Note: Wear gloves for every step to prevent contamination of specimen with DNAses or other agents that might inhibit or influence the reactions!

DNA Preparation (~ 1 h duration, ~ 20 min hands-on time [8 samples in parallel processed])

Background: The described InstantGene matrix DNA preparation method is fast and cheap. Alternative methods such as lysozyme/lysostaphin lysis are also suitable. The DNA is stored at -20°C until use.

- Suspend a loopful (2-3 colonies of a fresh overnight culture) of *Staphylococcus aureus* cells in 500 µl distilled water (use 1.5 ml reaction tubes that are numbered and labeled with "H_2O"), vortex, and centrifuge at 12.000 g for 1 min.
- Remove the supernatant and incubate the pellet with 100 µl (use 1000 µl blue tips for pipeting) of 6% InstantGene matrix solution (BIO-RAD; solution is ready to use on the running magnetic stirrer) for 20 min at 56°C.
- Vortex thoroughly (10 s) and heat for 8 min at 100°C.
- Vortex thoroughly and centrifuge at 12.000 g for 3 min.
- Transfer 80 µl of the supernatant into a new 1.5 ml reaction tube (use blue reaction tubes that are numbered and labeled with "DNA").

DNA Amplification (PCR reaction; ~ 2.5 h duration, ~ 30 min hands-on time)

Background: For amplification of the *Staphylococcus* protein A (*spa*) repeat region, a PCR is performed in a total volume of 50 µl containing cleaned DNA, 200 µM deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), 10 pmol of each primer, 5 µl of 10-fold concentrated PCR Buffer II (Applied Biosystems), $MgCl_2$ 1.5 mM, and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems). HPLC-cleaned primers spa-1113f (5'- TAA AGA CGA TCC TTC GGT GAG C -3') and spa-1514r (5'- CAG CAG TAG TGC CGT TTG CTT -3') are used for amplification. The primers are numbered from the 3' end of the primer on the forward strand of *S. aureus* (GenBank accession no. J01786; spa-1113f [1092-1113] & spa-1514r [1534-1514]). The thermal cycling reactions consist of an initial denaturation (10 min at 80°C) followed by 35 cycles of denaturation (45 s at 94°C), annealing (45 s at 60°C), and extension (90 s at 72°C), with a single final extension (10 min at 72°C). After the run has finished, the thermocycler cools the reaction at 4°C. The PCR products can be stored at -20°C. Facultatively, the PCR results can be controlled by running a gel electrophoresis (1.5% agarose, use post-amplification pipettes).

- Pipet 40 µl of the PCR master-mix for each PCR reaction into the numbered 0.5 ml reaction tube. The master-mix reaction tube is labeled with "MM-PCR" (containing dNTPs, primers, buffer, $MgCl_2$, and the *Taq* polymerase; all pipeting was done with filter tips). The master-mix should be stored at -20°C.
- Start the thermocycler program and, when the thermocycler has reached the initial denaturation temperature (80°C), place the reaction tubes into the thermocycler.
- Pipet (with filter tips) carefully 10 µl of the DNA (blue reaction tubes labeled "DNA") into the 0.5 ml reaction tube and mix a few times by up and down pipetting.
- Seal the reaction tubes thoroughly with the adhesive PCR film and close the thermocycler. The thermal cycling program continues automatically.

Purification of the PCR Product (~ 50 min duration, ~ 5 min hands-on time)

Background: To remove un-incorporated dNTPs and primers, the PCR reaction is purified by a cheap enzymatic method using exonuclease I (New England Biolabs) and shrimp alkaline phosphatase (Amersham Pharmacia Biotech; for preparing the stock and working solution it is referred to the separately distributed protocol). The purification reaction thermocycler program consists of 30 min incubation at 37°C, 15 min heat inactivation at 80°C, and 4°C cooling. The purified PCR products can be stored at -20°C. For use, both enzymes are stored on ice. Alternative purification methods such as column centrifugation or vacuum filtration are also suitable.

- Place new 0.5 ml reaction tubes in the post-amplification thermocycler that is cooling at 4°C.
- Transfer carefully 5 µl of each PCR product into the 0.5 ml reaction tubes (use post-amplification pipettes).
- Pipet 1 µl of exonuclease I (the working solution tube is labeled with "Exo I") enzyme and 1 µl of shrimp alkaline phosphatase (the working solution tube is labeled with "SAP").
- Start the purification reaction thermocycler program.

