

Molecular Analysis of Staphylococcal Superantigens

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Abstract

Staphylococcal superantigens (SAGs) comprise a large family of exotoxins produced by *Staphylococcus aureus* strains. These exotoxins are important in a variety of serious human diseases, including menstrual and nonmenstrual toxic shock syndrome (TSS), staphylococcal pneumonia and infective endocarditis, and recently described staphylococcal purpura fulminans and extreme pyrexia syndrome. In addition, these SAG exotoxins are being increasingly recognized for their possible roles in many other human diseases, such as atopic dermatitis, Kawasaki syndrome, nasal polyposis, and certain autoimmune disorders. To clarify the full spectrum of human diseases caused by staphylococcal SAGs, it is necessary to have assays for them. At present there are 23 characterized, serologically distinct SAGs made by *S. aureus*: TSS toxin-1 (TSST-1); staphylococcal enterotoxins (SEs) A, B (multiple variant forms exist), C (multiple minor variant forms exist), D, E, and G; and SE-like H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, and X. The most straightforward way to analyze *S. aureus* strains for SAGs is through polymerase chain reaction for their genes; we provide here our method for this analysis. Although it would be ideal to confirm that all of the same SAGs are produced by *S. aureus* strains that have the genes, antibody reagents for SAG detection are only available for TSST-1; SEs A–E and G; and enterotoxin-like proteins H, I, Q, and X. We provide a Western immunoblot procedure that allows in vitro quantification of these SAGs.

Key words Superantigens, *Staphylococcus aureus*, Toxic shock syndrome, Pneumonia, Infective endocarditis, Purpura fulminans, Extreme pyrexia

1 Introduction

Staphylococcus aureus causes a large number of human diseases, ranging from benign pimples and boils to life-threatening toxic shock syndrome (TSS), necrotizing pneumonia, infective endocarditis, purpura fulminans, and extreme pyrexia syndrome [1–7]. The organism has an array of cell-surface and cell-secreted virulence factors that allow it to cause illnesses [6]. The surface virulence factors allow the organism to colonize the host through adhesion to mucosal surfaces and resistance to phagocytosis. The secreted factors, including exoenzymes and exotoxins, allow the organism to interfere with normal immune system function, spread

into surrounding tissues, and access nutrients through cell damage. Among the secreted virulence factors that have known roles in serious human diseases are the superantigens (SAGs) [7, 8].

SAGs are simple protein exotoxins secreted by *S. aureus* strains, with one exception, as variable traits, meaning that some strains make the proteins, whereas others do not [7, 8]. The SAG family made by *S. aureus* includes TSST-1, the cause of nearly all menstrual TSS and half of nonmenstrual TSS [2, 9–11]; staphylococcal enterotoxin (SE) serotypes A, B n , C n (in which n denotes that multiple variant forms exist), D, E, and G; and SE-like serotypes H, I, and J–X [12]. Incidentally, there is no enterotoxin F, because this protein was renamed TSST-1 in 1984. Of these SAGs, SEs A–E and G have the ability to induce emesis in monkeys and are thus correctly referred to as SEs [12]. The remaining SAGs either have not been tested for emetic activity or lack emetic activity; and they are thus correctly referred to as enterotoxin-like proteins (SE-like G, H, and J–X) and TSST-1 [12]. All SEs and SE-like SAGs are able to cause nonmenstrual TSS illnesses [7, 8], but only SEG appears also to be associated rarely with menstrual TSS [13].

For purposes of this chapter, we provide methods to study TSST-1, SEs A–E and G, and SE-like proteins H–X. Their genes have been well characterized, and specific DNA primers may be made for their detection [12]. We also provide methods for quantifying production of selected SAG proteins to confirm gene function. These include tests for TSST-1; SEs A–E; and SE-like proteins G, H, and Q. Many of these proteins appear to be made in higher concentrations in vitro than the other SAGs, and they have been associated with human diseases [2, 7, 8, 10, 11, 13, 14]. Assays for other SAGs can be developed based on the techniques provided.

TSS is defined by the presence of fever, hypotension/shock, a red rash, desquamation of the skin on recovery, and a variable multiorgan component [15–17]. Many TSS patients do not meet all five of these defining criteria, which has led to the establishment of categories of illness including probable TSS and toxin-mediated disease [18, 19]. Thus, although it is clear that SAGs cause TSS, the spectrum of TSS illnesses remains to be defined completely. It is relatively easy to identify menstrual TSS, but there are large numbers of categories of nonmenstrual TSS-like illnesses, many of which are only now being associated with SAG production and *S. aureus*. Nonmenstrual TSS-like illnesses include illness associated with wounds, boils, and abscesses [19]; postsurgical [19]; post-respiratory virus TSS [5, 20]; purpura fulminans [5]; extreme pyrexia syndrome; necrotizing pneumonia [1, 14]; and recalcitrant erythematous desquamating disorder of patients with acquired immunodeficiency syndrome [21]. Other illnesses that may be associated with staphylococcal SAG production include infective endocarditis, atopic dermatitis and anaphylactic reactions [22, 23], nasal polyposis [3], mycosis fungoides

