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TITLE:

Application of DNA Fingerprinting Using the *D1S80* Locus in Lab Classes

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SUMMARY:

Here we describe a simple protocol to generate DNA fingerprinting profiles by amplifying the VNTR locus *D1S80* from epithelial cell DNA.

ABSTRACT:

In biological sciences, DNA fingerprinting has been widely used for paternity testing, forensic applications and phylogenetic studies. Here, we describe a reliable and robust method for genotyping individuals by Variable Number of Tandem Repeat (VNTR) analysis in the context of undergraduate laboratory classes. The human *D1S80* VNTR locus is used in this protocol as a highly polymorphic marker based on variation in the number of repetitive sequences.

This simple protocol conveys useful information for teachers and the implementation of DNA fingerprinting in practical laboratory classes. In the presented laboratory exercise, DNA extraction followed by PCR amplification is used to determine genetic variation at the *D1S80*

VNTR locus. Differences in the fragment size of PCR products are visualized by agarose gel electrophoresis. The fragment sizes and repeat numbers are calculated based on a linear regression of the size and migration distance of a DNA size standard.

Following this guide, students should be able to:

- Harvest and extract DNA from buccal mucosa epithelial cells
- Perform a PCR experiment and understand the function of various reaction components
- Analyze the amplicons by agarose gel electrophoresis and interpret the results
- Understand the use of VNTRs in DNA fingerprinting and its application in biological sciences

INTRODUCTION:

Molecular fingerprinting, also referred to as DNA fingerprinting, was introduced by Sir Alec Jeffreys while working in the Department of Genetics at the University of Leicester in 1984[1]. It is based on the 0.1% of the human genome that differs between individuals and is comprised of approximately three million variants. These unique differences in the genotype allow for the differentiation between individuals and can therefore function as a genetic fingerprint except for monozygotic twins. Consequently, DNA fingerprinting is used for the estimation of relatedness of different individuals that is applied for example in paternity testing or in population diversity studies. In our laboratory classes we aimed to convey the concept of DNA fingerprinting and allele frequencies. The method described here demonstrates a reliable and robust method for genetic fingerprinting by analyzing the variable number of tandem repeats (VNTR) at the *D1S80* locus. The method comprises the extraction of DNA from buccal mucosa epithelial cells and subsequent polymerase chain reaction (PCR) to amplify the *D1S80* locus, followed by visualization of fragment length differences on an agarose gel.

The *D1S80* VNTR minisatellite locus is highly variable and located at the telomeric region of chromosome 1 (1p36-p35). It was identified and described by Karsai and co-workers in 1990 as a locus with a core repeat unit of 16 bp, allowing for the separation of alleles differing by just one unit[2]. Furthermore, *D1S80* shows a high degree of variation with a mutation rate of approximately 7.77×10^{-5} [3]. *D1S80* has a high degree of heterozygosity[4,5] (e.g., 80.8% heterozygosity for Caucasians[6] and up to 87% heterozygosity for African-Americans[7]). Additionally, the *D1S80* locus is polymorphic in most populations, with typically over 15 different alleles carrying 14 to 41 repeats each. The frequency of *D1S80* alleles varies between populations. The allele with 24 repeat units (allele 24) is most frequent in European and Asian populations, whereas allele 21 is most common in African populations[4,7–10]. Consequently, the allele frequency distributions are diagnostic for different human populations and need to be taken into account for the estimation of relatedness (e.g., in paternity tests).

PCR based amplification of the *D1S80* VNTR locus has been a very useful method in forensic science, paternity tests, disease analysis and population diversity studies[11–14]. While in forensics today the use of VNTRs has been replaced by short tandem repeats, the *D1S80* VNTR locus is widely used in the determination of origins and genetic relations among and between populations[4, 8, 9, 11]. Furthermore, it is often used to teach DNA fingerprinting in practical

laboratory classes[15, 16]. The method described here represents a robust, cost-effective and easy-to-use method with a very high success rate in undergraduate laboratory classes. The purpose of this article is to provide an overview of the workflow for the molecular analysis of the human *D1S80* minisatellite locus from buccal mucosa epithelial cells. It includes demonstration of techniques, simplified protocols and practical suggestions described in previously published works[2, 17].

PROTOCOL:

NOTE: This protocol is only to be used if students or respective legal guardians agreed to the conductance of this protocol as genotypic profiles give insights into genetic relationships. DNA fingerprinting is a common molecular biology method mainly applied to population studies and forensic matters. Therefore, attention should be devoted to keep contamination risks as low as possible. To avoid contamination of the sample with DNA from an outside source or DNases, gloves should be worn, instruments should be thoroughly cleaned or sterilized and solutions should be filter-sterilized or autoclaved before usage.

1. Harvesting of buccal mucosa epithelial cells

CAUTION: Work with saliva and epithelial cells can lead to a transmission of infectious diseases. Therefore, standard and transmission-based precautions should be applied (e.g., the use of appropriate personal protective equipment).

NOTE: Include a positive control, which is provided by the instructor (e.g., DNA extracted by the instructor) and included in the downstream processing steps.

1.1. Wait at least 1 h after eating or brushing teeth before specimen collection.

1.2. Label an empty and sterile 2.0 mL microcentrifuge tube.

1.3. Remove one sterile buccal swab from the packaging and rub vigorously on the inside of the cheek for 30-40 times or 30-40 seconds to harvest buccal mucosa epithelial cells.

1.4. Place the tip of the collection swab into the previously labeled sterile microcentrifuge tube and break off the length of plastic that extends beyond the edge, either by hand or using sterile scissors.

1.5. Place the cap securely onto the tube, sealing the collection swab inside.

2. Extraction of genomic DNA from human cells

2.1. Before extraction, set a thermal mixer or a heating block to 65 °C for sample lysis.

CAUTION: Some chemicals used are classified hazardous. Read the safety data sheet carefully

and take appropriate safety measures before handling.

2.1.1. Prepare and sterilize by filtering or autoclaving all required buffers and solutions (lysis solution, 8 M potassium acetate, 2-propanol, 70% ethanol and elution buffer).

NOTE: All centrifugation steps should be performed at room temperature (20-30 °C) unless specified.

2.2. Add 500 μL of lysis solution (50 mM Tris/HCl, pH 8.0; 10 mM ethylenediamine tetra-acetic acid (EDTA); 2% sodium dodecyl sulfate (SDS)) to the buccal swab, making sure that the sample is completely immersed in the lysis solution.

2.2.1. Vortex vigorously for at least 5 s.

2.2.2. Incubate samples at 65 °C in a thermal mixer for 10 min.

2.2.3. Mix sample 3-4 times by pulse-vortexing for 5 s during incubation.

2.2.4. Remove swab from the lysis buffer, press the swab against the inside of the tube to obtain maximum sample volume.

2.3. Add 100 μL of 8 M potassium acetate to lysed cells.

2.3.1. Mix thoroughly by inverting the tube until there is a white precipitate.

2.3.2. Incubate for 5 min at room temperature.

2.4. Centrifuge the sample for 5 min at 18,000 *x g*.

2.5. Transfer 450 μL of the supernatant into a clean and sterile 1.5 mL microcentrifuge tube.

2.6. Add 450 μL of 2-propanol and mix thoroughly by inverting the tube (precipitation of the DNA).

2.7. Incubate for 5 min at room temperature.

2.8. Centrifuge for 5 min at 18,000 *x g*.

2.9. Discard the supernatant and invert the tube on a clean paper towel to dry the pellet and to avoid cross-contamination.

