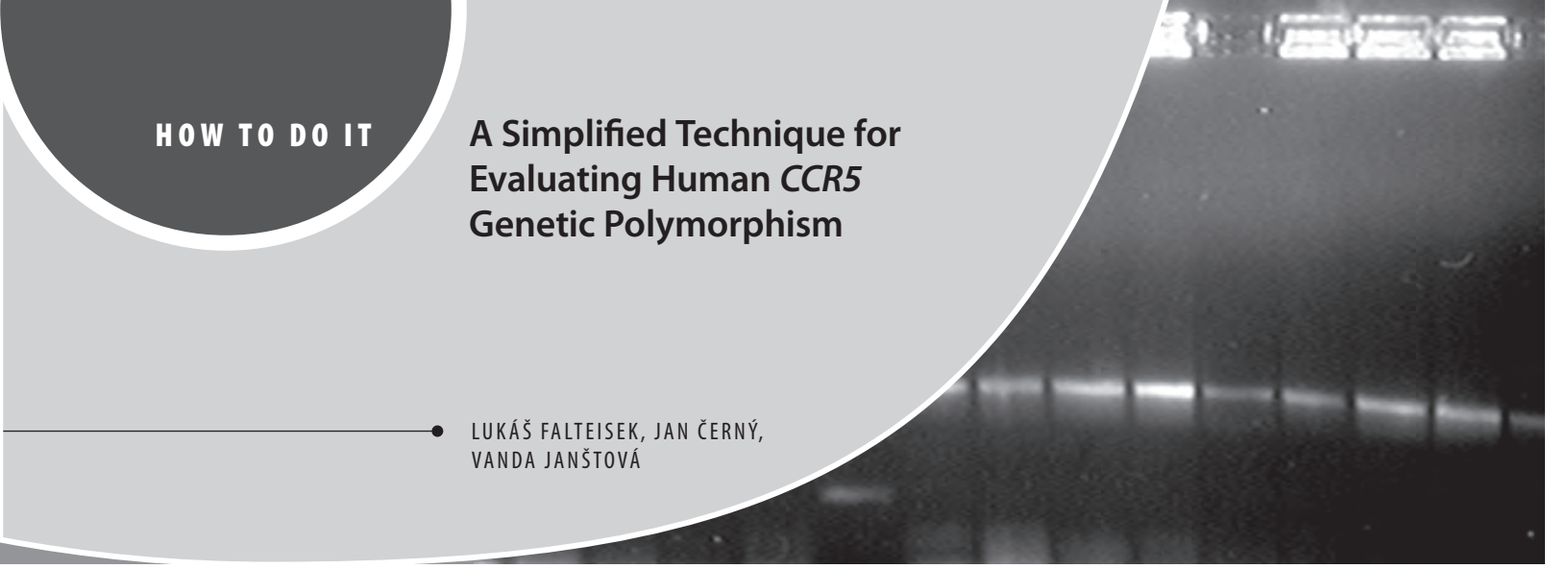
HOW TO DO IT

A Simplified Technique for Evaluating Human *CCR5* Genetic Polymorphism



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Abstract

To involve students in thinking about the problem of AIDS (which is important in the view of nondecreasing infection rates), we established a practical lab using a simplified adaptation of Thomas's (2004) method to determine the polymorphism of HIV co-receptor CCR5 from students' own epithelial cells. CCR5 is a receptor involved in inflammatory processes, which has been misused by some pathogens, including HIV, to enter host cells. As a result, a defective allele CCR5-Δ32 *has been enriched in some populations. The interesting story and hands-on work with their own tissue absorbed students in this 2-hour practical.*

Key Words: *PCR; polymerase chain reaction; CCR5; HIV resistance; genotyping; polymorphism; secondary school.*

Acquired immunodeficiency syndrome (AIDS) develops as a result of infection by human immunodeficiency virus (HIV), a virus that destroys various cells of our immune system. These cells are targeted because they express CD4, a membrane receptor primarily involved in recognizing the foreign, potentially dangerous elements in our bodies. HIV uses CD4 as its main receptor to infect the cells. Interestingly, CD4 is necessary but not sufficient to mediate virus entry.

