

Simple Protocol for Molecular Fingerprinting of Human Oral Microbiota Samples in Lab Classes[†]

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DNA fingerprinting is a major tool in identifying individuals and in evidence matching. However, this technique can be difficult to reproduce in practical classes. Here, we report on distinct PCR profiles obtained when amplifying saliva DNA of a score of distinct individuals with Random Amplified Polymorphic DNA (RAPD)-PCR primer BOXAIR. The RAPD-PCR method is simple and efficient for discrimination between bacterial strains and is used in this instance to obtain personalized fingerprints of each individual's oral microbiota. We present real results with undergraduate students confirming that this procedure is easily feasible in practical classes. Based on the results presented, we suggest a laboratory activity for undergraduate Molecular Biology or Microbiology students.

INTRODUCTION

Background

Bacterial typing by means of PCR fingerprinting is a well-established concept. It may be accomplished using primers directed at repetitive sequences that are interspersed throughout the genome, as in the case of the Repetitive Extragenic Palindromic (REP) sequence (1), the Enterobacterial Repetitive Intergenic Consensus (ERIC) sequence (1, 2), or the BOX elements (3); it may also employ primers that anneal randomly in several locations along the genome, such as Random Amplified Polymorphic DNA (RAPD) (4–8) and Arbitrarily Primed PCR (AP-PCR) (9, 10). When it comes to identifying human individuals through molecular means, primers that amplify hypervariable genomic regions, such as variable number tandem repeats (VNTR) and short tandem repeats (STR), are used (11). However, realistic molecular identification of human samples is impossible or tricky to perform in most undergraduate practical classes due to the fact that interindividual differences are very small and, as such, need capillary electrophoresis or at least polyacrylamide vertical gels to be revealed (12). On the other

hand, the application of the RAPD technique to produce distinctive banding patterns from individual human beings is significantly harder to achieve, taking into account the much lesser interindividual variability at the level of the human genome. Although the approach has been tried before (13), it has never been fully exploited (and remained fairly unknown to the large scientific audience).

With the objective of introducing in our lab classes the concept of DNA fingerprinting in the context of human individual sample discrimination, we tried to overcome the aforementioned limitations by using a commercial kit such as Bio-RAD BIOTECHNOLOGY EXPLORER Crime Scene Investigator PCR BASICS Kit. However, this approach is too unsophisticated in that one cannot use real-sample DNA but must use templates provided by the manufacturer, and the experiment results in just two bands per individual DNA, which is a simplistic result.

An interesting feature of human biological samples is the possibility of getting information about the individual's microbiome. The high degree of variability in microbiota between individuals is known and found to occur at different body sites, like the gut, skin, and the oral cavity (14). Specifically, human saliva is a good source of bacterial cells (15) and can be easily collected in a non-invasive manner. Recent studies have shown that the strains composing each human's salivary microbiota are exchanged during intimate kissing (16) or even cohabitation (17), leading to some, albeit transient, increase in similarity between subjects. However, the salivary microbiome was found to be unique enough to be suggested as a useful forensic tool (18, 19).

Combining this background with our experience in bacterial typing, we designed a laboratory activity to

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demonstrate whether random primers conceived for bacterial genotyping could be used to produce distinct patterns of amplification from the DNA of distinct human saliva samples.

After preliminary tests (Appendix 1), primer BOXAIR (20) was chosen. The robustness of our protocol with regard to changes in template DNA concentration was assessed (Appendix 2).

Intended audience

This activity was designed with our undergraduate Biomedical Sciences, Forensic Science, or Pharmacy majors in mind, to be done during their second-year laboratory classes within the courses of Microbiology or Molecular Biology following introductory biology and chemistry courses.

