

Molecular Analysis of Staphylococcal Superantigens

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Summary

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 pneumonias, and a recently described staphylococcal purpura fulminans. 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 17 well-characterized, serologically distinct SAGs made by *S. aureus*: TSS toxin-1; staphylococcal enterotoxins (SEs) A, B, C (multiple minor variant forms exist), D, E, and I; and SE-like G, H, J, K, L, M, N, O, P, and Q. In addition, SE-like proteins R, S, T, and U have been identified but remain poorly characterized. The most straightforward way to analyze *S. aureus* strains for the well-characterized 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 TSS toxin-1; SEs A–E; and enterotoxin-like proteins G, H, and Q. We provide a Western immunoblot procedure that allows in vitro quantification of these SAGs.

Key Words: Superantigens; *Staphylococcus aureus*; toxic shock syndrome; necrotizing pneumonia; purpura fulminans.

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, and purpura fulminans (1–7). The organism has an array of cell-surface and secreted virulence factors that allow it to cause illnesses (6). The surface virulence factors allow the organism to colonize the host, through

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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 as variable traits, meaning that some strains make the proteins whereas others do not (7,8). The SAG family made by *S. aureus* includes toxin-1 (TSST-1), the cause of nearly all menstrual TSS and half of nonmenstrual TSS (2,9–11); staphylococcal enterotoxin (SE) serotypes A, B, Cn (in which n denotes that multiple variant forms exist), D, E, and I; and SE-like serotypes G, H, and J–U (12). Incidentally, there is no enterotoxin F, because this protein was renamed TSST-1 in 1984. Of these SAGs, SEs A–E and I 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 are thus correctly referred to as enterotoxin-like proteins (SE-like G, H, and J–U) and TSST-1 (12). All SEs and SE-like SAGs are able to cause nonmenstrual TSS illnesses (7,8), but in addition to TSST-1, only SEG appears to be associated with menstrual TSS (13).

For purposes of this chapter, we provide methods to study TSST-1; SEs A–E and I; and SE-like proteins G, H, and J–Q. Their genes have been well characterized, and specific DNA primers may be made for their detection (12). We believe similar methods may be used for detection of the genes of enterotoxin-like proteins T and U when more information is available for these SAGs. We also provide methods for quantifying production of selected SAG proteins as measures to confirm gene function. These include tests for TSST-1; SEs A–E; and SE-like proteins G, H, and Q. 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, as they become recognized as more important, 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 TSS is caused by SAGs, 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)

and 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 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 first 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, where, 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).

- Control *S. aureus* strains for detection of SAg production: strain MN8 for production of TSST-1; CDC 11 for SEA; nonmenstrual TSS isolate MNHO for SEB; community-associated methicillin-resistant isolate MW2 for SEC and SEH (strain represents CDC USA 400 necrotizing pneumonia clones); isolate KSI1410 for SED; FRI 918 for SEE; MN6 for SE-like G; and MNNJ for SE-like Q (see **Note 2**). Production of the remaining SAg is not well characterized; thus, we do not present data related to detection of their proteins.
- SAGs SEA-E, SE-like H, and TSST-1: These may be purchased from Toxin Technology (Sarasota, FL), to serve as control proteins. The Schlievert 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**).
- Todd Hewitt medium (Becton, Dickinson, and Company, Sparks, MD), for growth of *S. aureus* strains.

2.2. Polymerase Chain Reaction

- Bacteria grown overnight in 25 mL of Todd Hewitt broth.
- DNeasy Tissue Kit (Qiagen, Valencia, CA), for DNA extraction.
- ReddyMix PCR Master Mix with loading dye (Abgene, Rochester, NY).
- Primers (see **Table 1**) from Sigma Genosys (St. Louis, MO). They are ordered DESALT and on a 0.05- μ mol scale. Once received, resuspend the primers in water to a final concentration of 100 pmol/ μ L (see **Note 3**).
- TempAssure 8-tube strips (0.8 mL), to hold the reaction mixtures (USA Scientific, Ocala, FL).
- PCR machine such as the Thermal Mastercycler (which is what we use) with gradient #5331 (Eppendorf, Hamburg, Germany).
- DNA gel:
 - Type 1 agarose (Sigma-Aldrich, St. Louis, MO).
 - Tris acetate EDTA (TAE) buffer made at a 10X concentration. The ingredients for the 10X buffer are 96.9 g of Tris base, 13.6 g of sodium acetate, 6.05 g of EDTA (disodium), and 2 L of distilled water.
- DNA gel apparatus QS-710 (International Biotechnologies, New Haven, CT).
- exACTGene 1 kb Plus DNA Ladder (Fisher, Pittsburgh, PA).
- Ethidium bromide solution made to a final concentration of 1 μ g/ μ L using powdered ethidium bromide dissolved in distilled water. This chemical is very hazardous and should only be weighed out under a chemical hood and handled with gloves.