(cutaneous T-cell lymphoma) [24], Kawasaki-like illness [25], and some cases of acute onset rheumatoid arthritis. To understand the role of staphylococcal SAgS in human illnesses, assays have been developed for their measurement. These include polymerase chain reaction (PCR)-based techniques to demonstrate the presence of the genes, followed by antibody-based assays for protein production. These latter antibody assays were previously unnecessary for measures of TSST-1 and SEs B and C, because there was a one-to-one correlation between the presence of the genes and production of high levels of protein by the strains [8, 26]. This is not the case with other SAgS, e.g., SEA-positive strains may make undetectable protein (<5 pg/ml to 5 µg/ml) *in vitro*, depending on the strain analyzed.

SAgS have an unusual mechanism of action in their activation of the immune system, and thus, they are often used as probes of immune system function. Such studies are likely to become even more important as the spectrum of SAg illnesses expands, including into autoimmunity. SAgS act by causing T-lymphocyte proliferation independent of the antigenic specificity of the T cell; rather, such proliferation is dependent on the composition of the variable region of the β -chain of the typical α - β T-cell receptor (TCR) [7, 27]. Thus, TSST-1 stimulates all human T cells with the β -chain TCR variable region 2 (V β TCR-2), but not other T cells. The consequence of this stimulation is that V β TCR-2 T cells may expand to become 60–70 % of all of a TSS patient's T cells during acute TSS, as opposed to 10 % normally. The ability of the SAgS to stimulate T cells also depends on the proteins' abilities to bind to major histocompatibility complex II (MHC II) molecules on the surface of antigen-presenting cells, most importantly macrophages [7]. The consequence of the cross bridging of V β TCR on T cells with MHC II on macrophages is massive cytokine release by macrophages (tumor necrosis factor- α [TNF- α] and interleukin-1 β) and T cells (TNF- β and interferon- γ) and consequent TSS illness [7]. Presumably, variations in amounts and types of SAgS made by *S. aureus* strains control the severity and type of illness produced.

2 Materials

2.1 *S. aureus* Strains

1. Control *S. aureus* strains for detection of SAg genes: TSS strain MN8 for TSST-1 and atopic dermatitis strain MN22 for all others; the latter strain is a naturally occurring clinical isolate with the genes for all SAgS except TSST-1 (*see* **Note 1**).
2. Control *S. aureus* strains for detection of SAg production: strain MN8 for production of TSST-1; CDC 11 for SEA; community-associated methicillin-resistant USA400 necrotizing pneumonia isolate c99-529 for SEB; community-associated

methicillin-resistant USA400 necrotizing pneumonia isolate MW2 for SEC and SEH; isolate KSI1410 for SED; FRI 918 for SEE; MN6 for SEG; and MNNJ for SE-like Q (*see Note 2*). Production of the remaining SAgS is not well characterized; thus, we do not present data related to detection of their proteins.

3. SAgS SEs A–E, SE-like H, and TSST-1: these may be purchased from Toxin Technology (Sarasota, FL), to serve as control proteins. It is important to assay these proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) since commercially available proteins sometimes have significant contaminants. The Schlievert’s laboratory makes all necessary SAgS to serve as controls. A detailed description of their production is beyond the scope of this chapter but has been provided elsewhere [4].
4. Todd Hewitt broth for growth of *S. aureus* strains.

2.2 Polymerase Chain Reaction

1. Bacteria grown overnight in 25 ml of THB.
2. DNeasy Tissue Kit (Qiagen, Valencia, CA) for DNA extraction.
3. Taq DNA polymerase (Qiagen) or Phusion high-fidelity DNA polymerase (NEB).
4. Primers (*see Table 1*) from Integrated DNA Technologies (IDT, Coralville, IA). They are ordered DESALT and on a

Table 1
PCR primers for SAg genes (TSST-1, SEs, and SE-like [SE/I]) presented from 5’ to 3’

Primer name	Primer sequence	Estimated size (bp)
SEA forward SEA reverse	GATTCACAAAGGATATTGTTGATAAATAT GTCCTTGAGCACCAAAATAATC	400
SEB forward SEB reverse	GTATGATGATAATCATGTATCAGCAA CGTAAGATAAACTTCAATCTTCACAT	625
SEC forward SEC reverse	GAGTCAACCAGACCCTATGCC CGCCTGGTGCAGGCATC	650
SED forward SED reverse	GCATTACTCTTTTTTACTAGTTTGGTA CCTTGCTTGTGCATCTAATTC	530
SEE forward SEE reverse	CTGAATTACAAAGAAATGCTTTAAGC GCCCTGCCTGAAGATCTA	420
SEG forward SEG reverse	AATTCCCAACCCGATCCTAAAATAG TCAGTGAGTATTAAGAAATACTTCCATTTTAATAC	708
SEI-H forward SEI-H reverse	TCACATCATATGCGAAAGCAG TAGACCAATCACCCTTTCC	357

