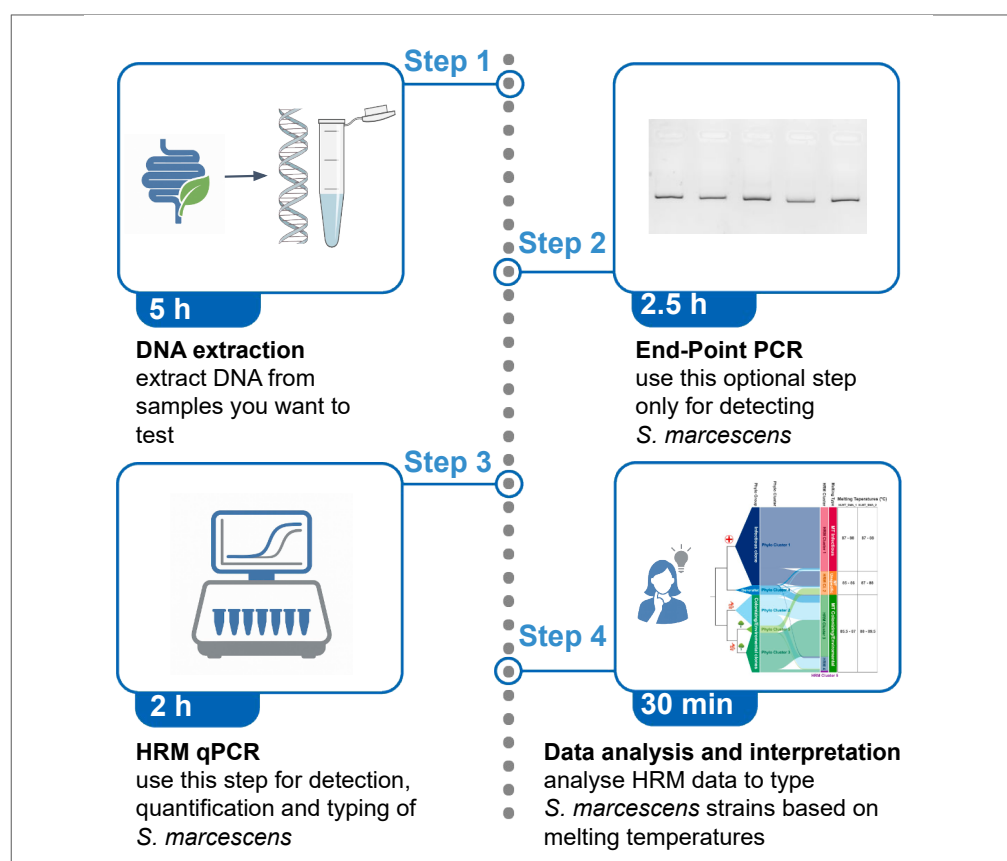


Protocol

Protocol for detection and typing of *Serratia marcescens* using a qPCR-based technique without cultivation or sequencing



High-resolution melting (HRM) qPCR is a molecular biology technique for quantifying DNA targets and discriminating them on the basis of their sequences. Hypervariable-locus melting typing (HLMT) exploits HRM for bacterial typing by targeting variable genetic loci. Here, we present an HLMT protocol for detecting *Serratia marcescens* clones directly from biological samples without cultivation. We describe steps for DNA extraction, endpoint PCR, and HRM qPCR. We then detail procedures for data analysis and interpretation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights
Guidance on
detecting and typing
Serratia marcescens
directly from samples
without cultivation

Steps for HRM qPCR
on unique
hypervariable loci,
compatible with
standard qPCR
instruments

Procedures for rapid,
cost-effective typing,
for outbreak control
and large screenings

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Protocol

Protocol for detection and typing of *Serratia marcescens* using a qPCR-based technique without cultivation or sequencing

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SUMMARY

High-resolution melting (HRM) qPCR is a molecular biology technique for quantifying DNA targets and discriminating them on the basis of their sequences. Hypervariable-locus melting typing (HLMT) exploits HRM for bacterial typing by targeting variable genetic loci. Here, we present an HLMT protocol for detecting *Serratia marcescens* clones directly from biological samples without cultivation. We describe steps for DNA extraction, endpoint PCR, and HRM qPCR. We then detail procedures for data analysis and interpretation.