Prerequisite student knowledge

As a prerequisite to successfully completing the protocol and interpreting the results, students should have some previous knowledge of the fundamental principles of molecular biology. Concepts of basic microbiology and of human-associated microbiota and microbiome and their individual variability need to be provided during previous theoretical classes, if not already covered elsewhere in the students' curriculum. In our experience, since RAPD is a peculiar example of PCR reaction employing just one primer randomly annealing to several genomic locations, it is pedagogically prudent for students to have some previous experience of standard two-primer PCR in order not to assume the one-primer model as the customary circumstance. Specific topics, such as DNA extraction, PCR, gel electrophoresis, and fingerprinting can be covered by the instructor in an introductory lecture, if they were not previously covered in the curriculum (estimated time about 2 hours). Some previous laboratory experience in pipetting and basic aseptic technique is also expected.

Learning time

Table 1 depicts our time organization for this activity. However, different scheduling is feasible with either shorter or longer blocks.

Learning objectives

By the end of this laboratory activity, students should be able to:

1. Describe and demonstrate how extraction and purification of genomic DNA works (if optional blocks 1 and 2 are included);
2. Show full knowledge of how PCR works in general and specifically in this type of setup (RAPD);
3. Explain the importance of proper handling of samples and reagents and of including negative controls

in DNA profiling, evaluate the practical limitations inherent in any identification method and explain the need for complex patterns in DNA profiling;

4. Interpret results properly;
5. Retain the concepts of human-associated microbiota and the related microbiome, the existence of intersubject variability at the level of the human microbiota/microbiome and the possibility of using such variability as a tool for individual discrimination by means of DNA fingerprinting.

PROCEDURE

Materials

A detailed description of the materials needed for each block is available in Appendix 7.

Student instructions

Students should be already used to working safely in a BSL2 (or BSL1, accordingly) laboratory environment. If they lack this previous training, appropriate instruction should be provided before starting this laboratory exercise. Also, a brief review on the topics of DNA extraction, PCR, gel electrophoresis, and fingerprinting is recommended. A students' protocol is provided in Appendix 4. Instructions on the optional blocks of DNA extraction and purity and concentration analysis are provided in Appendix 5.

PCR setup and run. Previously extracted DNA samples must be ready for the PCR experiment. In our classes, each group of three to five students picked up a DNA sample to work with. The reactions were carried out in a Bio-Rad MJ Mini TM Personal Thermal Cycler, in a total volume of 25 μ L (in 200 μ L tubes) per sample. A common reaction mix was prepared in a 1.5 mL tube, containing all common PCR ingredients (water, reaction buffer, heat-stable DNA polymerase, deoxynucleotide triphosphates [dNTPs] and primer). Table 2 contains a detailed description of the

TABLE 1.
Suggested scheduling scheme.

| Block | Activity / Task | Duration |
|-------|--|-----------|
| 1* | DNA extraction | 2 h |
| 2* | DNA purity and concentration analysis | 1.5 h |
| 3 | PCR setup (and run) | 1 h (3 h) |
| 4 | Product electrophoresis, visualization and photography, and discussion | 2 h |

Students meet once for each block attended. Block 3 takes about 3 hours, but students are not required to be present during the whole running of the PCR.

*These blocks are optional; the lecturer can choose not to address these topics within this module.

TABLE 2.
Typical recipe for each 25 μ L PCR reaction.

| Reagent | Stock Concentration | Final Concentration | Volume per Reaction (μ L) |
|--|----------------------|---------------------|--------------------------------|
| Buffer | 10 \times | 1 \times | 2.5 |
| MgSO ₄ | 20 mM | 1.5 mM | 1.9 |
| dNTP mix | 10 mM of each | 200 μ M of each | 0.5 |
| Primer BOXAIR (provided in our case by Stabvida, Portugal) | 100 μ M | 2 μ M | 0.5 |
| Taq polymerase | 5 U/ μ L | 1.5 U/ μ L | 0.3 |
| Nuclease-free water | — | — | 17.3 |
| DNA | 1 to 100 ng/ μ L | 2 to 8 ng/ μ L | 2 μ L* |

* DNA samples should be diluted appropriately so that 2 μ L corresponds to 50 to 200 ng DNA; individual DNA is added separately to each PCR tube and will not enter the common mix. dNTP = deoxynucleoside triphosphate.

concentrations and volumes of each reaction mix. Twenty-three microliters of the reaction mix were dispensed into each PCR tube, plus 2 μ L of each DNA sample. A negative control must always be included in the experiment, where nuclease-free water is substituted for the DNA, in order to prove the absence of external contamination. Students should be careful while handling the DNA polymerase, which must be kept on ice. PCR tubes containing the reaction mixes were loaded into the thermal cycler and the following program was used: 94°C 3 min + 30 cycles of 92°C 45 sec + 50°C 45 sec + 72°C 1 min followed by a last extension step of 5 min at 72°C.