Rare individuals had been repeatedly exposed to HIV but remained uninfected. Some of them were shown to have inherited a defective allele of *CCR5* (C-C chemokine receptor type 5 gene). CCR5, which was identified as a major co-receptor for HIV-1 (Choe et al., 1996), belongs to a family of receptors for chemokines – structurally related peptides that recruit leukocytes to inflammatory lesions, induce release of granule contents from granulocytes, regulate integrin avidity, and, in general, exhibit proinflammatory properties. CCR5 is predominantly expressed on T cells, macrophages, dendritic cells, and microglia (Blanpain et al., 2002). It interacts with its chemokine ligands RANTES, MIP-1β, and MIP-1α.

During the long-term interaction of human populations with particular pathogens, a variant, *CCR5*-Δ32 (a deletion of a 32-bp segment resulting in a nonfunctional receptor, unable to promote HIV-1 entry), has spread in some populations (Samson et al., 1996). *CCR5*-Δ32 is found in 5–14% of Europeans but is rare in Africans and Asians (Lucotte & Mercier, 1998). Plague and smallpox, which use CCR5 to enter host cells, have been proposed as pathogens driving the natural selection of the *CCR5*-Δ32 allele. This could explain the high occurrence of the mutation in the European population (Galvani & Slatkin, 2003). Individuals with the *CCR5*-Δ32 allele are healthy, without any obvious phenotype, which suggests that *CCR5* is largely dispensable. The interesting story behind CCR5 and *CCR5*-Δ32, together with a lack of pathological phenotype, makes this molecule an optimal educational marker (Thomas, 2004).

We wanted to get young people involved in thinking about HIV and asking nontrivial questions about AIDS. Because we believe that experimental work designed to deal with a particular topic leads to deeper understanding and long-term memory (Freedman, 1997; Thompson & Soyibo, 2002), and that this is dramatically enhanced when people's own body, experience, or memories are involved, we established a practical task – a simplified method of Thomas (2004), which allows students to determine the polymorphism of the HIV co-receptor CCR5 from their own epithelial cells (safe biological material).

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Our adaptation skips the DNA isolation step, making it a cheaper, faster, more reproducible method, suitable for a variety of teaching institutions. It can be accommodated in the typical time scheduled for practical courses at various schools.

Materials

Each student needs

- 1.5-mL microtube for collecting the buccal cells
- A toothpick or a teaspoon as a scraping tool
- 0.2-mL PCR microtube (the type depends on the cycler used)
- 2–200 µL micropipette tip

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Students will share

- 2–20 µL (or equivalent) micropipette(s)
- PCR premix – prepare as described in the Procedure section while students scrape their epithelia, and divide into PCR microtubes (48 µL per tube).
- 3% w/v agarose gel (prepare while PCR is running – or in advance – and store at 4°C) in TBE buffer: 89 mM Tris. HCl; 89 mM boric acid; 2 mM EDTA; pH 8.0. Add 3 g of agarose for every 100 mL of this buffer, and dilute GelRed 10,000 times in the gel. Count one well per sample (per student) and one for the DNA ladder for every 10 samples. It is important to use an appropriate DNA ladder that covers 100-bp and 200-bp lengths (see Procedure).

○ Safety Issues

Because collecting buccal cells using a toothpick is noninvasive, the only safety risk is using ultraviolet (UV) light. However, used toothpicks should be disposed of and destroyed so that they are not touched by another student. Always use a UV shield when analyzing the results, or take a picture of the gel and discuss the results in the picture. A nontoxic DNA stain (e.g., GelRed and Biotium) should be used. Alternatively, other, less sensitive DNA stains like crystal violet can be used to omit the UV excitation.