For complete details on the use and execution of this protocol, please refer to Alvaro et al.¹

BEFORE YOU BEGIN

This protocol was developed to detect and type *Serratia marcescens* without requiring a cultivation step. *S. marcescens* is a widespread environmental species that includes both opportunistic pathogens and infectious clones, the latter being significant nosocomial pathogens. These clones can rapidly spread within hospitals, leading to outbreaks with high mortality rates, particularly in neonatal intensive care units (NICUs).² Therefore, rapid identification of *S. marcescens* is critical for effective clinical management and infection control.

The protocol we describe here is based on High-Resolution Melting (HRM) analysis. HRM is an application of real-time PCR (qPCR) that enables the discrimination of bacterial lineages and clones on the basis of the melting temperatures of qPCR amplicons. The *S. marcescens* HRM protocol uses qPCR primers specifically designed to target genes unique to this species and to amplify highly variable genetic regions.¹ This strategy, which we have termed Hypervariable-Locus Melting Typing (HLMT), was previously introduced by our group for the detection and typing of other bacterial nosocomial pathogens.³

This HRM-based classification accurately distinguishes the *S. marcescens* infectious clone in 96% of strains.¹ Importantly, this cultivation-free protocol is applicable to DNA extracted directly from both environmental and clinical samples, facilitating rapid and cost-effective surveillance in diverse settings.



Although whole-genome sequencing (WGS) of bacterial isolates remains the gold standard for typing the infection-associated clone of *S. marcescens*, the time required for culturing and sequencing can pose a significant limitation, particularly given the rapid spread of this clone. In this context, our HRM protocol offers a valuable alternative for obtaining preliminary typing results quickly, while culturing and sequencing are still in progress. Moreover, the *S. marcescens* HRM protocol may be especially useful in low- and middle-income countries, as well as in settings where rapid or large-scale screenings are required.

The protocol is compatible with standard qPCR instruments, capable of HRM analysis with a temperature resolution of 0.5°C. In our laboratory, experiments were performed using the widely adopted Bio-Rad CFX-Connect machine (Bio-Rad, Hercules, CA). Notably, the use of more expensive qPCR platforms offering higher HRM temperature sensitivities is not necessary. The entire workflow can be completed in approximately five hours, with an estimated cost of 5\$ per sample when using three qPCR technical replicates.

Innovation

This protocol provides a novel approach for the rapid detection and typing of *Serratia marcescens* directly from environmental and clinical samples, without the need for prior bacterial culture. By using High-Resolution Melting (HRM) analysis of hypervariable genetic loci, it enables discrimination of infectious clones with high accuracy, while dramatically reducing the time from sample collection to preliminary results.

The workflow is designed for accessibility and efficiency. It uses standard qPCR instruments capable of HRM analysis, reducing the need for expensive sequencing platforms or specialized equipment. With a total processing time of approximately five hours and low per-sample cost, the method is suitable for both routine surveillance and outbreak investigations, including in resource-limited settings.

By combining speed, affordability, and broad applicability, this HRM-based method fills a critical gap between rapid molecular screening and confirmatory whole-genome sequencing. It provides actionable preliminary typing information that supports timely infection control decisions, early intervention, and improved patient safety. This cultivation-free strategy represents an innovative tool for hospital epidemiology, enabling high-throughput monitoring of *S. marcescens* infectious clones in diverse clinical and environmental contexts.

Institutional permissions

To establish the *S. marcescens* HRM protocol, we used microbial isolates and soil samples, from which DNA was extracted. These sample types do not require ethical approval. Additionally, we employed a previously established collection of human fecal DNA samples. This collection, described in a prior study, comprises DNA samples that were previously characterized by V3-V4 16S rRNA amplicon sequencing.⁴ We used this DNA collection (the same tubes of extracted DNA) because the relative abundances of *S. marcescens* were known from the 16S rRNA sequencing data. These human DNA samples were originally produced as part of a project on atopic dermatitis, conducted at the Department of Pediatrics, Vittore Buzzi Children's Hospital, Milan (Italy). The project received ethical approval from the Ethics Committee of ASST-Fatebenefratelli-Sacco (approval number 754, July 18th 2017). The study was conducted in accordance with the declaration of Helsinki, and all procedures followed the relevant guidelines and regulations. Written informed consent was obtained from the parents prior to enrollment of their children in the study.

Collect environmental or clinical samples from which you want to detect/type *S. marcescens*

⌚ Timing: 5 min (but can vary depending on the sample type and experimental design)

1. Collect samples you want to test with the *S. marcescens* HRM protocol.

Note: Some examples are given below.