(continued)

Table 1
(continued)

Primer name	Primer sequence	Estimated size (bp)
SEI-I forward SEI-I reverse	CAAGGAGATATTGGTGTAGGTAAC TTAGTTACTATCTACATATGATATTTCAACATC	657
SEI-J forward SEI-J reverse	CAGCGATAGCAAAAATGAAACA CCCTCTTCTAGCGGAACAAC	450
SEI-K forward SEI-K reverse	TGGATCAATGGAAATCAACAAAA CGGGCTACCCGAAAAATAAT	420
SEI-L forward SEI-L reverse	CTGTTTGATGCTTGCCATTG GCGATGTAGGTCCAGGAAC	370
SEI-M forward SEI-M reverse	GATGTTGGAGTTTTGAATCTTAGGAAC TCAACTTTCGTCCTTATAAGATATTTCTACATC	654
SEI-N forward SEI-N reverse	GATGTAGACAAAAATGATTTAAAGAAAAAATC TTAATCTTTATATAAAAAATACATCGATATGATAATTAG	684
SEI-O forward SEI-O reverse	AATGAAGAAAAATCCTAAAATTGAGG TTATGTAAATAAATAAACATCAATATGATAGTC	699
SEI-P forward SEI-P reverse	ACCAACCGAATCACCAGAAG GTTCAAAAGACACCGCCAAT	400
SEI-Q forward SEI-Q reverse	GATGTAGGGGTAATCAACCTTAG CTCTCTGCTTGACCAGTTCC	500
SEI-R forward SEI-R reverse	TACTATGGGGAATGTTGAATCC GGTATAAAGGGAACCAATCC	558
SEI-S forward SEI-S reverse	CTAACTCTTGAATTGTAGGTTCC CTCCACACAACCTATTATCAAACG	332
SEI-T forward SEI-T reverse	TCGGGTGTTACTTCTGTTTGC GGTGATTATGTAGATGCTTGGG	170
SEI-U forward SEI-U reverse	AAACCAGAACAATTGAATAAAGCG TTATTTTTTGGTTAAATGAACCTTCTACATTAATAG	714
SEI-X forward SEI-X reverse	TCTATGGGGGAACATTTGGA CCGCCATCTTTTGTATTATGA	420
TSST-1 forward TSST-1 reverse	GAAATTTTTCATCGTAAGCCCTTTGTTG TTCATCAATATTATAGGTGGTTTTTCA	655

0.05- μ mol scale. Once received, resuspend the primers in distilled deionized water to a final concentration of 50 pmol/ μ l (*see Note 3*).

- TempAssure single tubes, strips, or plates (0.2 ml) to carry out the reactions (USA Scientific, Ocala, FL).
- PCR thermocycler, no gradient required.

7. DNA gel:

- (a) Type 1 agarose.
- (b) Tris acetate EDTA (TAE) buffer or Tris borate EDTA buffer (TBE) made at a 50× or 5× concentration, respectively. The 50× TAE buffer is made with 242 g of Tris base, 57.1 ml glacial acetic acid, 100 ml of 0.5 M EDTA (pH 8.0), and distilled deionized water to a final volume of 1 l. The 5× TBE buffer is made with 54 g Tris base, 27.5 g boric acid, 20 ml of 0.5 M EDTA (pH 8.0), and distilled deionized water to a final volume of 1 l. The 0.5 M EDTA (pH 8.0) is made with 186 g EDTA per liter (pH to 8.0 with ~20 g/L of sodium hydroxide pellets).
8. DNA gel apparatus QS-710 (International Biotechnologies, New Haven, CT).
9. 1 kb plus DNA ladder.
10. Premixed ethidium bromide solution, 10 mg/ml (Bio-Rad). This chemical is a DNA intercalating agent and very hazardous. If made in the lab, ethidium bromide must be weighed out only under a chemical hood and handled with gloves.

2.3 Antisera

1. Polyclonal hyperimmune antisera against the SAGs SEs A–E, SE-like H, and TSST-1 (Toxin Technology, Sarasota, FL) (*see Note 4*). It is important to note that these commercially available antisera are made monospecific by various absorption techniques, and this may interfere with some assays. Hyperimmune antisera against SEG and SE-like Q are not commercially available, but we produce antisera in rabbits.
2. Antisera made by immunization of rabbits with the various purified SAGs. It is our experience that injection of 25 µg of SAG emulsified in incomplete Freund's adjuvant into the nape of the neck of rabbits every other week for three to four injections results in high-level serum antibody titers.