○ Procedure

Students took their own cheek epithelial scrapes (a wooden toothpick or a teaspoon were used as a safe and cheap tool) at least 1 hour after eating (to minimize the amount of irrelevant material). By rubbing the toothpick against the edge of the tube, they transferred the scraped material into a 1.5-mL microtube and then shook it down to the bottom of the tube by tapping. Using a 2–20 µL micropipette, they transferred ~2 µL of this sediment into a 200-µL microtube containing the polymerase chain reaction (PCR) premix. We strongly recommend checking to make sure there is opaque material of the right volume in the micropipette tip of each student. Some students may have more saliva in their samples, which is not a problem, as long as they take the opaque material from the bottom of the tube. The technique is robust enough to accommodate differences in the amount of entry material. The PCR premix was prepared by the teacher as follows: 1U LA polymerase (Top Bio), dNTP 100 µM each (Fermentas), 1 µL DMSO (Top Bio), 1× LA polymerase buffer (Top Bio), primers 50 pmol of each, distilled water to the total volume of 50 µL. We used primers described by Nischalke et al. (2004) synthesized by Sigma-Aldrich, 0.025 µmol, purification: desalt (orders can be made at http://www.sigmaaldrich.com/configurator/servlet/DesignTool?prod_type=STANDARD).

F: 5′ CAAAAAGAAGGTCTTCATTACACC 3′ (630-653 bp on the *CCR5* gene)
R: 5′ CCTGTGCCTCTTCTTCTCATTTCG 3′ (795-818 bp on the *CCR5* gene)

Immediately after mixing the PCR premix with the scraped epithelia, the samples were randomized; unlabeled properly locked tubes from all students were collected into one bag and scrambled.

Table 1. PCR was run as follows. Steps 2a to 2c were repeated 35 times.

Step	Temperature (°C)	Time
1. Initial denaturation	95	5 minutes
2a. Denaturation	95	20 seconds
2b. Annealing	60	20 seconds
2c. Elongation	68	20 seconds

See Table 1 for the PCR procedure. PCR resulted in an amplification of two products: 189 bp representing the wild type allele and 157 bp representing the *CCR5*-Δ*32* allele.

PCR products were mixed with a BFB sample buffer (50% glycerol; 0.13M EDTA; 0.12% bromphenol blue; pH 8.0), analyzed by agarose gel electrophoresis at 17 V/cm), and visualized by GelRed (Biotium, UV light 312 nm). We used GeneRuler DNA Ladder Mix (ready-to-use; Thermo Fischer Scientific, Waltham, MA) to determine the lengths of the PCR products.

○ Results

We tested the reproducibility of the simplified method with 200 students (48 from high schools and 152 from the Charles University in Prague – undergraduate level). Out of 200 students, 174 obtained the PCR product. As expected, most (116) of the students were wild type homozygotes (66.7%). We found three (1.7%) homozygotes for *CCR5*-Δ*32* polymorphism and 55 (31.6%) heterozygotes. All students scraped the epithelial buccal cells and added them to the PCR premix themselves, loaded the samples into agarose gels, evaluated "their" polymorphism (note that there was the randomization step at the beginning, ensuring that, although the samples belonged to the students from the group, none of the students were able to identify their own sample), and discussed the results and the background theory. We observed an extremely high interest during the whole procedure, which took 2 h in total (scraping the cells and PCR premix preparation 20 min, PCR and gel preparation 60 min, loading and running agarose DNA electrophoresis 30 min, followed by the discussion of the results). PCR offered a time gap to discuss the relevant virology, immunology, and other biology topics. Figure 1 shows results of six randomly chosen groups of students.

We performed this practical lab not only in the laboratory environment, but also in typical classrooms and at a summer camp. We tested the robustness of the technique and tried different PCR cyclers to scale down the cost. We conclude that the technique works well with all cyclers tested, even with the cheapest one on the local market (Thermal cycler BIOER TC-24/H, price $2300).