- a. **Soil Samples:** Collect soil in sterile containers using sterile tools to avoid contamination. Keep samples at 20°C–25°C and deliver them to the laboratory within 24 h. For longer storage, samples can be refrigerated at 4°C but should be processed within 48 h.
- b. **Bacterial Isolates:** Cultured isolates should be transferred into sterile tubes containing appropriate storage medium (e.g., nutrient broth with glycerol for freezing). Transport on ice or dry ice if frozen. Short-term transport at 4°C is acceptable if immediate freezing is not possible.
- c. **Fecal Samples:** Collect feces in sterile containers using clean tools. Store and transport at 4°C and process within 24–48 h to preserve sample integrity.
- d. **Rectal Swabs:** use sterile swabs with **Transwab** or equivalent transport medium containing charcoal to preserve bacterial viability and nucleic acids. Maintain at 20°C–25°C and deliver to the laboratory within 24 h. If delay is expected, refrigerate at 4°C up to 48 h.
- e. **Water Samples:** Collect water in sterile bottles, avoiding contamination. Transport at 20°C–25°C and analyze as soon as possible, preferably within 24 h.
- f. **Hospital Surface Swabs**⁵ (e.g., soap dispensers, tables) Use sterile swabs with **Transwab** or similar transport medium. Swab the surface thoroughly. Store and transport at 20°C–25°C and deliver to the laboratory within 24 h.

Extract DNA from samples

⌚ **Timing:** 1–5 h (but can vary depending on the sample type and kit used)

2. Extract DNA from the samples you collected in point 1.

Note: Adjust this step according to the type of sample. Consider that the efficiency of DNA extraction may vary significantly across different matrices.

Note: Select the appropriate DNA extraction kit based on the sample you want to extract and on the protocols in use in your laboratory. As an example, we list below the kits we use in our laboratory for the sample types listed above.

- a. Fecal samples: QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany).
- b. Soil samples: DNeasy PowerSoil Pro Kit (Qiagen).
- c. Bacterial isolates: DNeasy Blood & Tissue Kit (Qiagen).

Note: Follow the manufacturer's protocol for the chosen extraction kit.

⚠ **CRITICAL:** DNA yield and purity are essential for reliable downstream qPCR and HRM analyses. Check the 260/280 ratio is between 1.8 and 2.0; lower values indicate protein contamination, and higher values indicate RNA or other contaminants.

Order primers

⌚ **Timing:** Typically 1–2 working days (but can vary among companies and locations)

3. Order the two primer pairs listed below ([Table 1](#)) for the amplification of the *S. marcescens* genes SMDB11_2070 and SMDB11_3518. Primers were designed in our laboratory and synthesized by Eurofins Genomics.

Note: We choose a reputable primer synthesis company to ensure high-quality products and prompt delivery. Delivery times for synthesized primers may vary depending on the supplier and shipping destination.

Table 1. Primers used for PCR/qPCR/HRM assays

Primer pair	Target gene	Primer sequence	Amplicon size (nt)
HLMT_SMA_1	SMDB11_2070	HMLT_SMA_1_F: GAACYTGCGCATGATTATGCG HMLT_SMA_1_R: CGGWGACGACCTGCAGCTG	118
HLMT_SMA_2	SMDB11_3518	HMLT_SMA_2_F: ATGRCCGGGYAAGGCCATCGAT HMLT_SMA_2_R: TTCAGGGCGACCGCGTCG	134

Prepare standard series

⌚ Timing: 1 day

Note: Perform this step when setting up the *S. marcescens* HRM protocol for the first time in your laboratory. Generating standard curves is essential to ensure accurate quantification of the target genes in qPCR.

Note: This step requires access to *S. marcescens* genomic DNA. This DNA can originate from prior microbiological isolations conducted in your laboratory or from glycerol stock collections. Alternatively, *S. marcescens* cells are readily available for purchase from various commercial suppliers.

4. Generate standard curves by performing two endpoint PCR reactions on *S. marcescens* genomic DNA, one for each of the two primer couples.

Note: Table 2, below, lists the components contained in each tube of the end point PCR. We list the HiFi Taq DNA polymerase mix and water in use in our laboratory. However, any high fidelity Taq DNA polymerase and PCR grade water can be used. Note that cycling can change based on the Taq DNA polymerase used. We used an annealing temperature of 63°C for both primer pairs. Primers melting temperatures, as given by the manufacturer, are the following: 59.3°C and 63.1°C for the HLMT_SMA_1 couple; 61.8°C and 62.8°C for the HLMT_SMA_2 couple.