2.10. Incubate the DNA for 5 min at 65 °C in a heating block to dry the pellet completely.

2.11. To wash the DNA, add 500 μL of 70% ethanol.

2.12. Centrifuge for 1 min at 18,000 *x g*.

2.13. Discard the supernatant and invert the tube on a clean paper towel to dry the pellet.

2.14. Add 30 μL of resuspension buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) to the pellet.

2.15. Incubate the DNA for 10 min at 65 °C in a heating block to inactivate DNases.

3. Amplifying the *D1S80* VNTR locus using PCR

NOTE: Record the number of samples that will be used and prepare a worksheet with the required reagents and their volumes before collecting the necessary plastics and other materials. Label the sterile tubes/strips/plates to be used for the PCR with sample numbers. Remember to include a negative control using H_2O instead of DNA and a positive control (e.g., using the DNA provided by the instructor) to validate the PCR.

3.1. Prepare the 1x PCR master mix containing 10 μL of 5x PCR reaction buffer (containing 15 mM $MgCl_2$), 1 μL of deoxyribonucleotide triphosphate (dNTPs) (10 mM), 5 μL (10 pmol) of each primer pMCT118-f and pMCT118-r (forward - 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3', reverse - 5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3') according to Kasai et al.[2], 23.8 μL of ultrapure H_2O, and 0.2 μL of Taq DNA polymerase (5 U/μL).

NOTE: The primers pMCT118-f/pMCT118-r were designed on the flanking regions of the *D1S80* VNTR region amplifying the whole locus.

3.2. Label the PCR tubes/strips/plate with the sample numbers to be used.

3.3. Aliquot 45 μL of the master mix to each labeled PCR sample tube or well.

3.4. Add 5 μL of the DNA template to the master mix in each PCR tube/plate well to acquire a total volume of 50 μL. Change the pipette tip for every DNA sample to avoid cross contamination.

3.5. Include a no-template control (NTC) by using ultrapure H_2O instead of DNA.

3.6. Close PCR tubes/strips or seal plate and mix.

3.7. Centrifuge the PCR tubes/strips/plates for 20 s using a tabletop centrifuge.

NOTE: PCR conditions given in this protocol were optimized using the DNA Polymerase and the PCR thermal cycler used. In general, PCR conditions must be adapted to the DNA polymerase. The standard extension time for a Taq DNA polymerase is 1 min/kb.

3.8. Place the sample tubes/strips/plate in the thermocycler and incubate reactions using the following conditions (DNA polymerase extension time): 1 cycle of 95 °C for 2 min; 25 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s; 1 cycle of 72 °C for 10 min.

3.9. When the program is complete, remove the products from the thermocycler and store at 4 °C overnight or -20 °C until electrophoresis.

4. Agarose gel electrophoresis of PCR products

4.1. Prepare a 1.5% agarose gel.

4.1.1. Use 1.5 g of agarose powder and mix it with 100 mL of 1x Tris-acetate-EDTA (TAE) buffer solution in a flask.

4.1.2. Heat the mixture for about 1.5-2 min in a microwave oven (600 W). Swirl the contents and heat again, if necessary, to completely dissolve the agarose. Cool slightly and add 2 μL of PeqGreen to the agarose.

4.1.3. Pour the gel in a form using a comb with enough wells for all samples and add at least one molecular weight marker.

CAUTION: Boiling retardation may occur. Shake the flask carefully when resuspending the agarose which has not been dissolved yet.

NOTE: PeqGreen is a non-toxic dye for the detection of nucleic acids. It is applicable for staining of double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) as well as RNA. The sensitivity is comparable to ethidium bromide.

4.2. Remove the PCR products from 4 °C and centrifuge for about 10 s.

4.3. Load the wells of the gel with 10 μL sample. Do not overload the gel.

NOTE: The DNA polymerase buffer also includes compounds that increases sample density so that samples can be loaded directly onto gels without the need for a loading dye. This allows the sample to sink into the well and dyes help to track how far the DNA sample has migrated.

4.3.1. Add a molecular weight standard to the flanking wells (preferably a 50 bp molecular weight standard).

NOTE: Store the residual PCR samples at -20 °C in case the agarose gel electrophoresis has to be repeated.

4.4. Run the gel in 1x TAE running buffer at 150 V (constant) for approximately 40 min or until the lower yellow dye front that travels at around 50 bp reaches the lower end of the gel.

4.5. Image the gel while blue light or ultraviolet (UV) light is applied and record an image for fragment length analysis.

CAUTION: UV light can damage your eyes and skin. Always wear protective clothing and UV safety glasses when using a UV light box.

4.6. Dispose the gel in accordance with the institutional hazardous materials policy.

5. Analysis of fragment length results

NOTE: Use linear regression analysis to estimate the lengths of the fragments.

5.1. For sizing the *D1S80* PCR fragments, place a ruler on the gel photograph over the 50 bp molecular weight standard lane such that the top of the ruler lines up with the bottom of the well into which the sample was loaded (**Figure 1**).

NOTE: To calculate the linear regression equation for the molecular weight standard (50 bp molecular weight standard) a spreadsheet program was used.

5.2. Record the distance from the well (e.g., in cm) for each band of the 50 bp molecular weight standard in a table using a spreadsheet program (**Figure 2**).

5.3. Determine the log (e.g., base 10) of each fragment size of the 50 bp molecular weight standard and enter the log values into the table (**Figure 2**).

5.4. Plot each data point to a graph with the log of the band sizes on the vertical axis (y-axis) and the measured run distance from the top of the gel to each band of the molecular weight standard on the horizontal axis (x-axis) using a scatterplot (**Figure 3**).

5.5. Fit a trendline (linear regression line) and show the regression equation ($y = ax + b$) and the R^2 value on the graph (**Figure 4**).

5.6. Measure the distance migrated (e.g., in cm) for each *D1S80* amplicons (**Figure 5**).

5.7. Estimate the size of each *D1S80* amplicons using the regression equation: $y = ax + b$

where y = the log of the fragment size
a = the slope of the line (calculated in point 5)
x = the distance from the well (in cm)
b = the point where the regression line intercepts the y-axis (calculated in point 5)

NOTE: Since the y-value represents the log of the fragment size, the antilog (10^y) must be calculated to obtain the fragment size in bp of the *D1S80* amplicons.

6. Estimation of the number of repeat units in the alleles of the tested individuals

NOTE: The *D1S80* repeat unit is 16 bp in length. The smallest known allele for *D1S80* has 14 repeats. The amplicon scheme described here produces amplicons with flanking regions adding up an extra 145 bp to the final size.

6.1. Use **Table 1** to estimate how many repeat units are contained in each PCR fragment. The size extrapolated using the linear regression should be within 8 bp of any particular allele.