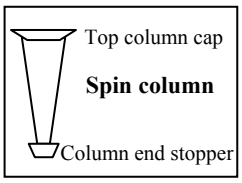
DNA Sequencing Reaction (~ 2.5 h duration, ~ 30 min hands-on time)

Background: The amplicons are sequenced using the ABI Prism BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (containing dNTPs, buffer, polymerase, and dye-labeled ddNTPs; Applied Biosystems). Each sequencing reaction contains 0.5 µl of the BigDye Terminator premix from the kit. The same primers used in the PCR are used for sequencing with an annealing temperature of 60°C. The sequencing master-mix contains either forward or reverse primer, premix, HPLC water, and 5x sequencing buffer (Applied Biosystems). The master-mix should be stored at -20°C. All sequencing reactions are performed with 25 cycles of denaturation (96°C, 10 s), extension (60°C, 4 min), and a thermal ramping of 1ºC/s. After the run has finished, the thermocycler cools the reaction at 4°C. The finished sequencing reaction products can be stored at -20°C.

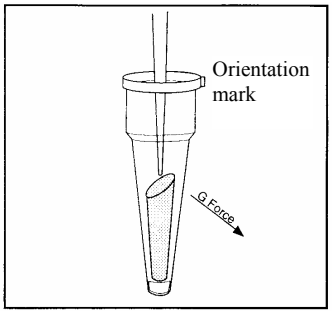
- Pipet 8 µl sequencing master-mix for each sequencing reaction in a new 0.5 reaction tube labeled either with forward or reverse. For the forward strand sequencing, use the forward master-mix (labeled "SEQ F"), for the reverse strand, use the reverse master-mix (labeled "SEQ R"). Avoid direct sunlight exposure!
- Add 2 µl of the purified PCR product (use post-amplification pipettes).
- Seal the PCR tubes thoroughly with the adhesive PCR film, close the post-amplification thermocycler, and start the thermocycler program.

Purification of the Sequencing Reaction (start preparation during sequencing reaction; ~ 2.5 h duration, ~ 30 min hands-on time)

Background: To remove unincorporated dye-labeled ddNTPs and salts, the sequencing products are purified using the Centri-Sep Spin Columns (Princeton Separations, Adelphia, NJ) for running on the ABI 3100 Avant Genetic Analyzer (Applied Biosystems). Alternatively, larger batches of sequencing products are purified with MultiScreen HV plates (Millipore, Ma, USA) loaded with Sephadex G50 Superfine columns (Amersham Biosciences) according to the instructions of the manufacturer (Millipore Tech Note TN053; i.e. including a 150 µl pre-rinse step necessary for capillary sequencers). The purified sequencing reaction products can be stored at 4°C for several days. For longer storage at -20°C and sending by mail, the purified sequencing reaction products should be dried.



- Gently tap the column to ensure that the dry gel has settled in the bottom of the spin column.
- Remove the top column cap, leave the column end stopper in place (see figure), and reconstitute the column by adding 700 µl HPLC water.
- Replace the top column cap and hydrate the gel by vortexing briefly (it is important to hydrate all of the dry gel). Place the rehydrated columns in an upright position.
- Allow at least 2 hours of room temperature hydration time before using the columns (reconstituted columns may be stored refrigerated at 4°C for several days).
- Remove large air bubbles from the column gel by briefly vortexing or inverting and sharply tapping the column.
- After the gel has settled and is free of bubbles, first remove the top column cap, then remove the column end stopper, and place the column into the wash tube (2.0 ml tube).
- To remove excessive fluid, shortly press and immediately remove the top column cap to force the fluid to start draining through the column filter into the wash tube. The column will stop draining on its own after about one minute. Discard the fluid and place the spin column again into the wash tube.
- Spin the open column and wash tube at 750 g for 2 min to remove further interstitial fluid (~ 200 – 300 µl). It is important to keep track of the position of the column using the orientation mark (see figure) molded into the column (e.g., all orientation marks point toward the outside of the rotor)!
- Discard the wash tube with the interstitial fluid. Do not allow the gel material to dry excessively. Therefore, process the sample within the next few minutes.
- Carefully dispense the complete sequencing reaction product (10 µl) directly onto the center of the gel bed at the top of the column, without disturbing the gel surface (see figure) using post-amplification pipettes.
- Place the column into a new 1.5 ml reaction tube and place both into the centrifuge. Maintain proper column orientation. The highest point of the gel media in the column should always point toward the outside of the rotor (see figure).
- Spin the column and reaction tube at 750 g for 2 min. The purified sample will collect in the bottom of the tube (~ 10 µl). Discard the spin column.



Further Processing of the Purified Sequencing Reaction

- Dry the sample in a vacuum centrifuge and then add 20 µl of HiDi formamide (Applied Biosystems) as loading media for sequencing electrophoresis on an ABI 3100 Avant Genetic Analyzer.

spa Sequence Analysis

- Analyze the *spa* sequence chromatograms using the Ridom StaphType software (Ridom GmbH).