⚠ **CRITICAL:** Ensure accurate pipetting of reagents to maintain reaction consistency. Use filter tips to avoid contamination.

- a. Prepare the two PCR master mixes (one for each couple of primer) for the desired number of samples, by combining the HiFi Taq DNA polymerase mix, amplification grade water and forward and reverse primers (refer to Table 2).
- b. Mix gently and distribute 19 μ L of each PCR master mix into PCR tubes already containing 1 μ L *S. marcescens* DNA (5-50 ng).
- c. Run on your thermal cycler the cycling program in Table 3.
- d. Load the PCR products into a 1.5% agarose gel in 1 $\times$ TBE buffer. The BenchTop 100 bp DNA ladder (Promega) was used in our laboratory as a molecular size marker.

Table 2. Reagents in one tube of endpoint PCR

Reagent	Volume (μ L) pr. sample	Final concentration in the PCR tube	Manufacturer
KAPA HiFi HotStart ReadyMix 2 $\times$	10	1 $\times$	Roche Diagnostics, Risch-Rotkreuz, Switzerland
Primer F 10 μ M	1	0.5 μ M	
Primer R 10 μ M	1	0.5 μ M	
Nuclease free water	7	-	Promega Corporation, Madison, Wisconsin, USA
Serratia DNA	1 corresponding to 5-50 ng of DNA		
Total	20		

Table 3. Cycling program for the endpoint PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	
Denaturation	95°C	30 s	35 cycles
Annealing	63°C	45 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	
Hold	4°C	∞	

- e. Excise the bands (118 bp for HLMT_SMA1; and 134 bp for HLMT_SMA2) using a sterile scalpel.
- f. Purify the excised bands using a dedicated kit.

Note: In our laboratory, we use the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA).

- g. Quantify the purified amplicons

Note: We used the Broad Range assay kit on the Qubit 3 Fluorometer (Qubit 3 Fluorometer, Thermo Fisher Scientific, Waltham, MA).

- h. Consider the two double stranded DNA fragments of 118 bp and 134 bp. Assume an average molecular weight of 660 g/mol per base pair and calculate the molecular weight of each fragment accordingly. Determine the number of molecules per nanogram of DNA using the calculated molecular weight and Avogadro's number. Measure the DNA concentration with the Qubit fluorometer (ng/μL) and multiply by the copy number per nanogram to obtain the final number of DNA fragment copies per microliter.
- i. For each of the purified bands, prepare serial 10-fold dilution, ranging from 10⁷ to 10 copies/μL, using nuclease-free water (Figure 1).

Note: Mix thoroughly at each dilution step.

Note: Store the diluted standards at –20°C until use.

△ CRITICAL: Optimize primer concentrations and/or annealing temperatures or number of cycles if PCR results are not optimal (e.g., low efficiency or presence of non-specific amplification products) to ensure efficient PCR amplification. Use fresh, high-quality DNA and avoid overloading the gel to prevent smearing and ensure clear band resolution.

Biosafety requirements

The molecular biology work described in this protocol does not have biosafety requirements and can be performed on the benchtop. The use of BSL2 microbiology facilities is required only if you work with *S. marcescens* isolates for deriving the DNA required for the preparation of the standard series or if samples you want to test with the *S. marcescens* qPCR and HRM protocol have these requirements.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
<i>Serratia marcescens</i> DNA	Alvaro et al. ¹	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad	1725274
PCR amplification-grade water	Promega	DW0991
Roche Diagnostics KAPA Hifi HotStart ReadyMix	Roche Diagnostics	07958927001 JKK2601
Critical commercial assays		
QIAGEN QIAcube Connect automated extractor	QIAGEN	9002864
QIAGEN DNeasy Blood & Tissue reagents	QIAGEN	69504
DNeasy PowerSoil Pro kit (50)	QIAGEN	47014
Zymoclean gel DNA recovery kit	Zymoclean	255307
Illumina NextSeq platform	Illumina	N/A
Deposited data		
<i>Serratia marcescens</i> genome assemblies	Alvaro et al. ¹	NCBI-PRJNA957961
Oligonucleotides		
HLMT_SMA_1_F: GAACYTGCGCATGATTATGCG	Alvaro et al. ¹	Ordered from Eurofins Genomics
HMLT_SMA_1_R: CGGWGACGACCTGCAGCTG	Alvaro et al. ¹	Ordered from Eurofins Genomics
HMLT_SMA_2_F: ATGRCCGGGYAAGGCCATCGAT	Alvaro et al. ¹	Ordered from Eurofins genomics
HMLT_SMA_2_R: TTCAGGGCGACCGCGTCG	Alvaro et al. ¹	Ordered from Eurofins Genomics
Software and algorithms		
Bio-Rad CFX Manager	Bio-Rad	1845000
Other		
Sequence data, analyses, and resources related	This paper	N/A
Bio-Rad CFX Connect real-time PCR system	Bio-Rad	1855201
Bio-Rad T100 thermal cycler	Bio-Rad	1861096