6.2. Record the genotype of each tested individual as a combination of allele repeat size numbers.

REPRESENTATIVE RESULTS:

Using the described protocol, *D1S80* VNTR marker analysis was performed on human genomic DNA extracted from buccal mucosa epithelial cells harvested by swab sampling (**Figure 6**). Following the amplification using PCR, a typical representation of an agarose gel containing the *D1S80* amplicons is shown in **Figure 1**. Lane 1 shows the 50 bp molecular weight standard. Next to the molecular weight standard, PCR products from eight student samples are visualized. Lane 10 shows the NTC in which water was used instead of template DNA. Most of the analyzed samples clearly display two bands, representing individuals heterozygous for the *D1S80* locus. Lane 2 and Lane 9 show a single band representing individuals homozygous for the *D1S80* locus. Lane 5 is showing an ambiguous single band which is much wider compared to the other bands. This could be a result of two *D1S80* alleles differing in only one repeat unit. For further analysis, the sample in lane 5 will be considered as a single band, representing a homozygous individual.

Downstream gel electrophoresis fragment analysis performed on verified PCR products was used for size calling of the *D1S80* VNTR locus of different student samples. Interpretation of the amplicon sizes obtained by PCR analysis relies on the molecular weight standard used. The distance from the well for each band of the molecular weight standard was measured (**Figure 1**) and recorded (**Figure 2**). Based on the size and migration distance the size of the individual bands can be calculated using a linear regression analysis. The log (base 10) was determined for each band in the student samples (**Figure 5**) and the respective antilog represents the number of bp for each amplicon. The number of repeat units can be calculated according to the amplicon size obtained (**Table 2**).

For each student sample the amplicon sizes were calculated by linear regression and each band could be easily assigned to a particular allele as shown in **Table 2**. The information on the *D1S80* allele sizes can then be used to determine allele frequencies amongst the small population subset of undergraduate students. The 28-repeat allele is the most frequent among the students due to two homozygous individuals for this repeat allele (individuals 1 and 4). The second most frequent allele is the 18-repeat allele, which is carried by the homozygous individual 8 and by the heterozygous individual 6. Low frequency alleles are the 21-, 26- and 30-repeat alleles found only once in individuals 6, 5 and 3, respectively. The allele frequency patterns of the *D1S80* VNTR locus

of the small student population can further be compared to the allele frequency of larger human populations, for example populations originating from different continents[4, 7–10].

Among the students, individuals 2 and 3 share the 24-repeat allele, individuals 2 and 7 share the 20-repeat allele and individuals 5 and 7 share the 23-repeat allele. Interestingly, the two *D1S80* homozygous individuals (individual 1 and 4) share the 28-repeat allele. Matching alleles between two individuals can indicate a potential relatedness. However, shared alleles can also occur by chance. Thus, it is crucial to determine the likelihood of an allele match between two individuals. The likelihood depends on the allele frequency of individual alleles in the general population and statistical approaches should be employed to attach a statistical weight to VNTR marker analysis such as the Bayesian approach. In summary, the presented fingerprinting method can be utilized in hands-on class practicals to teach the use of VNTRs in DNA fingerprinting and its application in biological science.

FIGURE AND TABLE LEGENDS:

Figure 1: Representation of an agarose gel after electrophoresis of *D1S80* amplification products with a ruler placed beside the 50 bp molecular weight standard lane. Lane 1 and 11 contain the 50 bp molecular weight standard, while lanes 2-9 contain PCR reaction products. Lane 10 contains the no template control (NTC).

Figure 2: Determination of DNA fragment migration distance and Log of each fragment size of the 50 bp molecular weight standard. The distance from the well (in cm) for each band of the 50 bp molecular weight standard is recorded and the log (base 10) for each fragment is determined. Values are entered into a table using a spreadsheet program.

Figure 3: Scatter plot generated by plotting DNA fragment migration distance of the molecular weight standard on the x-axis against the log (base 10) of the size of each fragment on the y-axis.

Figure 4: Exploitation of the linear regression line and regression equation as well as the R^2 value for the data.

Figure 5: Determination of the size of the *D1S80* amplicons. The distances from the well for each fragment is entered into the regression equation. The antilog of the y-value represents the fragment size in bp.

Figure 6: Suggested timeline and workflow for *D1S80* VNTR analysis. The entire *D1S80* VNTR analysis procedure presented here, from buccal epithelial cell harvesting to fragment length evaluation, may be completed within a single working day.

Table 1: Amplicon size for each *D1S80* VNTR locus.

Table 2: Allele composition of the different individuals tested. The number of repeat units can

be approximated according to the amplicon size obtained by linear regression analysis.

DISCUSSION:

Here we described a simple and cost-efficient method for implementing molecular fingerprinting in undergraduate practical classes.

To screen for genetic variation at the *D1S80* locus, human biological sample collection along with DNA extraction and analysis is required. It is essential that the ethical use of human biological specimens is ensured throughout the whole process. Sample management is controlled within a comprehensive regulatory framework which ensures the correct use of samples and associated data[18] (e.g., consent for the use of human biological material). Participants must be properly informed about the use of their samples, the risk of discovery of anomalies in genetic relations (e.g., for related individuals), privacy protection and intentions for future storage of the biological specimens and data. All donors (students or colleagues) must give consent freely and understand the right to withdraw without giving reason. In general, it is indispensable to make oneself familiar with the respective guidelines and regulations for human sample management before conducting this lab class.

For the collection of buccal mucosa epithelial cells in undergraduate laboratory courses, care must be taken to avoid contamination of the DNA samples with DNA from the operator or with DNases. It is recommended that lab coats, gloves and protective glasses are always used as well as sterile swabs and microcentrifuge tubes. Buccal swab-based cell harvesting is considered a convenient and cost-effective method for the collection of genetic material suitable for PCR-based VNTR analysis, as it is relatively inexpensive and noninvasive. Beside buccal swabs, saliva is the most common oral sampling method for medical research. It has been shown that buccal swabs contain a higher proportion of epithelial cells than saliva, making them more reliable in providing sufficient quantity and quality of DNA for PCR-based *D1S80* VNTR analysis[19, 20]. Additionally, buccal swabs can be mailed out after self-collection overcoming geographical impediments when used, for example, in population diversity studies[21].

DNA extraction has become relatively easy due to the development of extraction kits from different companies. Nevertheless, the use of those kits may not be suitable in laboratory classes due to limited financial resources. Here, we presented an easy, fast and cost-efficient method for DNA extraction from human samples without the need of a commercially available DNA extraction kit. The DNA extraction method described does not use organic solvents, instead cellular proteins are removed by increasing the salt concentration using an 8 M potassium acetate solution. This method also allows for handling of several samples at once and yields in sufficient DNA for several genotype analyses for each sample.

PCR is a common technique in many molecular laboratories. While generally trouble-free, there are pitfalls that may complicate the reaction producing spurious results, which have been discussed elsewhere[22]. PCR conditions presented here have appeared very robust in the face of poor template DNA quality, but lack of PCR amplification was nevertheless occasionally observed when using templates with extremely low DNA concentrations due to poor buccal epithelial cell

harvesting. The DNA primers used in this study can be ordered from different companies, such as those cited in the table of materials at the end of this paper. Special care must be taken when working with DNA primers in order to avoid contamination with DNases or DNA from the operator. PCR products should also be kept cold when not in use.

Another critical step is the agarose gel electrophoresis. Fragments differing by only one repeat unit of 16 bp may not be separated in the gel electrophoresis and thus appear as a single band. In this case, a proper determination of the number of repeat units of the *D1S80* alleles tested cannot be assured. Therefore, the run time of the gel should be increased while the voltage must be reduced and the gel concentration may be increased to provide a better resolution and separation of the single bands.

