1] Cmr - Chloramphenicol resistant
[2] Kmr – Kanamycin resistant

Table S2: Primers used in this study

Primer name	Sequence 5'-3'
SEB complementation	
pCM29-seb-kpnI-For [1]	CACAGGTACCAAAGGAGATAAAAAATGTATAAGAGATTA
pCM29-seb-EcoRI-Rev [1]	CACAGAATTCTCACTTTTTCTTTGTCGTAAG
SEC deletion*	
pKOR-sec-upstream-For [2]	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**AGGCACAGCAATGTGTTCA
pKOR-sec-upstream-Rev[3]	GCTAGCACGCGTCTCCTTCATCCAACATTCCC
pKOR-sec-downstream-For[3]	ACGCGTGCTAGCGAGTGAAGATAGAAGTCCACCTTACA
pKOR-sec-downstream-Rev [2]	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GCAAGCATCAAACAGTTACAAC
SEC-screen-For	GAAATCCTCTGTTTCTCCTTGAG
SEC-screen-Rev	CTATAAATATGGTTCTAACTCTC
SEC complementation	
pCM29-sec-kpnI-For [1]	CCGGTACCGTGTATCTAGATACTTTTTGGGAA
pCM29-sec-EcoRI-Rev [1]	CCGAATTCTTATCCATTCTTTGTTGTAAGGTGG

* Primers design was based on primers previously described (14)
[1] Restriction sites (indicated in the primer name) are underlined in the primer sequence.
[2] attB sites used for recognition by BP Clonase are shown in boldface
[3] Overhang for splice PCR for fragment indicated are underlined in the primer sequence

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