MATERIALS AND EQUIPMENT

End-Point PCR		
Reagents	Final concentration	Amount
KAPA Hifi HotStart ReadyMix	1 ×	10 µL
Primer F	0,5 µM	1 µL
Primer R	0,5 µM	1 µL
Nuclease free water	N/A	7 µL

Use the mix immediately for End-Point PCR. Store the reagents listed in the Table at –20°C till the expiration date specified by the manufacturer.

qPCR		
Reagents	Final concentration	Amount
SsoAdvanced Universal SYBR Green	1 ×	7,5 µL
Primer F	0,25 µM	0,4 µL
Primer R	0,25 µM	0,4 µL
Pcr amplification grade water	N/A	5,7 µL

Use the mix immediately for qPCR. Store the reagents listed in the Table at –20°C till the expiration date specified by the manufacturer.

STEP-BY-STEP METHOD DETAILS

Note: Below, we present a detailed step-by-step protocol for the detection and typing of *S. marcescens* clones. Detection can be performed using either End-Point PCR or qPCR. qPCR also enables bacterial quantification through comparison with the standard curve. Finally, typing of *S. marcescens* is conducted via HRM analysis following qPCR.

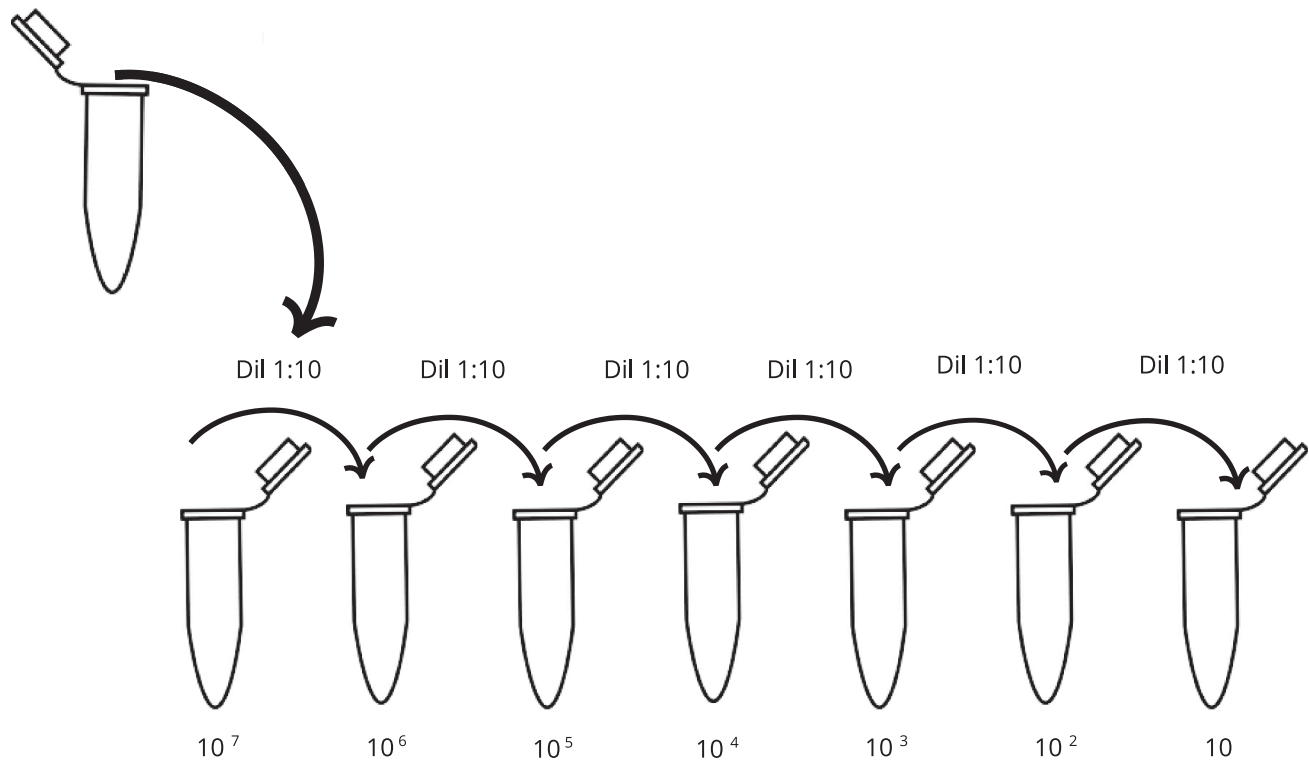


Figure 1. Workflow of serial dilutions for setting up standard series for qPCR

Endpoint PCR for *S. marcescens* detection

⌚ Timing: 2.5 h

1. Use this step for rapid detection of *S. marcescens* in samples when quantification and typing are not required.
2. Prepare the master mix for End-Point PCR and set the cycling conditions as described in the “before you begin – prepare standard series” section. Include a negative (no template) control, to exclude contamination, and a positive control to ensure that the reaction is working. As a template for the positive control, consider using e.g., the *S. marcescens* DNA you used for preparing the standard series.

⚠ **CRITICAL:** Ensure accurate pipetting of reagents to maintain reaction consistency. Use filter tips to avoid contamination.

3. Run the PCR and analyze the products via gel electrophoresis to confirm *S. marcescens* presence. See the “before you begin – prepare standard series” section.

Quantitative PCR and subsequent HRM analysis

⌚ Timing: Approximately 2 h

Note: The qPCR described below allows *S. marcescens* detection and quantification through comparison of threshold cycles (Ct) of “unknown” samples with those of the standard curve. The subsequent HRM analysis allows the typing of *S. marcescens* clones.

Table 4. qPCR/HRM master mix

Reagent	Volume (μL) pr. sample	Final concentration in the PCR tube	Manufacturer