It is worth mentioning that not all alleles for an VNTR locus contain complete repeat units. Non-consensus alleles (microvariants) that contain incomplete repeat units are common at most VNTR loci and their sizes fall in between sizes of alleles with full repeat units. These microvariants are barely detectable by agarose gel electrophoresis. In contrast, techniques like polyacrylamide gels or capillary electrophoresis can resolve alleles that differ by one to a few repeat units or microvariants[12, 23–26]. However, the latter techniques are less suitable for undergraduate laboratory classes as they have many disadvantages including use of hazardous compounds, complex preparation and lack of equipment. For the fragment size determination, special care must be taken when measuring the distance of the migrated DNA fragments. If the bands are too diffuse to be measured precisely, the calculation of the *D1S80* allele fragment size by linear regression may be incorrect resulting in the wrong estimation of repeat unit numbers. In that case a re-run of the agarose gel electrophoresis after optimizing the gel conditions is advisable as previously described by Lorenz and co-workers[22].

The entire *D1S80* VNTR analysis procedure presented here, from buccal epithelial cell harvesting to fragment length evaluation, may be completed in a single working day. This protocol is a robust, cost-efficient and easy-to-use method suitable for undergraduate practical laboratory classes.

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The authors have no conflict of interest to disclose.

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Figure1

Click here to access/download;Figure;fig 1.pdf

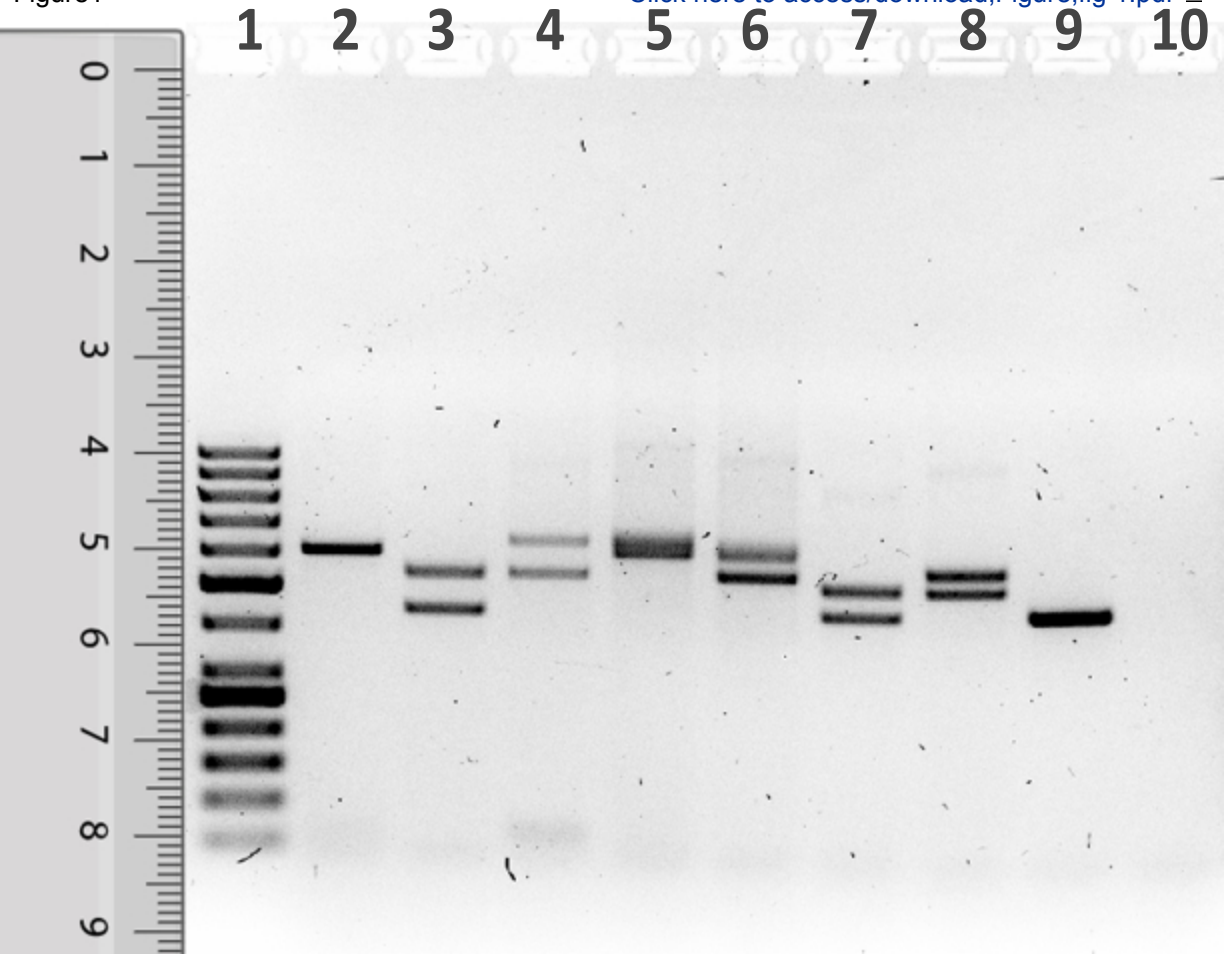
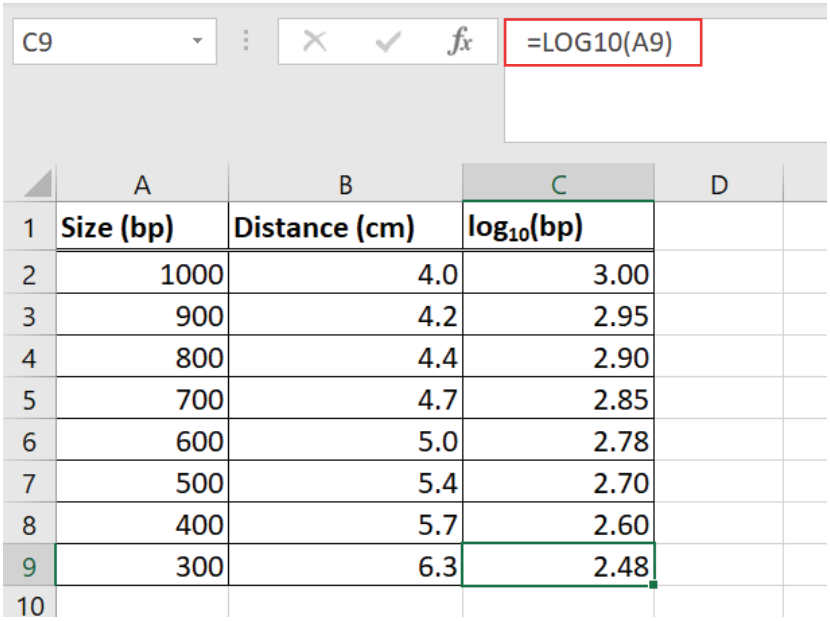