○ Considerations

We evaluated the technique on a large group of students and concluded that identification of the polymorphic status was possible in 87% of samples. A minority of PCR reactions (13%) failed to give a detectable product. This can have several reasons. Some of the students were not able to scrape enough epithelia or transfer it to the PCR vial. Also, there are the known polymorphisms *Y176C* and *T177A* in the genomic region complementary to the 5′ end of our

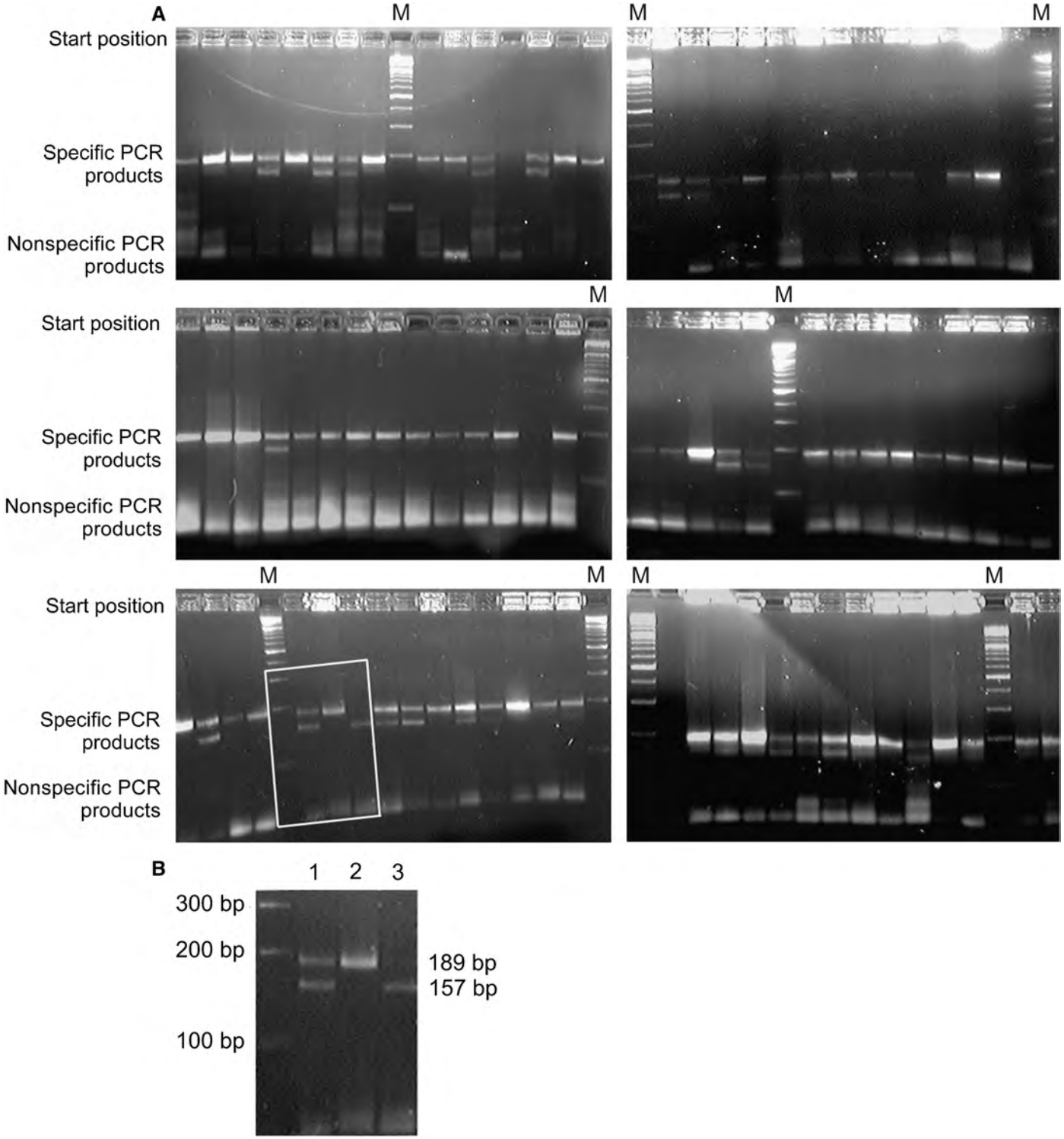

Figure 1. (A) An example of six typical experiments. Note that at least one heterozygote was identified in each group. There is genomic DNA visible in some wells at the loading site. The position of molecular weight markers is marked "M" in each gel. (B) Part of a gel (highlighted by a rectangle in A), which shows in lane 1 a heterozygote with two bands – 198 bp representing the wild type allele and 157 bp representing the *CCR5-Δ32* allele; in lane 2 a wild type homozygote; and in lane 3 a *CCR5-Δ32* homozygote.

forward primer (Zhang et al., 1997). Different scraping techniques were tested on several volunteers. These tests showed that careful scraping is a critical step for the effectiveness of the method. For some people, it is easier to obtain enough epithelial material from a lingual scrape, which is also a good template for PCR. The sample should not contain larger compact epithelium particles. Centrifugation of the scraped material is not recommended. The frequency of the *CCR5* truncated form gives, for a typical group of students (15–25),

an almost 100% chance of identifying a polymorphism among tested individuals (at least in the European population and emigrants from Europe; Lucotte & Mercier, 1998).

The major advantage of choosing *CCR5* as a gene of interest is the fact that every student has met the issue of HIV and AIDS, and the topic is extremely important epidemiologically. In our experience, students easily understood the aim of this practical course. However, it is crucial to help students interpret the results of the genotyping properly. The important fact, which must be stressed several times, is that being a *CCR5*-Δ*32* homozygote does not mean being resistant to HIV, which can use various co-receptors to enter the host cell. For example, the syncytial