SsoAdvanced Universal SYBR Green Supermix 2x	7.5	1x	Bio-Rad, Hercules, California
Primer F 10 μM	0.4	0.25 μM	
Primer R 10 μM	0.4	0.25 μM	
PCR amplification-grade water	5.7		Promega Corporation, Wisconsin, USA
<i>Serratia marcescens</i> DNA	1 5-50 ng when working with bacterial cultures 1 1-10 ng for environmental DNA		
Total	15		

Note: Standard qPCR instruments, capable of HRM analysis with a temperature resolution of 0.5°C, work fine with this protocol. In our laboratory, we used the CFX Connect Real -Time PCR Detection System (Bio-Rad, Hercules, CA)

- Amplify each *S. marcescens* DNA sample in triplicate, using both primer pairs HLMT_SMA_1 and HLMT_SMA_2 (see “before you begin - prepare standard series” section).

△ CRITICAL: Always run technical triplicates for each sample to ensure reproducibility and reliable quantification.

- Prepare the master mix for qPCR as shown in Table 4.
- Run the qPCR cycling program listed in Table 5.
- Analyze the melting curve (70°C–95°C in 0.5°C increments) for HRM typing of *S. marcescens* clones.

Note: Classify the strain as: (1) “MT infectious” if melting temperatures of both primer pairs are between 87°C and 88°C; (2) “MT Colonising/Environmental” if HLMT_SMA_1 melting temperature is between 85.5°C and 87°C, and HLMT_SMA_2 melting temperature is between 88°C and 89.5°C; (3) “MT Unspecific” if HLMT_SMA_1 melting temperature is between 85°C and 86°C, and HLMT_SMA_2 melting temperature is between 87°C and 88°C. More details about the groups are given below (in Data Analysis) and are reported in.¹

△ CRITICAL: Ensure accurate pipetting of reagents to maintain reaction consistency. Use filter tips to avoid contamination.

Note: Table 4, above, lists the components contained in each tube of qPCR. We list the SYBR Green mix and water in use in our laboratory. However, any other equivalent reagent can be used.

Data analysis

- Use End-point PCR to detect the presence of *S. marcescens* in a sample.

Note: To this end, visual inspection of the PCR gel can provide evidence of the *S. marcescens* presence in the sample (see figure S1 in Alvaro et al., 2024). We recommend carefully verifying the amplicon size on the gel to minimize false positives resulting from non-specific amplification. A sample should be considered colonized by *S. marcescens* only when both independent end-point PCR experiments yield consistent positive results.