2.3. Antisera

- Polyclonal hyperimmune antisera against the SAGs SEA-E, SE-like H, and TSST-1 (Toxin Technology, Sarasota, FL) (see **Note 4**). Hyperimmune antisera against SE-like G and Q are not commercially available, but we produce antisera in rabbits.
- Antisera made by immunization of rabbits with the various purified SAGs. It is our experience that injection of 25 μ g 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.

Table 1
PCR Primers for SAGs Presented from 5N to 3N

Primer name	Primer sequence	Approximate size (bp)
SEA Forward	ATTGTTTTGGGGGAGTTTGAAGTT	400
SEA Reverse	TACATTGCGTTTTATTGGTTGCTC	
SEB Forward	GTATGATGATAATCATGTATCAGCAATA	640
SEB Reverse	CGTAAGATAAACTTCAATCTTCACATC	
SEC Forward	GAGTCAACCAGACCCTATGCC	610
SEC Reverse	CGCCTGGTGCAGGCATC	
SED Forward	GAGACTAGCCGCAATCTATCC	650
SED Reverse	GCTGCATTTAGTAATGCTGGCTG	
SEE Forward	GGTAGCGAGAAAAGCGAAG	450
SEE Reverse	GCCTTGCTGAAGATCTAGCTC	
SEG Forward	TGAATGCTCAACCCGATCCTAAAT	580
SEG Reverse	CAACCAAAAACTTGTATTGTTCTTTTCA	
SEH Forward	TTCACATCATATGCGAAAGCAGAA	620
SEH Reverse	CAGATTTTAAAGTTTTATTGTCTTCA	
SEI Forward	CGTATGCTCAAGGTGATATTGGTG	580
SEI Reverse	AAAACTTACAGGCAGTCCATCTCC	
SEJ Forward	TTTAGGATCCCTACAGAACCAAAGG	900
BamHI		
SEJ Reverse <i>NcoI</i>	GTTTCCATGGATAGCAAAAATGAAAC	650
SEK Forward	GTGTCTCTAATAATGCCAGCGCTC	
SEK Reverse	TTTGGTAGCCCATCATCTCC	450
SEL Forward	CACCAGAATCACACCGCTTA	
SEL Reverse	TCCCCTTATCAAAACCGCTAT	800
SEM Forward	TTTTGCTATTTCGCAAAATCATATCGCA	
SEM Reverse	TCAACTTTCGTCCTTATAAGATATTTCTAC	720
SEN Forward	TGAGATTGTTCTACATAGCTGCAA	
SEN Reverse	AATTAGATGAGCTAACTGTTCTATTATCAC	950
SEO Forward	TAGTGTAACAATGCATATGCAAAATG	
SEO Reverse	ATTATGTAAATAAATAACATCAATATGATA	660
SEP Forward	GGAAGCTAAAGCAGAGACAC	
SEP Reverse	CCCGTTTCATATGAAGTGCCACC	500
SEQ Forward	GCTTCAAGGAGTTAGTTCTGG	
SEQ Reverse	CTCTCTGCTTGACCAGTTCCGGTG	625
TSST-1 Forward	GAAATTTTTCATCGTAAGCCCTTTGTTG	
TSST-1 Reverse	TTCATCAATATTTATAGGTGGTITTTTCA	