2.4 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 40 % Acrylamide/Bis, 37.5:1 (2.6 % C) from Bio-Rad. Acrylamide is neurotoxic; care should be taken when working with this compound.
2. TEMED.
3. 1.5 M Tris–HCl, pH 8.8 for resolving (lower) gel. Store at room temperature.
4. 0.5 M Tris–HCl, pH 6.8 for stacking (upper) gel. Store at room temperature.
5. 10 % solution of ammonium persulfate (APS) in deionized distilled water. Prepare fresh each time (for adequate gel polymerization).
6. 10 % SDS in deionized distilled water.

7. Running buffer (4 l): 12.12 g Tris base, 57.04 g glycine, 2 g of SDS, and deionized distilled water up to 4 l. The final concentrations are 0.05 M Tris base, 0.4 M glycine, and 0.1 % (w/v) SDS. Store at room temperature.
8. Sample buffer (2×): 0.125 M Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol, and 0.001 % (w/v) bromophenol blue in deionized distilled water. Sample buffer can be made more concentrated if a larger sample volume is desired.
9. Prestained molecular weight markers (SeeBlue® Plus2; Invitrogen, Carlsbad, CA).
10. SDS-PAGE and Western transfer apparatus: we use a Bio-Rad mini PROTEAN® 3 Cell and Western transfer apparatus (Hercules, CA).

2.5 Western Immunoblotting

1. Transfer buffer: for transfer buffer without methanol, dissolve 6 g of Tris base and 7.3 g of glycine in up to 2 l of deionized distilled water. For transfer buffer with methanol, dissolve 6.04 g of Tris and 28.8 g of glycine in 1.6 l of deionized distilled water, and add 200 ml of methanol and deionized distilled water to a final volume of 2 l.
2. Immunoblot polyvinyl difluoride (PVDF) membrane (Bio-Rad) and methanol to equilibrate the PVDF membrane.
3. Tris-buffered saline (TBS): 0.61 g Tris base, 4.38 g NaCl, and deionized distilled water to a final volume of 500 ml. For TBS-Tween-20 (TBST), add 250 µl of Tween 20 to a 500-ml volume of TBS. Final concentrations are 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, and 0.5 % (v/v) Tween-20.
4. Blocking solution: 1 % (w/v) bovine serum albumin (BSA) in TBST. For example, add 0.3 g of BSA to a 30-ml volume of TBST.
5. Primary antibody: usually 10–30 µl in 20 ml of TBST.
6. Secondary antibody: usually 10–20 µl in 20 ml of TBST. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich).
7. Alkaline phosphatase (AP) buffer: 20-ml volume per blot containing 1 ml of 1 M Tris-HCl, pH 9.6, 80 µl of 1 M MgCl₂, and 19 ml of deionized distilled water. AP buffer can be made in larger volumes (e.g., 0.5–1 l) and stored at room temperature.
8. NBT substrate: nitroblue tetrazolium dye to a final concentration of 2 mg/ml of water. Store at –20 °C.
9. BCIP substrate: 5-bromo-4-chloro-3 indolyl phosphate to a final concentration of 5 mg/ml of dimethylformamide (DMF). Dissolve 0.05 g of BCIP in 10 ml of DMF and store at –20 °C covered in foil (light sensitive). Note that DMF is volatile and toxic; review the chemical safety information before handling this product.

2.6 Density Scans

1. Image J 1.34S program from NIH (this can be downloaded from <http://rsb.info.nih.gov/ij/>).

3 Methods

For assessing the presence of the genes for all known SAGs and the proteins for SEs A–E and G, SE-like H and Q, and TSST-1, strains to be analyzed plus controls are cultured aerobically (with shaking at 200 rpm) at 37 °C until the organisms are well into stationary phase. Most SAGs are made either during the exponential phase (from an initial absorbance of 0 to 1.0 at 600 nm wavelength; examples are SEA and SE-like K) or post exponential phase (from 1.0 to approx. 3.0 absorbance at 600 nm; examples are SEB, SEC, and TSST-1). We use THB as the growth medium.