Product electrophoresis, visualization and photography. Electrophoresis was performed using 1.5% (w/v) agarose gels and an electrophoresis buffer suited for the separation of small DNA fragments (such as the SGTB buffer by GRiSP, Portugal, or TBE) with ethidium bromide (0.5 μ g/mL). Also in this block, the instructor can choose whether students prepare the agarose gel or whether it is supplied ready to use. Students must be particularly careful when handling hot agarose and ethidium bromide (see safety issues). Three microliters of loading buffer were added to each PCR reaction tube. Fourteen microliters of each mix (corresponding to half of the product) were run in an electrophoresis apparatus at 155 V during 40 min, along with a low-molecular-weight DNA marker (such as 100-bp ladder GRS Universal Ladder, GRiSP, Portugal). The resulting bands were visualized and photographed using a Bio-Rad Universal Hood II Gel Imager. At this point, students were asked to interpret the results, giving a plausible explanation for what they observed in the photographs.

Faculty instructions

If the students perform the DNA extraction, each student providing a saliva sample should sign an informed consent form approved by the local Ethics Committee, the institution's IRB (institutional review board) should approve

the lab protocol, and samples should then be stored and discarded according to local rules.

If blocks 1 and 2 are opted in, when the concentration of the DNA is checked, it is advisable to pay attention to the differences between the values obtained in the different samples. If a high discrepancy in the DNA concentrations is observed, adjustments may be necessary, e.g., dilutions may be needed for samples with higher DNA concentrations, usually > 200 ng/ μ L. This procedure may be particularly useful when a common PCR reaction mix is employed, so that all groups can use the same volume of DNA sample in the PCR reaction mix in the next step of the activity.

If the lecturer chooses not to address the topic of DNA extraction, work needs to be performed ahead of time to get DNA extracted from saliva samples from different volunteer donors. The activity can be performed by students in groups or individually. In our classes, we typically split the class into groups of three to five students. In this situation, each group must be provided with one DNA sample to work with or, if the DNA extraction is performed, one member of each group will offer the saliva sample for the group.

When the PCR reaction mixes are prepared, the instructor must make sure that negative controls are included, in order to confirm the absence of contamination. It is crucial to avoid contamination of the reaction mix with environmental or human biological material. Teachers can take advantage of this protocol to stress the importance of careful collection and handling of samples, especially in case of forensic applications. It is highly recommended that the instructors emphasize the importance of an accurate pipetting process. Although it is often advised to perform the preparation of PCR mixes on ice and to use a hot-start procedure, we found that this is not really necessary if the preparation is reasonably quick and samples are put to cycle straightaway. However, students must be careful when handling the DNA polymerase, and the stock has to be kept on ice constantly.

We found that this procedure is reasonably easy to perform with undergraduate students, and, in the end, clearly

different patterns are observed for each DNA sample. However, it is not unusual for one or another sample to not result in amplification, or to observe some amplification in the negative control. Students must be encouraged to find plausible explanations for these unexpected results: wrong handling of the materials and samples are the usual causes in our experience.

We suggest a final discussion to sum up the meaning of the results obtained.

Suggestions for determining student learning

In order to assess student learning outcomes, questions were designed and included in an evaluation test administered at the end of the activity (Appendix 6). However, alternative evaluation schemes, such as lab reports or oral presentations of results, are also possible, according to local routine and the instructor's preference.