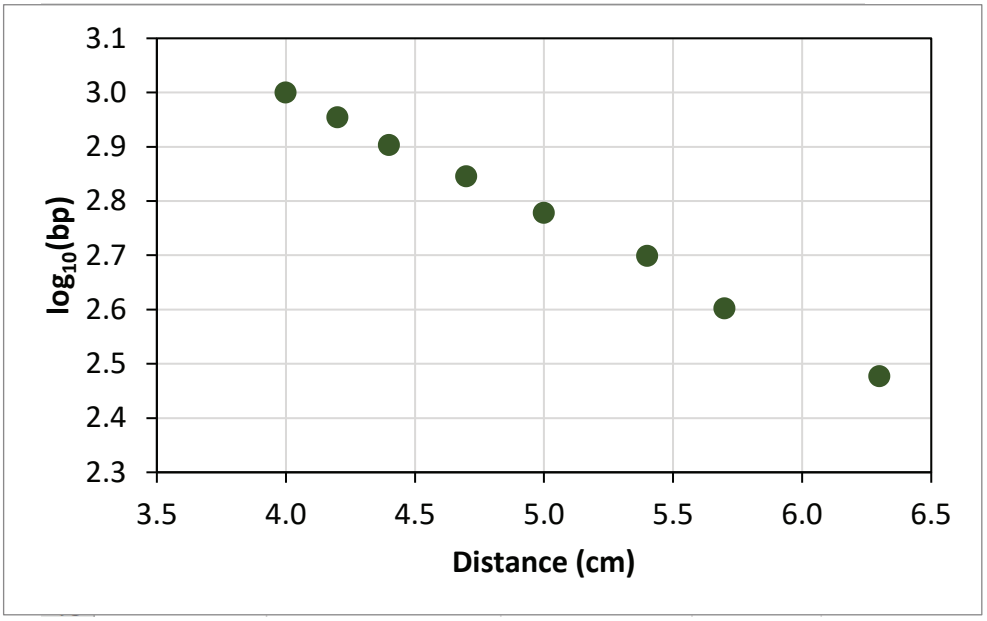
Figure2

Click here to access/download;Figure;fig2.pdf



	A	B	C	D
1	**Size (bp)**	**Distance (cm)**	**$\log_{10}$(bp)**	
2	1000	4.0	3.00	
3	900	4.2	2.95	
4	800	4.4	2.90	
5	700	4.7	2.85	
6	600	5.0	2.78	
7	500	5.4	2.70	
8	400	5.7	2.60	
9	300	6.3	2.48	
10				

Figure3



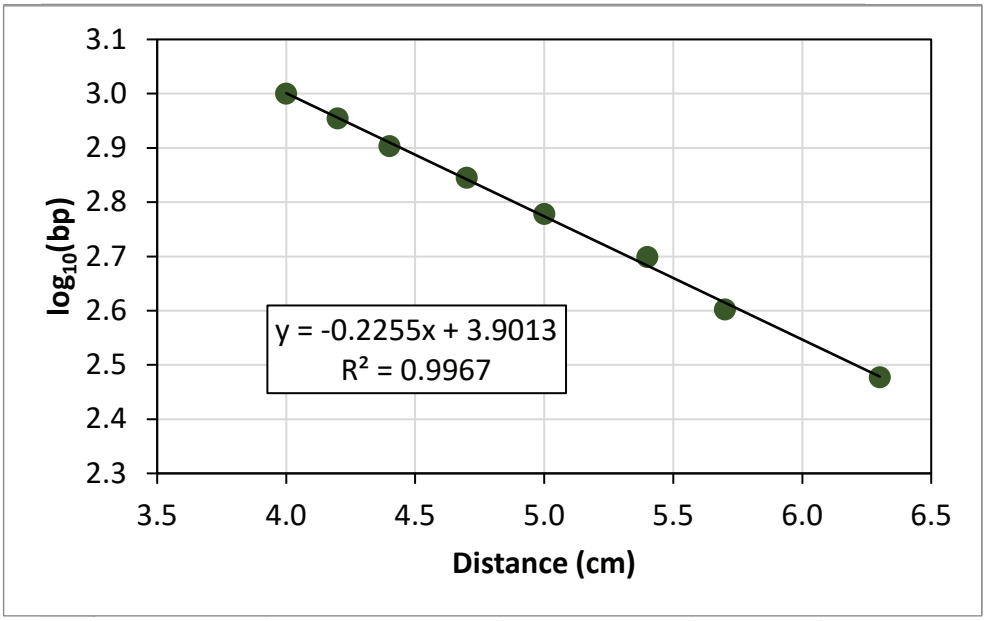
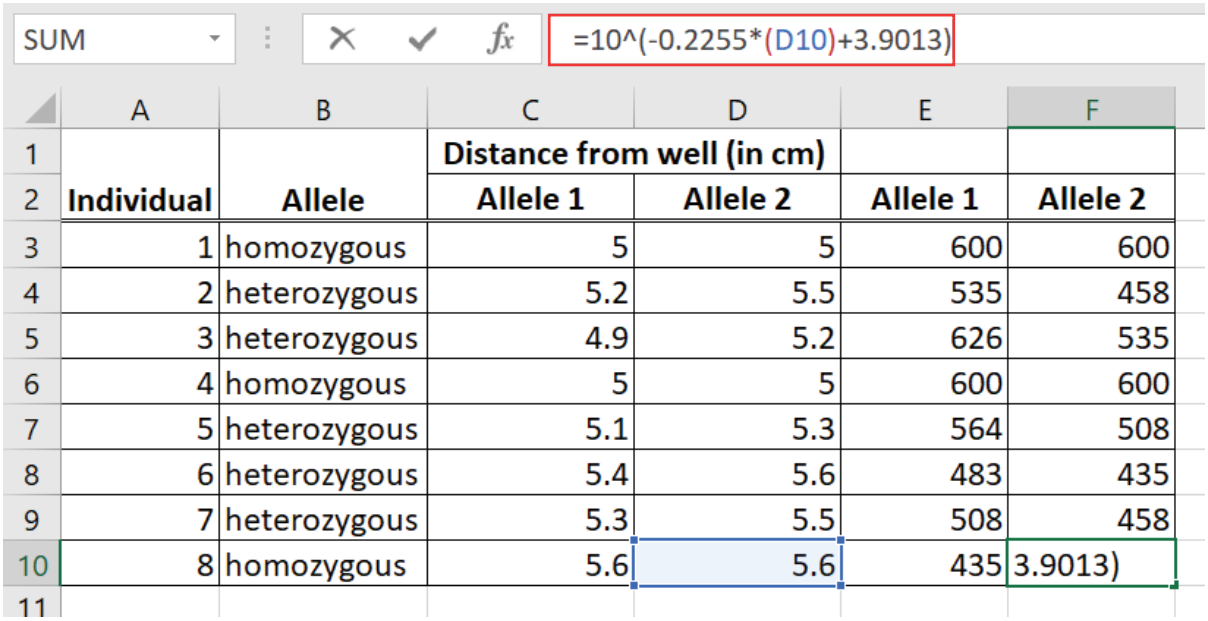