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. 30% Acrylamide aqueous solution (29 g of acrylamide, 1 g of bis-acrylamide 100 mL). Acrylamide is neurotoxic, so care should be taken when working with this compound.
2. TEMED.
3. Lower (separating) buffer: 1 M Tris-HCl, pH 8.8, 20% (w/v) sodium dodecyl sulfate (SDS). Store at room temperature.
4. Upper (stacking) buffer: 1 M Tris-HCl, pH 6.8, 20% (w/v) SDS. Store at room temperature.
5. Ammonium persulfate: Prepare fresh each time a 10% solution in water.
6. Running buffer: 0.05 M Tris base, 0.4 M glycine, 0.1% (w/v) SDS. Store at room temperature.
7. Sample buffer (2X): 0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue in water.
8. Prestained molecular weight markers (SeeBlue® Plus2; Invitrogen, Carlsbad, CA).
9. SDS-polyacrylamide gel electrophoresis (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: 0.025 M Tris base (do not adjust the pH), 0.05 M glycine.
2. Immunoblot polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and methanol to equilibrate the PVDF membrane.
3. Tris-buffered saline with Tween-20 (TBST): 0.01 M Tris-HCl, pH 7.5, 0.05 M NaCl, 0.5% (v/v) Tween-20.
4. Blocking solution: 1% (w/v) bovine serum albumin (BSA) in TBST.
5. Primary antibody: 10 µL/30 mL of TBST.
6. Secondary antibody (10 µL/30 mL of TBST): Alkaline phosphatase-conjugated antirabbit IgG (Sigma-Aldrich).
7. Substrate: Buffer (20 mL) containing 0.05 M Tris-HCl, pH 9.6, 0.004 M MgCl₂, 66 µL of nitroblue tetrazolium dye (2 mg/mL in water) and 33 µL of 5-bromo-4-chloro-3-indolyl phosphate (5 mg/mL in dimethyl formamide).

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 SEA-E; SE-like G, 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 Todd Hewitt broth as the growth medium.

3.1. Polymerase Chain Reaction

1. Grow bacteria overnight in 25 mL of Todd Hewitt medium 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 rpm 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 µL 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 µL of EB buffer each time, elute the DNA twice with 100 µL of water each time.
2. After the DNA is extracted in a final volume of 200 µL, make the PCR reaction mixture. The ReddyMix PCR Master Mix must stay on ice during the preparation. To perform PCR of the genes, add the following reagents to a 0.2-mL tube to make a 50-µL total volume reaction: 47 µL of ReddyMix PCR Master Mix, 1 µL of the forward primer of the gene to be amplified, 1 µL of the reverse primer of the gene to be amplified, 1 µL of bacterial DNA. These values may be scaled down by half if needed. Also include a positive control bacterial DNA sample and a no-template negative control.
3. After all reaction mixtures are made, tightly close the caps of the tubes, and insert the tubes into an Eppendorf Thermal Mastercycler. The cycle settings are as follows: 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min for 35 cycles; 72°C for 3 min; and a hold at 4°C until the DNA gel is prepared and ready to be subjected to electrophoresis.
4. To make the DNA gel, dilute 10X TAE buffer to 1X with distilled water. Add 3.3 g of agarose to 300 mL of 1X TAE buffer to give a final gel concentration of 1.1%. Microwave the mixture until it becomes clear. Allow the hot gel mixture to cool on the bench for 5 min. Pour the gel into the gel tray until the bottom of the tray is covered with 0.75–1 cm of gel. Immediately add 10 µL of ethidium bromide to the mixture to give a final concentration of approx 0.5 µg/mL and stir in before the gel solidifies. 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. Then completely submerge the gel in the same 1X TAE buffer that was used to prepare the DNA gel.

6. Add DNA ladder (10 μ L) to the leftmost lane, and add 10 μ L of PCR products individually to additional wells. 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* from a recently emergent clone (referred to as USA 300 by Centers for Disease Control) that caused necrotizing pneumonia for the presence of SAg genes. The strain is positive only for the SE-like Q gene (see **Note 6**).

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

1. Inoculate *S. aureus* strains into 10 mL Todd Hewitt broth 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 SAg while killing the *S. aureus* cells. We have found that this 80% final concentration of ethanol will precipitate 100% of SAg 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 1000g 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 amount (100 μ L) of the clarified supernates with 100 μ L of SDS-PAGE sample buffer, and leave at room temperature overnight to denature the proteins.

3.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

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 0.75 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 lower separating gel by mixing 1.75 mL of acrylamide solution, 1.9 mL of lower buffer, 1.35 mL of distilled water, 10 μ L of TEMED, and 70 μ L of ammonium persulfate for each gel (see **Note 8**). Pipet the solution into the gel apparatus, leaving space for the stacking gel. We overlay the gel with distilled water. This gel takes approx 30 min to polymerize.
2. Pour off the overlay solution and prepare the stacking gel.
To make the stacking gel solution, mix 0.18 mL of acrylamide, 0.24 mL of upper Tris buffer, 0.9 mL of distilled water, 5 μ L of TEMED, and 10 μ L of ammonium persulfate per each gel.