Sample data

Examples of some electrophoresis results obtained by students in our classes are shown in Figure 1. Each image contains the results from the groups of one class. Individual banding patterns were observed throughout the groups. Lane 3 of Figure 1E is empty. This is an example of a case where the DNA amplification failed, probably due to pipetting errors, or possibly due to the degradation of the DNA sample. Students were called to discuss the results and this particular situation turned out to be useful in order to explain the sensitivity of the PCR process and the importance of being rigorous and accurate during the preparation of the reaction mixes.

Safety issues

For this laboratory activity, students should wear standard laboratory protection (i.e., lab coat, closed-toed shoes, and gloves) at all times, and, in general, ASM Guidelines for Biosafety in Teaching Laboratories should be followed: if the protocol starts with Block 2, BSL1 will be appropriate. If Block 1 is included in the activity and fresh saliva is handled by students, then BSL2 will be needed; local safety standards for handling potentially infectious human specimens should be followed, and safe disposal of waste should be implemented according to local regulations. When working with hot agar solutions, thermal gloves should be always used. If a microwave oven is used, students should be warned about the danger of a superheated agarose solution suddenly boiling over out of the flask. When working with ethidium bromide, use of gloves is mandatory. Special care should be taken by students when using a UV transilluminator: skin and eyes must be protected from UV radiation in case some of it escapes from the apparatus (use safety goggles or glasses). Students should be taught and made aware of proper disposal of each type of material, especially in the

case of stain-containing gels and buffers, which are subject to local regulations.

DISCUSSION

Field testing

The activity presented here was introduced in the past few years as a component of Molecular Biology or Microbiology lab classes offered with Pharmacy, Biomedical Sciences, and Forensic Sciences courses. The size of each class varied between five and sixteen students, who were divided into working groups of one to five (our whole sample totals five classes and 55 students).

In order to assess student reaction to this activity and whether they considered it useful as a learning tool, anonymous questionnaires were administered after completion of the protocol. Students were asked to rank each of the statements provided on a scale of 1 ("totally disagree") to 5 ("fully agree"). In addition, there was a space for free comments, although no-one used it. The results are compiled in Table 3. Most students agreed that this practical activity encourages participation in the learning process, helps understanding key concepts in Molecular Biology, and fosters the understanding of the concept of personalized molecular profile. More than 90% of the students agreed that the experiment helped them to understand the concept of individual specificity of the human oral microbiota. The vast majority of the 55 students rated the experiment as useful and enjoyable.

Overall, the experiment proposed here is an easy and cheap alternative to standard fingerprinting techniques that was performed by undergraduate students with no special difficulty and yielded clear and easy-to-understand outcomes.

Evidence of students learning

In order to assess that the learning objectives were accomplished, a posttest was administered to each class, which showed that in general the main concepts informing this module had been understood and retained (Grade statistics are shown in Fig. 2; questions and examples of students' answers can be found in Appendix 6, with our suggestion for a scoring rubric linked to student learning objectives). For a more scientific assessment of student learning, we suggest a pre-/posttest results comparison.

Possible modifications (optional)

The proposed laboratory activity described here is flexible and can be easily adapted for alternative scenarios. One option refers to the inclusion or not of the DNA extraction step in the protocol (blocks 1 and 2), as previously addressed.

Additionally, the distribution of the class is flexible. In our approach, the students were normally split into groups