3.1 Polymerase Chain Reaction

1. Grow bacteria overnight in 25 ml of THB at 37 °C with shaking at 200 rpm. Add the grown culture (1.5 ml) to a microcentrifuge tube and spin down at $14,000 \times g$ for 2 min. Aspirate the supernate, and extract the DNA from the pellet according to the protocol found on page 33 of the DNeasy Tissue Handbook provided by the supplier of the kit. There are two deviations from the DNeasy method:
 - (a) Add 20 μ l of lysostaphin to the DNeasy enzymatic lysis buffer (*see* **Note 5**). Lysostaphin can be purchased from Sigma-Aldrich and is resuspended in distilled water to a final concentration of 1 mg/ml (it can be stored frozen with repetitive freeze/thaws at –20 °C until used). The enzymatic lysis buffer is made fresh for each batch of bacteria grown.
 - (b) Instead of performing elutions twice with 200 μ l of EB buffer each time, elute the DNA twice with 100 μ l of water each time.
2. After the DNA is extracted in a final volume of 200 μ l, make the PCR reaction mixture, and keep it on ice during the preparation. To perform PCR of the genes, add 1 μ l of bacterial DNA to a 0.2-ml tube to make a 50- μ l total volume reaction. Follow the manufacturer's instruction for the addition of primers, reaction buffer, dNTPs, and polymerase, as they vary depending on the polymerase of choice. The PCR reaction can also be performed in 30- μ l volumes. Include positive and negative control bacterial DNA samples.
3. After all reaction mixtures are made, tightly close the caps of the tubes, and insert the tubes into PCR thermocycler. Calculate the annealing temperature optimal for the primer pair according to the polymerase used (Taq usually requires lower annealing temperatures than Phusion). The New England Biolabs (NEB) website provides annealing temperatures for a primer pair according

to the polymerase of choice. Follow the cycle settings of the polymerase used for a total of 30 cycles. The initial DNA denaturation step can be carried out for 1–3 min, and the final elongation step can be carried out for 4 min. The extension time will depend on the polymerase used and the expected DNA fragment size, estimate accordingly. When the cycles are completed, keep the reaction mixture at 4 °C until the DNA gel is prepared and ready to be subjected to electrophoresis.

4. To make the DNA gel, dilute 50× TAE or 5× TBE buffer to 1× with distilled water. Prepare 0.8 % agarose gels by adding 4 g of agarose to 500 ml of 1× TAE or TBE buffer. Microwave the mixture until it becomes clear. Allow the hot gel mixture to cool on the bench until bottle is cool to the touch. It is important that the gel mixture is cool enough so ethidium bromide vapors are not created when added to the gel. Add 20 µl of ethidium bromide (from 10 mg/ml premixed solution) to 500 ml of TBE or TAE gel mixture. Pour the gel into the gel tray until the bottom of the tray is covered with 0.75–1 cm of gel. Add a comb to the gel with the appropriate number of wells (samples + ladder).
5. Once the gel has completely solidified, remove the comb, and place the gel in the gel-running apparatus. Completely submerge the gel in the same 1× buffer used to prepare the DNA gel (addition of ethidium bromide to the buffer is not required, but it increases the quality of detection of the DNA fragments).
6. Add DNA ladder (2–4 µl) to the leftmost lane, and add 10 µl of PCR products individually to additional wells (DNA loading dye should be added to the mixture if not included in the polymerase kit). DNA loading dye (6×) can be made with 25 mg of bromophenol blue, 25 mg of xylene cyanol FF, and 1.5 g of Ficoll to a final volume of 10 ml. Attach the gel apparatus cathode and anode to a power supply, and electrophorese the samples at approx. 100 V for 1 h.
7. Take the gel to an ultraviolet (UV) light box and expose to UV light. The UV light will cause the DNA to luminesce, allowing detection of the PCR products. Products under 100 bp in length are assumed to be degraded DNA or remnants of primers. Figure 1 shows the results of testing a strain of *S. aureus* (FRI913) for the presence of SAg genes. The strain is positive for the SEA, SEC, SEE, TSST-1, and SE-like L, Q, and X genes (see Note 6).

3.2 Growth and Preparation of *S. aureus* for SAg Protein Assays

1. Inoculate *S. aureus* strains into 10 ml THB in 125-ml Erlenmeyer flasks, and culture aerobically until stationary phase.
2. Place the samples in 50-ml conical centrifuge tubes (Falcon polypropylene; Becton, Dickinson, and Company), and add 40 ml of absolute ethanol (4 vol) to the cultures to precipitate