Table 5. qPCR/HRM program

Step	Temperature	Time	Repetition
Initial denaturation	95°C	2 min	
Denaturation	95°C	7 s	40 cycles
Annealing	63°C	7 s	
Extension	72°C	15 s	
HRM starting	95°C	2 min	
HRM range	From 70°C to 95°C	5 min	Followed by 0.5°C increments with fluorescence data acquisition

9. Use qPCR to obtain information on both the presence and quantification of *S. marcescens* in the sample.

Note: We recommend considering a sample as colonized when the threshold cycle (Ct) values are below 35 for both qPCR. If one or both Ct values exceed 35, confirmation via end-point PCR is strongly advised to rule out potential false positives or low-level contamination.

10. Use post-qPCR HRM analysis to enable the typing of *S. marcescens* strains.

Note: Based on their melting temperatures, strains can be classified into three categories, referred to as Melting Types (MT): MT Infectious, MT Colonising/Environmental and MT Unspecific.¹ MT Infectious strains are typically associated with hospital-acquired infections, whereas those of the MT Colonising/Environmental are frequently linked to gut colonization and/or environmental niches. Finally, MT Unspecific strains exhibit melting profiles not clearly distinguishable between infectious and colonizing/environmental origins. This classification aligns with the clinical context of isolation, where strains from sterile compartments are considered infection-associated and those from non-sterile compartments are considered colonizing.

- a. Classify the strain as “MT infectious” if the melting temperature of both HLMT_SMA_1 and HLMT_SMA_2 are between 87°C and 88°C;
- b. Classify the strain as “MT Colonising/Environmental” if HLMT_SMA_1 melting temperature is between 85.5°C and 87°C, and HLMT_SMA_2 melting temperature is between 88°C and 89.5°C;
- c. Classify the strain as “MT Unspecific” if HLMT_SMA_1 melting temperature is between 85°C and 86°C, and HLMT_SMA_2 melting temperature is between 87°C and 88°C.

More details about the groups and their respective temperatures are reported in.¹

EXPECTED OUTCOMES

The protocol allows the specific detection of *S. marcescens*. As shown, no cross-reactions are produced by other bacterial species, even close to *S. marcescens*.¹

In detail, no amplification bands are observed when amplifying, by any of the two end-point PCR, other *Serratia* species, under the conditions described in the present protocol. The *Serratia* species tested are the following: *Serratia odorifera*, *Serratia ficaria*, *Serratia liquefaciens*, *Serratia rubidaea*.¹

Further, no amplification bands are observed when amplifying, by any of the two end-point PCR, complex DNA samples (as those extracted from feces and from soil¹) provided that these samples are not colonized by *S. marcescens*. For example, in our case, we tested this point on fecal and soil DNA samples, previously characterized by 16S rRNA sequencing and known to be *S. marcescens*-free, and observed no amplification bands.¹ In other words, none of the numerous bacterial species present in these samples appeared to cross react with the *S. marcescens*

primers. As shown in,¹ amplification from these samples, using the primers described in this protocol, is obtained only when spiking them with *S. marcescens* DNA or intact cells. This point is very important because it provides evidence that the protocol can be used for the direct detection of *S. marcescens* using DNA extracted from complex environmental or clinical samples, avoiding the cultivation step.

The observed limits of detection for this protocol are the following:

- DNA extracted from *S. marcescens* isolates subjected to end-point PCR: 10^4 genome copies for the HLMT_SMA_1 primer couple and 10^3 genome copies for the HLMT_SMA_2 primer couple;
- Mixed samples, obtained by spiking a *S. marcescens*-free fecal DNA with known amounts of *S. marcescens* DNA, and then subjected to qPCR: 10 genome copies for both primer couples;
- Mixed samples, obtained by spiking a *S. marcescens*-free soil with different amounts of *S. marcescens* intact cells, and then subjected to PCR: 10^5 cells (10^4 for some *S. marcescens* clusters), for both primer couples. Concerning qPCR, the analysis was successfully conducted till the soil sample spiked with 104 cells.

For the setting up of the HMR-based typing of *S. marcescens*, we used a study dataset composed of 230 *S. marcescens* isolates of various origin. As better discussed in,¹ the protocol sensitivity and specificity were respectively 86% and 97%. More in detail, the protocol provided a classification (either “MT Infectious” or “MT Colonising/Environmental”) for 197/230 strains (about 86%). Among these, the HRM-based classification correctly distinguished the “Infectious clone” in 190/197 (96%) of the strains.