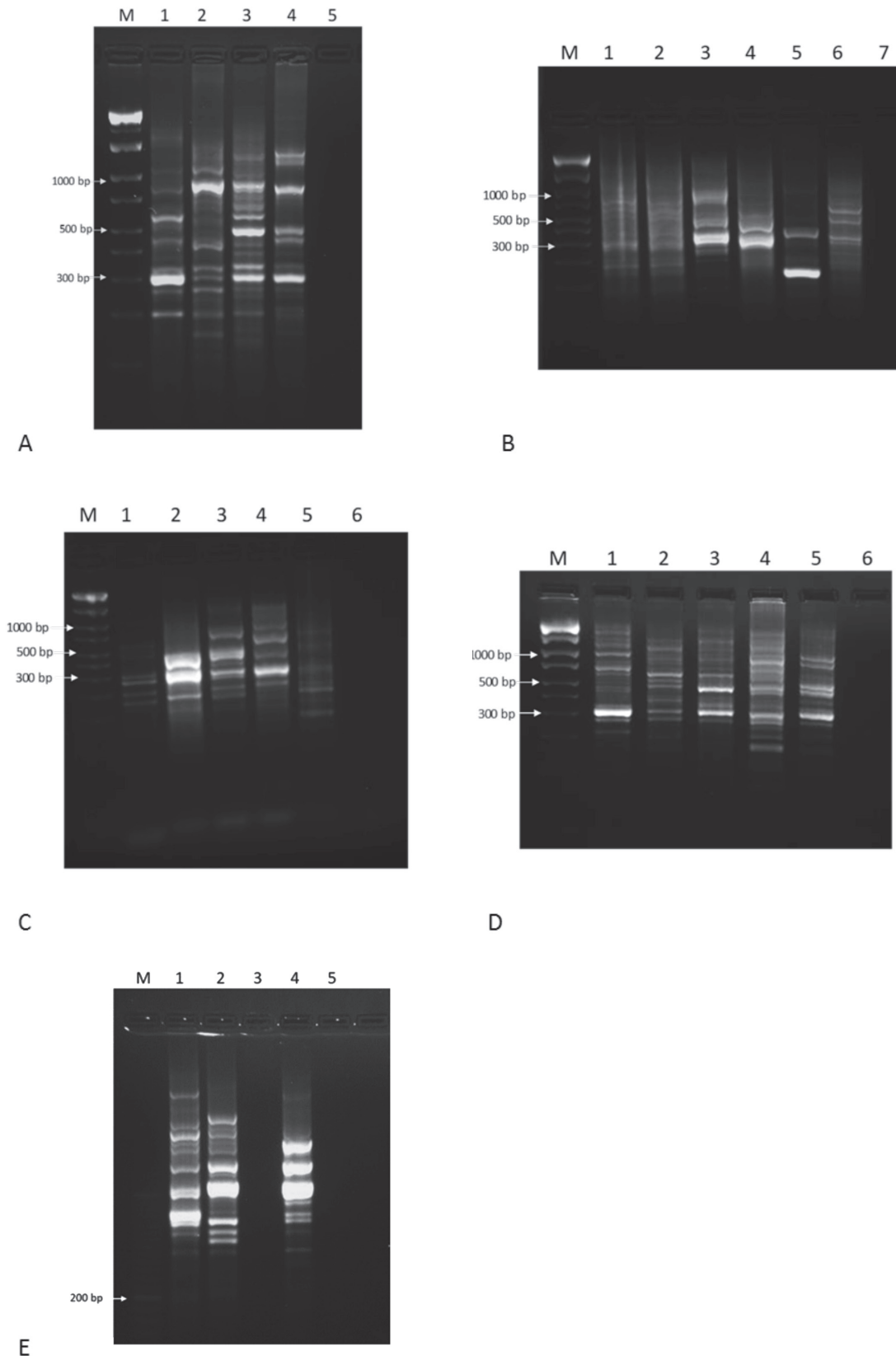


FIGURE 1. Results obtained by five groups of students (A to E). A) 1, 2, 3, 4 = individual saliva DNA samples. 5 = water negative control. B) 1, 3, 4, 5, 6 = individual saliva DNA samples. 7 = water negative control. 2 = same sample as n° 1, loaded twice by mistake. C) 1, 2, 3, 4, 5 = individual saliva DNA samples. 6 = water negative control. D) 1, 2, 3, 4, 5 = individual saliva DNA samples. 6 = water negative control. E) 1, 2, 3, 4 = individual saliva DNA samples. 5 = water negative control. M = molecular weight marker.