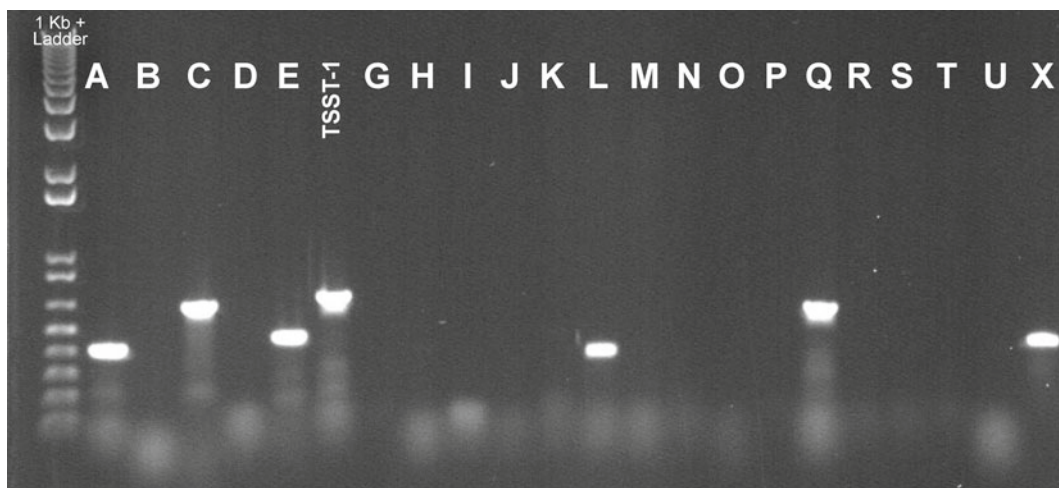


Fig. 1 PCR screen for the presence of superantigens using chromosomal DNA from the *S. aureus* strain FRI 913

SAGs while killing the *S. aureus* cells. We have found that this 80 % final concentration of ethanol will precipitate 100 % of SAGs in as little as 2 h; the treated cells may remain at room temperature or 4 °C for months without losing the ability to be resolubilized.

3. Collect the precipitates by centrifuging at $1,000 \times g$ for 15 min. Pour off the ethanol, and place the samples flat under a laminar flow hood for 30 min to dry off the remaining ethanol (*see Note 7*).
4. Resuspend the samples in 1 ml of distilled water (10 times concentrated relative to the original supernate concentration), and centrifuge in a microfuge ($14,000 \times g$ for 5 min).
5. Treat a small volume (100 μ l) of the clarified supernates with 100 μ l of 2 $\times$ SDS-PAGE sample buffer, and boil for 3–5 min, or leave at room temperature overnight to denature the proteins.

3.3 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

As stated previously, we use the Bio-Rad mini PROTEAN 3 Cell and Western transfer apparatus. The gel apparatus is equipped with a minigel system that is 1.0 mm or 1.5 mm thick. The glass slides are carefully washed with soap and water, then rinsed with ethanol, and dried. The apparatus is assembled and the gels are poured.

1. Prepare the stacking (upper) gel and the resolving (lower) gel by following the amounts and order listed in Table 2 (*see Note 8*). Volumes are calculated for two gels. Mix and pour the resolving gel first; when done polymerizing, mix and pour the stacking gel. Alternatively, prepare the stacking gel without TEMED and add when ready to be poured.

Table 2
Preparing the stacking (upper) gel and the resolving (lower) gel

	1.0 mm			1.5 mm resolving	
	Stacking	resolving			
Percent gel	4 %	12 %	16 %	12 %	16 %
40 % Bis-acrylamide	0.3 ml	4.8 ml	6.4 ml	7 ml	8 ml
0.5 M Tris-HCl, pH 6.8	750 µl	–	–	–	–
1.5 M Tris-HCl, pH 8.8	–	4.0 ml	5.0 ml	4.0 ml	5.0 ml
10 % SDS	30 µl	160 µl	160 µl	200 µl	200 µl
10 % APS	30 µl	200 µl	200 µl	250 µl	250 µl
ddH ₂ O	1.874 ml	6.82 ml	5.22 ml	7.83 ml	6.525 ml
TEMED	10 µl	20 µl	20 µl	25 µl	25 µl
Final volume	3 ml	16 ml	16 ml	20 ml	20 ml

Final volumes are enough for two gels

2. Pour the solution with a 10- or 5-ml pipet into the glass plates leaving space for the stacking gel and comb (leftover gel is thrown away after it polymerizes in the tube). To make a smooth line, the gel can be overlaid with saturated butanol or distilled water. Let the gel polymerize for at least 15 min (if butanol is used, wash it off with distilled water), and dry between plates with filter paper.
3. Pour the stacking gel, put the comb in place, and allow the gel to polymerize for ~30 min. Use leftover gel to check for correct polymerization. Gels can be stored with the combs for a few weeks wrapped in paper towel and soaked in SDS-PAGE running buffer (usually in a resealable bags).
4. Carefully remove the comb, rinse the wells with running buffer to remove unpolymerized gel from the wells (otherwise it will interfere with loading of the sample), and place the gel in the apparatus for electrophoresis. The wells can be easily rinsed with the use of a 12-ml syringe and an 18-gauge needle.
5. Add running buffer to the upper and lower chambers, according to the manufacturer's instructions, and 20 µl of each sample to the wells using a pro pipet equipped with extended tips. Control SAg samples loaded at 10.0, 1, 0.1, 0.01, and 0.001 µg/ml should be loaded for quantification. Molecular weight markers are loaded as recommended by the supplier. The unknown samples can be diluted 10- and 100-fold to ensure that the SAg concentrations are in the range of appropriate quantification.