strains of HIV-1 and HIV-2 use another chemokine receptor, CXCR4, as a co-receptor (Zhang et al., 1997). Thus, the *CCR5*-Δ*32* allele does not provide complete resistance to HIV infection, not even for homozygotes. Students should not conclude that they do not have to protect themselves against HIV infection. Also, they should be reminded of other sexually transmitted diseases. The main problem with many genotyping experiments is that students might obtain unwanted delicate information about their possible health risks. We have chosen the *CCR5* gene because there is no evidence of any disease associated with *CCR5*-Δ*32* in humans (although more severe progress of some experimental infections in mice has been reported; Glass et al., 2005). Moreover, we randomized the samples as described above, which meant that students were working with a particular sample, not knowing whom in the group it belonged to. This enabled them to analyze and discuss the results of the whole group without the problem of making wrong conclusions about being immune to an HIV infection. It also helped to attract the students to the general issues (e.g., population genetics and the application of the Hardy-Weinberg equilibrium to allele frequencies identified in a particular agarose gel).

○ Conclusion

Our simplified protocol makes the identification of *CCR5* polymorphism cheap and high-throughput, independent of the laboratory environment, and therefore useful for providing this exciting knowledge to large numbers of students.

It gives an excellent starting point for topics like

- genetics of populations (the Hardy-Weinberg law);
- spreading of alleles;
- mutations providing some positive effect as well as a negative one (compare with sickle-cell anemia);
- a drug and therapy design (some promising anti-HIV drugs in clinical trials block CCR5; Hütter et al., 2009);
- transplantation of peripheral blood stem cells from a homozygous *CCR5*-Δ*32* donor to an HIV-1 positive patient, who had no viral rebound 20 months after the transplantation and the end of antiretroviral therapy; and
- an overall discussion of PCR.

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Resources

http://evolution.berkeley.edu/evosite/relevance/IA2HIV.shtml

http://youtu.be./9leO28ydyfU

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