TABLE 3.
Questionnaire questions and answer statistics^a.

| | Average ^b | SD | Mark ≥ 4 |
|---|----------------------|------|---------------|
| The general organization of the experiment was appropriate | 4.44 | 0.54 | 98.2% |
| The experiment's aims were clear | 4.35 | 0.73 | 85.4% |
| The experimental method was clear | 4.47 | 0.60 | 94.6% |
| This type of lab work encourages active student participation | 4.09 | 0.73 | 81.8% |
| This experiment helps understand important concepts in molecular biology | 4.25 | 0.58 | 92.7% |
| My understanding about DNA, replication, and PCR improved after this experiment | 4.07 | 0.79 | 76.4% |
| I was eager to see the final results | 4.35 | 0.70 | 87.3% |
| This experiment worked well in my class | 4.44 | 0.57 | 96.4% |
| This experiment allowed me to understand that oral microflora is different in each individual | 4.67 | 0.65 | 91.7% |
| This experiment deals with important concepts in molecular biology | 4.33 | 0.58 | 94.6% |
| This experiment allowed me to understand the concept of personalized molecular profile | 3.93 | 0.81 | 78.2% |
| I got to understand how research in this field is performed | 4.09 | 0.78 | 81.8% |
| This type of experiment inspired my interest in molecular biology | 3.84 | 0.96 | 70.9% |
| After observing and interpreting the results, my doubts were clarified | 4.22 | 0.76 | 80.0% |
| This experiment is a useful learning instrument | 4.38 | 0.68 | 89.1% |
| I liked performing this experiment | 4.36 | 0.78 | 90.9% |

^aTotal number of students = 55.

^bStudents were asked to rank each of the statements between 1 (totally disagree) and 5 (fully agree).

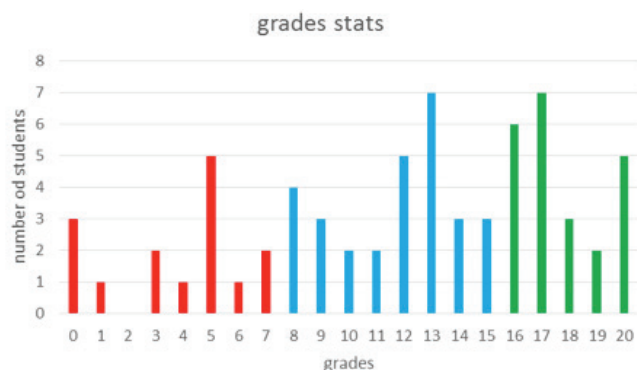


FIGURE 2. Histogram of grades (0 to 20) from 67 students. 0 to 7 (red) = not proficient; 8 to 15 (blue) = proficient; 16 to 20 (green) = highly proficient. Questions and students' answer examples can be found in Appendix 6, with our suggestion for a scoring rubric linked to student learning objectives. Authorization for using students' grades was obtained from our internal Ethics Committee.

of three to five, but the activity can also be performed individually, with one sample per student, which, in case of opting for running the four blocks, would be the student's personal sample. This will depend on class size and the resources available, as well as the specific objectives of the instructor.

As an alternative scenario, the instructor can simulate a forensic case by selecting one of the DNA samples without disclosing its identity to the students and asking them to match this sample (corresponding to "the biological specimen obtained on the crime scene") by comparing its PCR

banding profile with that of all the students sampled ("the suspects cohort").

If the laboratory is equipped for also safely extracting DNA from the volunteers' blood samples (enough DNA can be extracted from a drop of blood obtained non-invasively by finger pricking), the proposed PCR conditions can be used with DNA from blood as well. With this procedure, students could run the PCR products from each donor's saliva and blood side by side. Since DNA obtained from a healthy subject's blood should be just of human origin, in this case they should be able to observe that the interindividual differences between the band patterns from saliva samples (corresponding to a mix of human/microbial metagenome) are much more obvious than those from blood samples.