6. Completely assemble the apparatus with electrodes, and turn on the apparatus.
7. Electrophorese the samples at 150–180 V until the bromophenol blue marker dye reaches the bottom of the resolving gel (*see Note 9*). Turn off the apparatus. The gels are now ready to be used for Western immunoblotting.

3.4 Western Immunoblotting for SAgS

Resolved proteins are transferred to PVDF membranes for Western immunoblotting. These directions assume use of the Bio-Rad mini PROTEAN 3 Cell and Western transfer apparatus.

1. Equilibrate the PVDF membrane by wetting in absolute methanol. Pour 1× Western transfer buffer in a glass dish, and place two sponges (included with the apparatus) and two pieces of 3MM paper cut to the size of the PVDF membrane in the glass dish (per gel).
2. Remove the SDS-PAGE gel from the apparatus, separate the glass plates containing the gel with a spatula, remove the stacking gel with a spatula, and place the plate with the gel attached in the glass dish with transfer buffer. Remove the gel from its glass plate using gentle agitation and a spatula, and then place on a piece of wetted 3MM paper. In order, place the following on the transfer cassette: one piece of wetted sponge, the piece of 3MM paper with attached gel, the PVDF membrane, the second piece of 3MM paper, and the second piece of wetted foam. Be careful not to allow air bubbles to become trapped between the 3MM paper with gel, the PVDF membrane, and the second piece of 3MM paper, to ensure uniform transfer. Set up the cassette with the black side down, and add the stack toward the clear side. When inserted into the transfer apparatus, the black side is toward the cathode (which is marked as black).
3. Fill the transfer tank with transfer buffer, place a magnetic stir bar at the bottom of the apparatus, and insert the closed cassette.
4. Place the transfer apparatus onto a magnetic plate (to help dissipate heat), and perform electrotransfer of the samples onto the PVDF membrane for about 1 h at 200 mA at 4 °C. Ice packs fitted to the transfer apparatus are available for transfers done at room temperature. Transfers done in buffer without methanol require 70–80 min (45–60 min is enough for transfers done in buffer containing methanol).
5. Disassemble the apparatus, and remove the PVDF membrane. Nitrocellulose and Nytran membranes can also be used, but PVDF membranes are more durable and useful for protein sequencing (*see Note 10*).
6. Quick rinse the membrane twice with 30-ml volumes of TBS in a large petri dish to remove residual detergent.

7. Block the PVDF membrane for at least 30 min with a 30-ml volume of TBST containing 1 % BSA, and gently shake on a platform shaker at room temperature (*see Note 11*). Alternatively, membranes can be block overnight at 4 °C.
8. Quick rinse the membrane twice with 30-ml volumes of TBST and add primary antibody (usually 10–30 µl) in TBST-1 % BSA. Shake for at least 30 min at room temperature.
9. Perform three, 5-min washes with 30-ml volumes of TBST on a platform shaker.
10. Add secondary antibody (usually 10–20 µl) in a 30-ml volume of TBST-1 % BSA. Shake for at least 30 min at room temperature.
11. Perform three 5-min washes with 30-ml volumes of TBST on a platform shaker.
12. Quick rinse twice with 30-ml volumes of TBS to remove residual detergent, and incubate membrane for 5 min in a 30-ml volume of AP buffer.
13. Discard AP buffer, and place the membrane in a new large petri dish (a disposable container is required as the substrates are corrosive). Add a 30-ml volume of AP buffer containing 1 ml of NBT and 400 µl of BCIP (AP buffer plus substrates can be prepared up to 1 h in advance and kept at room temperature). Agitate the membrane at room temperature until the standard SAg samples have reacted, turning the membrane slightly purple (the time to stop the reaction is just when the background also begins to turn purple). Stop the reaction by pouring copious distilled water on the membrane.
14. Remove the membrane from the substrate solution, put in between paper towels to dry, and keep away from the light (the membrane will continue to develop in the light as long as the membrane is wet). The membrane can be air-dried quickly by placing the membrane sandwiched in between paper towels on the laminar flow hood.
15. Photograph the PVDF membrane, and upload the image onto a computer. Fluorescent imaging systems, like the Odyssey, can be used to scan the membrane for protein quantification.

3.5 Determination of Density

For measuring SAgS, we use the NIH imaging program, Image J, to capture the density of the protein bands. To do this, we use the program to draw around each stained band and determine its density. The background density is determined from three regions of the PVDF membrane that do not contain protein and subtracted from the SAg values.

It is important to note that *S. aureus* makes protein A, which has the ability to bind IgG nonspecifically and thus will also come through in the membranes. The protein A bands are not present in

the same molecular weight range as the SAg and thus, do not interfere with band quantification. Standard amounts of SAg are used to prepare a standard curve by plotting band density versus the logarithm of the SAg concentration. This typically gives a straight line with an R^2 value >95 . The amount of SAg made by the unknown samples is derived from the graph.