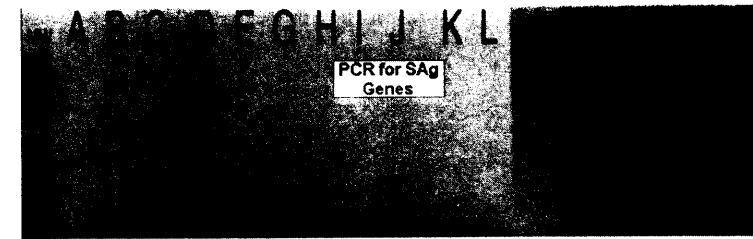


Fig. 1. PCR analysis of DNA from *S. aureus* strain that caused necrotizing pneumonia.

3. Pour the gel, put the comb in place, and allow the gel to polymerize for 30 min.
4. Carefully remove the comb, and place the gel in the apparatus for electrophoresis.
5. Add running buffer to the upper and lower chambers, and add 20 μ L of each sample to the wells using a pro-pipet equipped with extended tips. We electrophorese control SAg samples of 10.0, 1, 0.1, 0.01, and 0.001 μ g/mL, molecular weight markers, as recommended by the supplier, and then 20- μ L amounts of the unknown samples (these samples can also 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 80 V until the bromophenol blue marker dye reaches the bottom of the separating gel (see **Note 9**). Then turn off the apparatus. The gels are now ready to be used for Western immunoblotting.

3.4. Western Immunoblotting for SAg

The samples that have been separated by SDS-PAGE, including unknowns, prestained molecular weight markers, and purified standard SAg, 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. Wet the PVDF membrane in absolute methanol and then place in a glass dish containing Western transfer buffer. At the same time, place two pieces of foam (included with the apparatus) and two pieces of 3MM paper cut to the size of the PVDF membrane in the glass dish.
2. Remove the SDS-PAGE gel from the SDS 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 foam, 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. We 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 and insert the closed cassette.
4. Allow electrotransfer of the samples onto the PVDF membrane for about 1 h, beginning with 100 V and monitoring the amperage until it shifts from approx. 250 mA at the start and rises to 350 mA.
5. Disassemble the apparatus and remove the PVDF membrane. Nitrocellulose and Nytran membranes can also be used, but we use PVDF membranes because they are durable and useful for protein sequencing (*see Note 10*).
6. Place the PVDF membrane in 30 mL of TBS (two times) to wash the membrane. There is no incubation with the TBS; add it immediately and then pour off.
7. Block the PVDF membrane for 30 min by adding 30 mL of TBST containing 1% BSA and gently agitating on a shaking platform (*see Note 11*).
8. Directly add the primary antibody, and agitate the membrane for 30 min.
9. Pour off the primary antibody and agitate the gel two times for 5 min each with TBST.
10. Add secondary antibody in 30 mL of TBST, and incubate with agitation for 30 min.
11. Pour off the secondary antibody, and wash the PVDF membrane twice (5 min each) with TBST and then once with TBS (5 min).
12. Add alkaline phosphatase buffer with added substrate (30 mL total), and agitate the PVDF membrane until standard SAg samples have reacted, turning the membrane purple. The time to stop the reaction is just when the background also begins to turn purple.
13. Remove the PVDF membrane from the substrate solution, rinse with distilled water, and air-dry.
14. Photograph the PVDF membrane and visualize the image on a computer.

3.5. Determination of Density

For measuring SAGs, we use the NIH imaging program 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 stain the PVDF membranes. The protein A bands are not present in the same molecular weight range as the SAGs and, thus, do not interfere.

Standard amounts of SAg are used to prepare a standard curve by plotting band density vs the logarithm of the SAg concentration. This typically gives a straight line with an R^2 value >0.95. 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°C . To store strains, grow the organisms in Todd Hewitt broth until stationary phase; usually overnight will suffice. Collect the cells by centrifugation (1000g, 15 min), and suspend them in 1/10 vol with Todd Hewitt broth supplements with 10% rabbit blood (for lyophilization) or Todd Hewitt broth 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 SAGs are produced by the strains and not quantified, double immunodiffusion may be used instead of Western immunoblotting. It is our experience that the antisera and control SAGs available from Toxin Technology are suitable for such analyses. Double immunodiffusion is performed after growing the strains in Todd Hewitt broth and concentrating the culture fluids by treatment with 4 vol of ethanol 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 μL of 50–100 $\mu\text{g}/\text{mL}$) and samples to be tested (20 μL) are added to adjacent wells. The slides are incubated for 4 h at 37°C in a humidified chamber or overnight at room temperature. The slides are read for precipitation lines forming lines of identity with control SAGs. 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 not specifically 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 yr 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. Other SAg testing requires assays for both the genes and the proteins: the proteins may or may not be made by the strains, and the proteins may be made in very low concentrations by the strains.

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 mo) 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 de-aerating 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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