Figure5

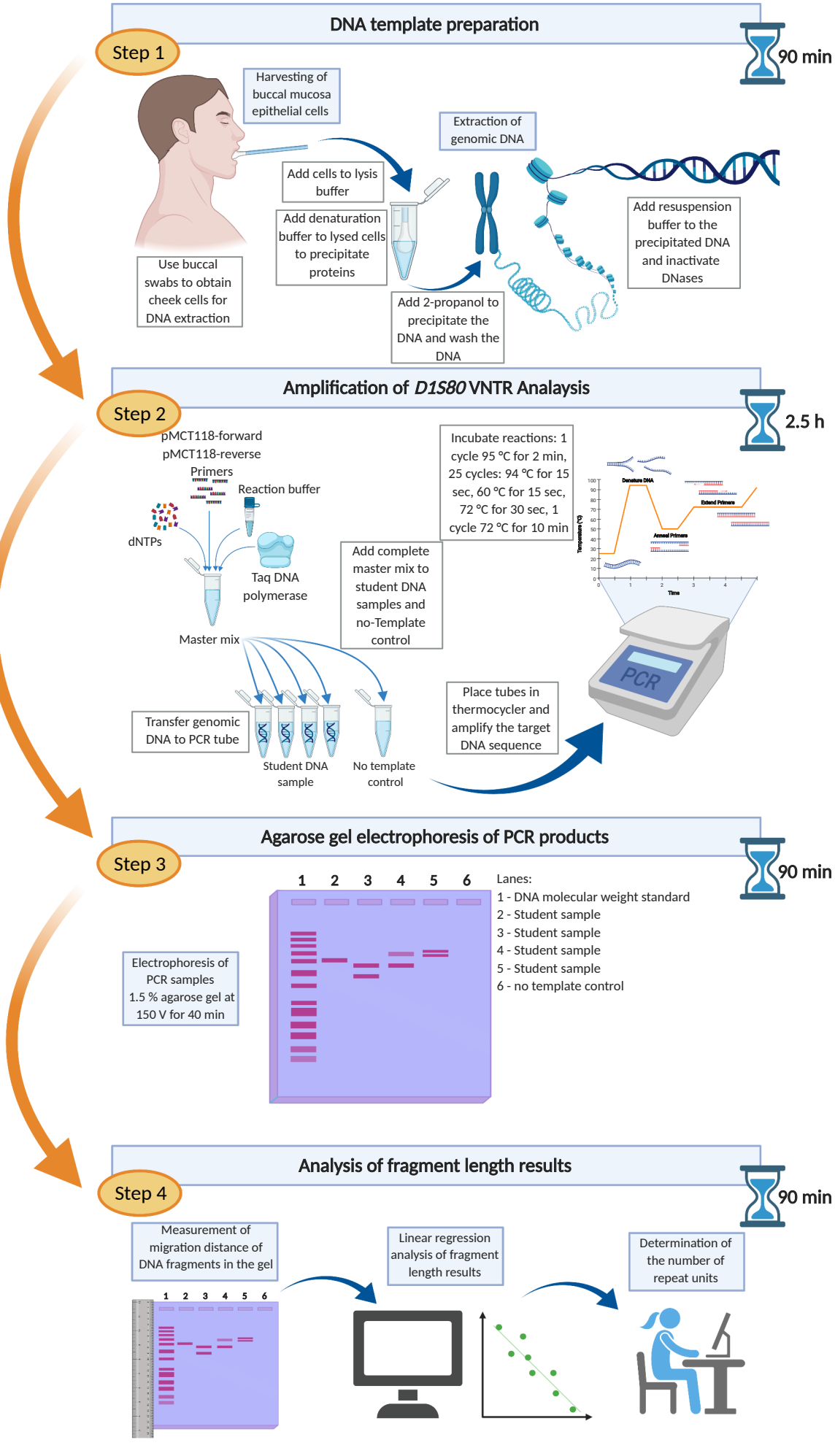
Click here to access/download;Figure;Fig5.pdf



	A	B	C	D	E	F
1			Distance from well (in cm)			
2	**Individual**	**Allele**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
3	1	homozygous	5	5	600	600
4	2	heterozygous	5.2	5.5	535	458
5	3	heterozygous	4.9	5.2	626	535
6	4	homozygous	5	5	600	600
7	5	heterozygous	5.1	5.3	564	508
8	6	heterozygous	5.4	5.6	483	435
9	7	heterozygous	5.3	5.5	508	458
10	8	homozygous	5.6	5.6	435	3.9013)
11						

Figure6

Click here to access/download;Figure;Fig6.pdf



Allele size (in bp)	Number of repeats
369	14
385	15
401	16
417	17
433	18
449	19
465	20
481	21
497	22
513	23
529	24
545	25
561	26
577	27
593	28
609	29
625	30
641	31
657	32
673	33
689	34
705	35
721	36
737	37
753	38
769	39
785	40
801	41

Individual	Allele	Distance (in cm)	Size (in bp)	Number of repeat units
1	homozygous	5	600	28
2	heterozygous	5.2 ; 5.5	535, 458	24, 20
3	heterozygous	4.9 ; 5.2	626, 535	30, 24
4	homozygous	5	600	28
5	heterozygous	5.1 ; 5.3	564, 508	26, 23
6	heterozygous	5.4 ; 5.6	483, 435	21, 18
7	heterozygous	5.3 ; 5.5	508, 458	23, 20
8	homozygous	5.6	435	18

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL microcentrifuge tube	Sarstedt	72,706	autoclaved
2 mL microcentrifuge tube	Sarstedt	72,695,500	autoclaved
2-propanol	Fisher chemical	PI7508/17	
5x PCR buffer	Promega	M7845	
Acetic acid	Sigma/Merck	A6283-500ML	
Agarose	BioBudget	10-35-1020	
Buccal swabs	BioBudget	57-BS-05-600	
Centrifuge	Eppendorf		5430
Combs	BioRad		
dNTPs (10 mM)	Invitrogen	R0192	
EDTA	Carl Roth	8043.1	
Ethanol	Honeywell	32221-2.5L	
Gel caster	BioRad		
Gel chamber	BioRad		SubCell GT
Gel Doc XR+	BioRad		
Geltray	BioRad		SubCell GT
GeneRuler 50 bp DNA Ladder	Thermo Fisher	SM0371	
Go-Taq Polymerase	Promega	M7845	
Heating block	Eppendorf	Thermomixer	1.5 mL and 2 mL
Microwave	Sharp		R-941STW
peqGreen	VWR	732-3196	
Pipettes	Gilson		1000 µL, 200 µL, 20 µL, 2 µL
Potassium acetate	Arcos Organics	217100010	
PowerPac power supply	BioRad		100-120/220-240V
Primers	Sigma/Merck		
Scale	Kern		PCB 2.5 kg
SDS	Arcos Organics	218591000	
Shaker	IKA labortechnik		IKA RH basic
Tabletop centrifuge	myFuge		
Thermal Cycler	BioRad		C100 Touch
Tris	VWR	103156x	

Vortex mixer	LMS	VTX-3000L

Heinrich Heine Universität Düsseldorf

We thank the reviewers for their constructive criticism. We greatly appreciate your time and efforts to thoroughly review the manuscript. Please find below our detailed response to the specific recommendations of the reviewers as well as to the editorial and production comments (both in **bold**) in blue. An electronic version of the revised manuscript is being uploaded on the JoVE website, along with a marked-up version in which all revisions have been noted using ‚track changes'.

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: We have carefully proofread the manuscript and corrected all spelling and grammar issues.

2. Please revise the title to be more concise: Molecular Analysis of the Human D1S80 Minisatellite Locus from Buccal Mucosa Epithelial Cells
Please reflect the revised title in the video as well.
Response: As suggested, the title has been revised to make it more concise. The title was changed to ‚A Guide for the Application of DNA Fingerprinting Using the Human *D1S80* Locus in Lab Classes' in the written manuscript as well as in the associated video.