4 Notes

1. *S. aureus* strains may be stored indefinitely lyophilized or frozen at -20 or -80 °C. To store strains, grow the organisms in THB until stationary phase; usually overnight will suffice. Collect the cells by centrifugation ($1,000 \times g$, 15 min), and suspend them in 1/10 vol with THB supplemented with 10 % rabbit blood (for lyophilization) or THB containing 10 % glycerol (for freezing). The *S. aureus* cells may then be lyophilized in ampoules or frozen.
2. If the investigator wishes to assess only whether or not SAg are produced by the strains and not quantify the SAg, double immunodiffusion may be used instead of Western immunoblotting. It is our experience that the antisera and control SAg available from Toxin Technology are suitable for such analyses. Double immunodiffusion is performed after growing the strains in THB and concentrating the culture fluids by treatment with 4 vol of ethanol to precipitate SAg and resuspension to one-tenth the original volume. Microscope slides are used to prepare the double immunodiffusion gels. These are coated with 4 ml of 0.75 % agarose melted in phosphate-buffered saline (0.005 M sodium phosphate, pH 7.2; 0.15 M NaCl). After the slides have solidified, wells 4 mm in diameter and 4 mm from each other are punched in the agarose in a hexagonal pattern. Control SAg (20 μ l of 50–100 μ g/ml) and samples to be tested (20 μ l) are added to adjacent wells. The slides are incubated for 4 h at 37 °C in a humidified chamber or overnight at room temperature after addition of 20 μ l of antiserum to a center well. The slides are read for precipitation lines forming lines of identity with control SAg. In this assay, staphylococcal protein A does not interfere with the reactions, because rabbit IgG does not precipitate with protein A, even though it will react with rabbit IgG. It is our experience that this assay can be used with 100 % effectiveness to detect SEB, SEC, SED, SEE, SE-like H, and TSST-1 qualitatively.
3. When PCR is performed, the investigator may wish to use multiplex analysis in which multiple assays are performed in the same reaction tube, with agarose gel electrophoresis used to separate the different samples based on size. We do not

routinely perform such assays. However, such analyses are possible if the researcher uses primers of different sizes. Some of the primer combinations given in Table 1 can be used for this purpose.

4. It is our experience that the antisera and SAGs purchased from Toxin Technology are stable when stored at 4 °C as long as they do not become contaminated. We have retained such preparations for up to 1 year without loss of activity. If the researcher wishes to store samples frozen, the preparations should not be repetitively frozen and thawed.
5. Lysostaphin is required for lysis of *S. aureus* cells. Despite the fact that many textbooks state that Gram-positive bacteria can be easily lysed with lysozyme, this is not the case with staphylococci.
6. In our experience, it is always best to determine the SAG gene composition of the strains to be tested prior to performing assays for the proteins. This allows conservation of expensive antisera and control SAGs (purchased from Toxin Technology). If the investigator wishes to test only for SEB, SEC, and TSST-1, only assay for either the genes or SAG proteins needs be performed. We find that there is a one-to-one correlation with the presence of the genes and their production. These proteins typically are made in concentrations of 5–100 µg/ml of culture fluid when cells are grown aerobically. Other SAG testing requires assays for both the genes and the proteins: the proteins may or may not be made by the strains, and if made, the proteins may be present in very low concentrations.
7. It is unnecessary to dry the samples completely. We allow air to blow across the tubes until excess ethanol is removed and the surface of the residual pellet has a dull finish.
8. Ammonium persulfate is inactivated by oxygen; thus, vigorous mixing is strongly discouraged. If the gels do not polymerize, it is possible that the ammonium persulfate is too old (we purchase new ammonium persulfate every 6 months) or that the compound is inactivated by exposure to air. In the latter case, this can be overcome by mixing the gel in a side-arm flask and deaerating the solution while mixing. This can be done by connecting the side-arm flask to a vacuum for about 2 min while mixing.
9. The molecular weights of all SAGs are between 22,000 and 30,000. This corresponds to a gel region with minimal other contaminating staphylococcal proteins. It is worth noting that the SAGs separated by this method will be near the gel bottom. Thus, the researcher may wish to electrophorese the samples until the bromophenol blue dye is only three-fourths of the way to the bottom.

10. We use PVDF membranes for Western immunoblotting for two reasons: first, they are sturdy and resist tearing, and second, we often electrophorese second samples with the intent of staining the PVDF membrane with Coomassie brilliant blue dye to allow visualization of all proteins. In this case we may wish to cut out and partially destain proteins for submission for direct protein sequence analysis. This is easily accomplished with the use of PVDF membranes.
11. Three percent gelatin may also be used in place of 1 % BSA. In that case, be sure to incubate with blocking solution at 37 °C to prevent the gelatin from solidifying.

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