SUPPLEMENTAL MATERIALS

- Appendix 1: Preliminary tests
- Appendix 2: Method's robustness test with different DNA concentrations
- Appendix 3: Comparisons between RAPD profiles obtained from DNA from blood or saliva
- Appendix 4: Students' protocol
- Appendix 5: Student instructions on DNA extraction and purity and concentration analysis
- Appendix 6: Final evaluation test example and assessment rubric
- Appendix 7: Detailed materials list

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REFERENCES

1. Versalovic J, Koeuth T, Lupski JR. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 19:6823–6831.
2. Shangkuan YH, Yang JF, Lin HC, Shaio MF. 2000. Comparison of PCR-RFLP, ribotyping and ERIC-PCR for typing *Bacillus anthracis* and *Bacillus cereus* strains. *J Appl Microbiol* 89:452–462.
3. Tacão M, Alves A, Saavedra MJ, Correia A. 2005. BOX-PCR is an adequate tool for typing *Aeromonas* spp. *Antonie van Leeuwenhoek*, 88:173–179.
4. Wang G, Whittam TS, Berg CM, Berg DE. 1993. RAPD (arbitrary primer) PCR is more sensitive than multilocus enzyme electrophoresis for distinguishing related bacterial strains. *Nucleic Acids Res* 21:5930–5933.
5. Rajasundari K, Ilamurugu K, Logeshwaran P. 2009. Genetic diversity in rhizobial isolates determined by RAPDs. *African J Biotechnol* 8:2677–2681.
6. Shangkuan YH, Lin HC. 1998. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella typhi* and other *Salmonella* species. *J Appl Microbiol* 85:693–702.
7. Saunders GC, Dukes J, Parkes HC, Cornett JH. 2001. Interlaboratory study on thermal cycler performance in controlled PCR and random amplified polymorphic DNA analyses. *Clin Chem* 47:47–55.
8. Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res* 20:5137–5142.
9. Welsh J, McClelland M. 1990. Fingerprint genomes using PCR with arbitrary primers. *Nucleic Acids Res* 18:6531–6535.
10. Ménard C, Brousseau R, Mouton C. 1992. Application of polymerase chain reaction with arbitrary primer (AP-PCR) to strain identification of *Porphyromonas (Bacteroides) gingivalis*. *FEMS Microbiol Lett* 95:163–168.
11. Butler JM. 2005. *Forensic DNA Typing*, Second ed. Elsevier Academic Press, Burlington, MA.
12. McNamara-Schroeder K, Olanon C, Chu S, Montoya MC, Alviri M, Ginty S, Love JJ. 2006. DNA fingerprint analysis of three short tandem repeat (STR) loci for biochemistry and forensic science laboratory courses. *Biochem Mol Biol Educ* 34:378–383.
13. Baransel A, Dulger HE, Tokdemir M. 2004. DNA amplification fingerprinting using 10 × polymerase chain reaction buffer with ammonium sulfate for human identification. *Saudi Med J* 25:741–745.
14. Ursell LK, Clemente JC, Rideout JR, Gevers D, Caporaso GJ, Knight R. 2012. The interpersonal and intrapersonal diversity of human associated microbiota in key body sites. *J Allergy Clin Immunol* 129:1204–1208.
15. Dawes C. 2003. Estimates, from salivary analyses, of the turnover time of the oral mucosal epithelium in humans and the number of bacteria in an edentulous mouth. *Arch Oral Biol* 48:329–336.
16. Kort R, Caspers M, Graaf A Van De, Egmond W Van, Keijsers B, Roeselers G. 2014. Shaping the oral microbiota through intimate kissing. *Microbiome* 2:1–8.
17. Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-lyons D, Caporaso JG, Knights D, Clemente JC, Nakielny S, Gordon JI, Fierer N, Knight R. 2013. Cohabiting family members share microbiota with one another and with their dogs. *Elife* 2:e00458:1–22.
18. Siqueira JF, Fouad AF, Rôças IN. 2012. Pyrosequencing as a tool for better understanding of human microbiomes. *J Oral Microbiol* 4:1–15.
19. Hasan NA, Young BA, Minard-Smith AT, Saeed K, Li H, Heizer EM, McMillan NJ, Isom R, Abdullah AS, Bornman DM, Faith SA, Choi SY, Dickens ML, Cebula TA, Colwell RR. 2014. Microbial community profiling of human saliva using shotgun metagenomic sequencing. *PLoS One* 9 (5): e97699.
20. Versalovic J, Schneider M, Bruijn FJ de, Lupski JR. 1994. Genomic fingerprint of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* 5:25–40.