Changes to be made by the Author(s) regarding the video:
1. The institutional citation numbers are floating too high above the text. Consider bringing them down closer to the letters.
Response: As suggested, the institutional citation numbers in the title card were brought down closer to the letters.

2. 01:14 Hold on this close up of the oral swab for another moment so we can acknowledge it as viewers.
Response: We froze the frame for several seconds.

3. 01:06, 03:16, 04:00 The chapter title cards should fade out to the next shot just as they fade in from the previous shot."
Response: We agree and changed the fading effect accordingly.

4. Please upload a revised high-resolution video here: https://www.dropbox.com/request/KKe3dHJ9hiA4HZTnAkwW?oref=e
Response: The video in its revised version has been uploaded using the above mentioned link. The video file is called "JoVE62305_revised.mp4".

Reviewers' comments

Heinrich Heine Universität Düsseldorf

Reviewer #1

Manuscript Summary:
The manuscript by Siebers et al. is interesting in that it describes in detail the methodology for VNTR fingerprinting of human individuals during a university lab class and numerical interpretation of the results. I guess the main novelty of the final publication will be the associated video.

Reviewer's comments to the Author:

Major Concerns:

(1) The main question is about the novelty of this report. The paper by Jackson et al. 2006 seems to describe pretty much the same lab class (as far as I can see from the abstract, I have no access to the full paper). But there are no shared authors between the two papers. So, I shall leave the decision on whether the limited originality of this MS is enough for publishing by JoVE to the journal's editors.

Response: As far as we know novelty is not a requirement for publishing in JoVE. In our article we focused on scientific validity and a high degree of usefulness to the scientific community with respect to educational purposes.

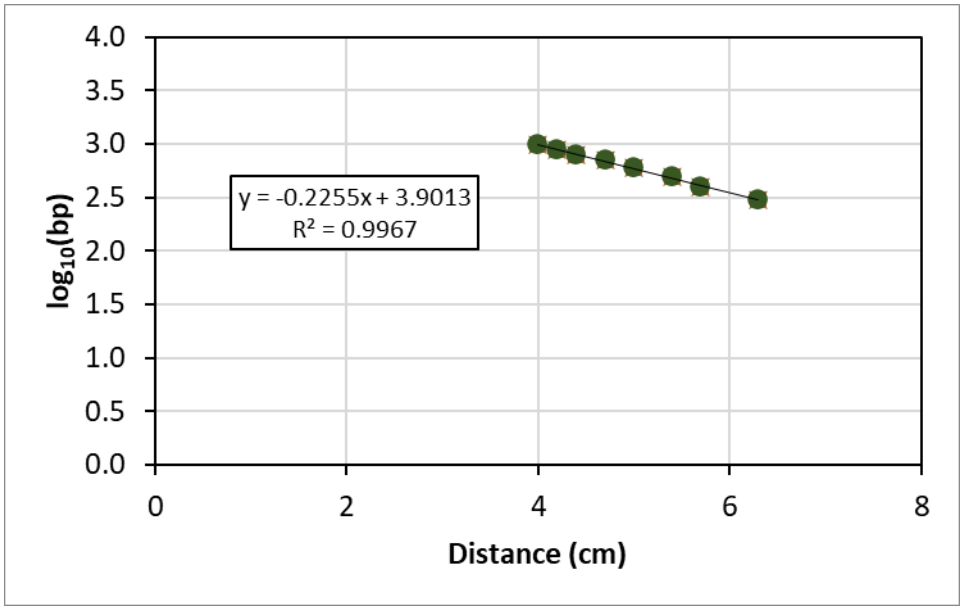
Minor Concerns:

(2) Since human fingerprinting is mainly applied to forensic matters, better attention should be devoted to procedures used to keep contamination risks as low as possible: gloves and possibly masks and hair-caps should be worn; thoroughly cleaned (or sterilized) scissors should be employed to cut swab stems; solutions should be filter-sterilized and/or autoclaved.

Response: To address this comment, a short section was added concerning the risk of sample contamination during VNTR analysis. Refer to L113-117 in the revised version.

(3) There's a problem with Figure 5: the line depicting the regression results shown in the picture doesn't match the equation. Something wrong happened and needs to be corrected.

Response: We did not find any error in our regression equation. However, we see why this impression can arise from looking at Figure 5. Both axes in Figure 5 do not start from 0, but rather show a zoom into the plotted data. We replotted the data by rooting the graph in (0,0), demonstrating that our equation and especially the y-intercept are correctly calculated. Nevertheless, we decided against replacement of Figure 5 with the new one shown below as the data points and the regression line are not so well recognizable.



(4) I introduced a series of corrections and suggestions to the text in order to make it clearer and easier to read. Some better care with spelling and more generally the English language would be expected...
Response: We greatly appreciate the effort and integrated the corrections and suggestions to the manuscript. The manuscript has been thoroughly proofread, sincerely hoping to meet with your requirements.

(5) I would use italics for locus/loci, but I am not sure about JoVE's policy. I also think that dNTPs should be spelled out on first occurrence.
Response: Referring to JoVE's policy we italicized all Latin words and nomenclature in our manuscript. The term locus/loci is derived from Latin but has been incorporated in the English language. Therefore, we did not italicize this term in accordance with the way JoVE handled this issue in the past.
As suggested, dNTPs were spelled out on first occurrence. Refer to L208 in the revised version.

(6) Three reference are written as First Author et al. in the reference list. I am not sure about JoVE's policy: they need to be checked out. There seems to be some more general inconsistency in the reference style in the list.
Response: We reassured with the Jove style guide that for six or more authors only the first author's name followed by et al. should be listed in the references. This is the case for the three references Reviewer #1 has pointed out. Detailed review of our reference list did not reveal any inconsistency as far as we are aware.

Reviewer #2
Manuscript Summary:
This manuscript provides an excellent example of using PCR to genotype a highly-variable

minisatellite in humans. It is well-written, with the protocol well-explained, and it will be very useful in teaching genetics to students. Thanks to the authors - I am thinking about how to incorporate this kind of practical study in our own curriculum!

Reviewer's comments to the Author:
I have no concerns.

Response: We appreciate the comment from the Reviewer #2 and wish our best for incorporating this protocol in the classroom.

Reviewer #3
Manuscript Summary:
Overall very good procedure for application to an undergraduate class. Well thought out and with potential to have success.

Response: Thank you.

Major Concerns:
none

Reviewer's comments to the Author:

Minor Concerns:
(1) Should include a positive control on the extraction and that was performed by the instructor and including in the downstream steps. Since these are students, they may not be able to successful perform the different steps in the procedure so if you end up with no product at the end, would be hard to pinpoint if an error occurred at the sample collection, extraction, or making/running/visualization of the gel steps.
Response: As suggested, we have inserted a specific note in the protocol section to include a positive control that was performed by the instructor. Refer to L126-127 in the revised version.