LIMITATIONS

One of the main limitations is the fact that the protocol may classify some isolates as “undetermined” when the combinations of HLMT_SMA_1 and HLMT_SMA_2 melting temperatures do not fit any of the tree Melting Type described above. It could happen for instance in case of Horizontal Gene Transfer (HGT).

Another possible limitation is linked to the repeatability of HRM assay, which could affect the portability of HRM-based typing protocols. As shown,¹ the HLMT protocol presented here has 94% of repeatability and 88% of portability. These results show that although both repeatability and portability are not perfect, they can even be suitable for large screenings when a large number of samples are included.

This protocol was implemented using one of the most widely adopted qPCR platforms, the Bio-Rad CFX Connect Real-Time PCR system. While this instrument is not among the most sensitive available, it represents a typical standard in many laboratories. Like most conventional qPCR systems, it has an HRM temperature resolution of 0.5°C, which may limit its ability to discriminate between strains with closely similar melting temperatures. Higher-resolution instruments can overcome this limitation. Nevertheless, our previous results have shown that, even with a standard system as the Bio-Rad CFX Connect, the protocol successfully distinguished the three major *S. marcescens* groups associated with distinct clinical and environmental contexts. Therefore, the protocol is fully compatible with commonly available qPCR equipment.

It is finally to be noticed that the qPCR on soil mixed samples evidenced some technical problems, likely associated with the efficiency of DNA extraction, and/or with the presence of strong inhibitors of DNA amplification in this specific sample. For this reason, when dealing with complex and “difficult” matrices, we suggest our protocol to be used for detection and just preliminary typing. In these cases, a more precise typing result would be obtained on the isolated strains.

TROUBLESHOOTING

Problem 1

Poor quality and/or yield in DNA extraction when applying the *S. marcescens* HRM protocol on complex materials (e.g., soil,¹ feces,¹ soap dispenser⁵).

Potential solution

We recommend using commercial kits, specifically designed and optimized for the type of sample under analysis. Moreover, we suggest checking that the amount of sample to be processed does not exceed the maximum input recommended by the DNA extraction kit in use. For instance, the kits employed in our laboratory for stool DNA extraction require not exceeding 220 mg of fresh or frozen stool. In general, when dealing with complex and “difficult” matrices, we suggest using our protocol for detection and only preliminary typing. In these cases, a more accurate typing result would be obtained on isolated strains.

Problem 2

No DNA amplification following end point PCR.

Potential solution

If DNA amplification fails, assess both the quantity and quality of the extracted DNA using a spectrophotometer. Excessive DNA input and/or poor DNA quality may inhibit the qPCR reaction. Try diluting DNA samples before PCR: this can also help in the case PCR inhibitors are present.

Problem 3

Unclear classification of Melting Types (MTs).

Potential solution

Compare melting curves with reference strains; repeat qPCR in technical triplicates. If melting temperatures do not fit defined ranges, classify as “MT Unspecific” and consider repeating with isolated DNA.

Problem 4

Sample cross-contamination or false positives.

Potential solution

Use dedicated pipettes, sterile filter tips, and separate areas for pre- and post-PCR work. Include negative controls and avoid overloading DNA in gel electrophoresis.

Problem 5

Multiple or irregular melting curves in HRM analysis.

Potential solution

If DNA has been extracted directly from the sample, i.e., without strain isolation, multiple or irregular HRM curves could be due to the presence of multiple *Serratia marcescens* strains, or cross reactions or contamination. To exclude contamination, check DNA quality, use fresh high-quality primers, optimize primer concentration and annealing temperature, and ensure the qPCR instrument is properly calibrated.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Francesco Comandatore (francesco.comandatore@unimi.it).

Technical contact

Further information regarding specific details of the steps for this protocol should be directed to and will be fulfilled by the technical contact, Simona Panelli (simona.panelli1@unimi.it).

Materials availability

This study did not generate new unique reagents. All primers were designed in-house and synthesized by Eurofins. Materials described in this protocol are commercially available and listed in the [key resources table](#).

Data and code availability

This protocol does not generate new datasets or code. The experimental data and script resources supporting this protocol are derived from or are available in the associated publication and its supplementary materials. Additional materials may be provided by the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

Experimental design: F.C., S.P., and G.Z.; bioinformatics analyses: F.C., L.S., and D.M.M.; molecular biology experiments: G.B. and C.B.; paper writing: F.C. and G.B.; funding and supervision: F.C. and G.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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