(2) I would also recommend adding to the classroom discussion about non-consensus alleles (microvariants) as they are common at most loci and with the low resolution offered with the agarose gel (as opposed to polyacrylamide gels or capillary electrophoretic).
Response: We have added information about non-consensus alleles in the discussion section as suggested by reviewer #3. It now reads:

It is worth mentioning that not all alleles for an VNTR locus contain complete repeat units. Non-consensus alleles (microvariants) that contain incomplete repeat units are common at most VNTR loci and their sizes fall in between sizes of alleles with full repeat units. These microvariants are barely detectable by agarose gel electrophoresis. In contrast, techniques like polyacrylamide gels or capillary electrophoresis can resolve alleles that differ by one to a few repeat units or microvariants[12, 23–26]. However, the latter techniques are less suitable for undergraduate laboratory

classes as they have many disadvantages including use of hazardous compounds, complex preparation and lack of equipment.

Reviewer #4

In this study, the authors report A Simple Method for the Molecular Analysis of the Human D1S80 Minisatellite Locus from Buccal Mucosa Epithelial Cells in Practical Laboratory Classes.

This method not really original because already described by many publications and private companies or structure which produces peadogocigal tools. The protocol is given for schools in the same objectives, here are some examples:
https://www.bioutils.ch/protocoles/10a-la-pcr-locus-d1s80
https://worldwidescience.org/topicpages/v/vntr+locus+d1s80.html
ttps://www.minipcr.com/product/minipcr-forensics-lab-d1s80-vntr/
http://babec.org/wp-content/uploads/2016/02/D1S80_Teacher.pdf

The Cold Spring Harbour Laboratory has already described this for High School, an Undergraduate by a 48-minute video available on YouTube
https://dnalc.cshl.edu/resources/dnalc-live/watch/20200515-forensic-dna-profiling-part-i-17196

Nevertheless this article is well written, clear and also pedagogical. To be more original and to fit more with the regulation should be completed by specific chapter on the regulations and safety procedures. So for this article I suggest:

Reviewer's comments to the Author:

Major remarks:

(1) A chapter in which the authors define the regulation to use this experiment European and specific regulation concerning the use of DNA, the destruction of the sample, analysis,
human biological sample should be considered as a human sample according to the regulation of each country .

Response: As suggested, we discussed the guidelines and regulations for human sample management in the European Union. Refer to L411-421 in the revised version. It now reads:

To screen for genetic variation at the *D1S80* locus, human biological sample collection along with DNA extraction and analysis is required. It is essential that the ethical use of human biological specimens is ensured throughout the whole process. Sample management is controlled within a comprehensive regulatory framework which ensures the correct use of samples and associated data[18], *e.g.* consent for the use of human biological material. Participants must be properly informed about the use of their samples, the risk of discovery of anomalies in genetic relations (*e.g.* for related individuals), privacy protection and intentions for future storage of the biological specimens and data. All donors (students or colleagues) must give consent freely and understand the right to withdraw without giving reason. In general, it is indispensable to make oneself familiar with the respective guidelines and

regulations for human sample management before conducting this lab class.

(2) This protocol is forbidden to be used par persons from the same family, whatever the degree of kinship (especially if it is parent/child or brother/sister) because it can reveal anomalies (example paternity problems as describe in the summary).
Response: We agree that greater emphasis should be placed on the legal basis especially when it comes to such confidential data. We included a note in the protocol section to stress that this analysis can only be performed if the student and/or legal guardian agrees to the analysis. Refer to L110-111 in the revised version. Further, we included a section in the discussion referring to the guidelines and regulations for human sample management and on this occasion mention again, that students (and/or legal guardians) must consent to this analysis before conducting this class (L411-421 in the revised version).

Minor remarks:
(3) A note should be added concerning safety measure concerning the use of the different reagents. In this period due to the COVID, this protocol cannot be used because it uses saliva.
Response: We totally agree. When working with students we are very anxious to reduce risk by replacing materials, chemicals, equipment and methods with something that is less hazardous. For this specific lab class we reduced risk by replacing ethidium bromide with PeqGreen, the avoidance of organic solvents for DNA extraction, the preference of agarose gel electrophoresis over other techniques that require the use of toxic compounds.
We included a note to stress that it is necessary to familiarize with the safety data sheet of the single chemicals used and to take the respective precautions. Refer to L148-149 in the revised version.
Furthermore, in L122-124 we added a note that working with saliva and epithelial cells can lead to a transmission of infectious diseases.

(4) There is not a real conclusion in this article
Response: The conclusion of this article is that it represents a practicable protocol for DNA fingerprinting for (undergraduate) lab classes which is using cost-efficient chemicals and which can be performed within one day. We further show representative results and give insights on how to interpret or discuss the data (allele frequencies and shared alleles). The goal is to motivate students to think about the results and convey the technique of DNA fingerprinting.

Technical remarks:
(5) Lines 158, 167, 174
The centrifuge steps to be more easily used
17950 should be 18000
17450 should be 17500 or better 18000,
for pedagogical reason easier applicable.
These differences are not important for this protocol

Response: A suggested, the centrifugation steps were rounded up to 18000 x g to make it easier applicable.

(6) Line 232
NOTES: PeqGreen Prefer less toxic and should be considered as a CMR
Response: We reassured that PeqGreen is used in accordance to the Regulation (EC) No 1272/2008 [CLP] of the European Parliament and the Council of 16 December 2008 on classification, labeling and packaging of substances and mixtures. It is classified as not harmful and is therefore not subjected to any labeling obligations of the particular national legislations.

(7) Concerning the protocol PCR:
Line 214
the information does not fit with the figure 7 steps 2 (30 cycle/ 25 cycles) and in order to optimize the results generally no less than 35 cycles are used.
Response: We were not able to find any inconsistency in cycle number either in the manuscript, video or in Figure 7. According to our specific experimental settings, best results were obtained by repeating the PCR extension step 25x times. But we agree, the PCR conditions should be adjusted with respect to the particular settings used. We adapted this chapter to make it more generally valid. It now reads:

NOTE: PCR conditions given in this protocol were optimized using the Go-Taq® DNA Polymerase (Promega) and the C1000 Touch PCR thermal cycler (Bio-Rad Laboratories). In general, PCR conditions must be adapted to the DNA polymerase. The standard extension time for a Taq DNA polymerase is 1 min/kb.

(8) Some technical detail seems not applicable in reality.
Elongation step for fragments which are longer than 500 pb 30 sec is not enough (using a standard polymerase 1000 nuc/sec).
Response: We fully agree that the elongation step must extend 30 sec for fragments longer than 500 bp using a standard Taq polymerase. Due to the restricted time quota during the lab class we tested different elongation times and number of extension cycles beforehand and chose the experimental setup shown in the manuscript obtaining best results in a short period of time. We adapted this chapter to make it more generally valid (see response above).

(9) In this protocol it will be nice to have a positive control to validate the PCR (a positive control for example from previous PCR).
Response: As also noted by reviewer #3 we included a respective note. Refer to L126-127 in the revised version.

(10) lines 332 to 342
In representative results, this part must be totally rewritten because it is not the aim to find likehood between individuals, in a wrong or bad context, it could be wrong interpreted.

Heinrich Heine
Universität
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Response: To address this comment, we included a section on allele frequencies in the results section. These allele frequencies can be used further to compare to allele distributions of different human populations (L353-362 in the revised version). We are also very cautious about the interpretation of likelihood between individuals and stress that students and legal guardians have to give consent to perform this protocol (L110-111, L411-421).

Conclusion:
This article and video not really original, but it could be published and presented if this document integrates more the regulations and ethics points concerning the use of DNA and biological samples and more focus on ethics on the exploitation of the results. So in this case, with these explanations it will be the original major point of this article.