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Dear Student,

In this issue of the journal we offer two reserach articles. The first article, *The Spring Force Constant and the Coefficient of Kinetic Friction Determination* by Zobon et. al, is the work of a group of students that describes an experiment conducted in their Physics class. The second article, *Construction of the Disruption Casette For the Deletion of the EST3 Gene in. C. parapsilosis* by Molina and Steinberg, is the result of research conducted by Mr Alejandro Molina, a former Hostos student, during his summer research internship at the Weill Cornell Medical College.

We hope that you will enjoy both of these articles, and follow these authors in sharing your reaserch experiences on the pages of the *Hostos Journal of Students Research*.

Editorial Board

A Physics Lab Experience: The Spring Force Constant and the Coefficient of Kinetic Friction Determination

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ABSTRACT

A body is elastic if it returns to its original shape and size after being deformed. A spring can be defined as an elastic member, which exerts a resisting force when its shape is changed. For elongations that are not too great, it has been observed that when a spring is stretched the applied force is directly proportional to its elongation. This relationship is well understood as Hooke's law. This study focuses on determining the force constant of three different springs with different stiffness as well as the coefficient of kinetic friction between two surfaces; wood on lead. The stiffer the spring is the bigger is its force constant. The coefficient of kinetic friction is universal and depends only on the surfaces that are in contact, no matter what force you apply.

INTRODUCTION

A spring can be defined as an elastic member, which exerts a resisting force when its shape is changed. A body is elastic if it returns to its original shape and size after being deformed (1). This study focuses on generating a model relating the force applied to a spring and the distance the spring stretches from its original length, or rest length. This relationship is well understood as Hooke's law (1) and it states that the elongation of a spring is proportional to the applied force. The spring will return to its rest length when the force is removed so long as the elastic limit has not been exceeded. This proportionality constant is called force constant (k). The main focus of this work was to determine the force constant of three different springs with different stiffness. Also, by using the force constant for two of the springs, we will determine the coefficient of kinetic friction between two surfaces.

PART I: THE FORCE CONSTANT DETERMINATION

The objective of this physics project was to determine the force constant (k) of three different kind of springs.

Method: Apparatus

5 different masses, 3 springs, a spring holder and a meter rule.

Experimental procedure

The spring was placed on a vertical holder. Different bodies with different masses were placed on the spring and the displacements from the initial position to the final position were measured. There are only two forces acting on the body attached to the spring. They are the elastic force and the gravitational force. Newton's second law was used to calculate the force constant (Figure 1).

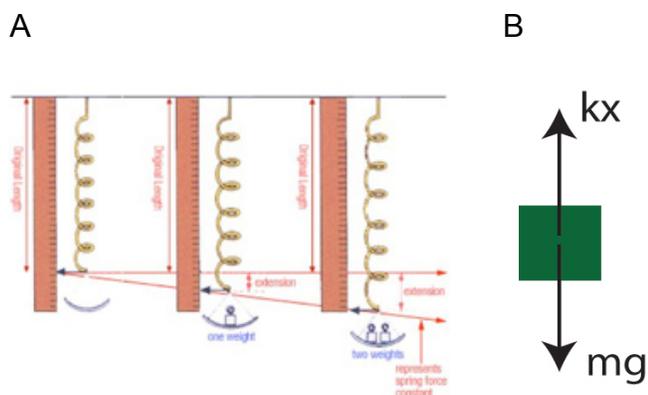


Figure 1. A body attached to a hanging spring.*

A) A body is suspended from the spring. It is in equilibrium when the upward force exerted by the stretched spring equals the body's weight. B) Free-body diagram for the body.

*Courtesy of www.ncsu.edu/PER/SCALEUP/LabSample.pdf (2)

Results

The two forces acting on the body attached to the spring, the elastic and gravitational forces, are shown in the free-body diagram in Figure 1B. The body suspended from the spring is in equilibrium when the upward force exerted by the stretched spring equals the body's weight (Newton's first law of motion). Mathematically, this can be expressed as

$$\sum_{i=1}^2 F_i = F_{\text{elastic}} + (-w) = 0 \quad (1)$$

where F_{elastic} represents the elastic force; $F_{\text{elastic}} = kx$, where k is the force constant and x is the stretched distance. w stands for the weight of the bodies (different masses) placed on the spring. After substituting the expression of elastic force into Eq. 1, we obtained the following equation.

$$w = mg = kx \quad (2)$$

This equation (Eq. 2) shows that the force constant (k) can be determined from a linear relationship between the body's weight (w) placed on the spring and the corresponding stretched distance. If we plot weight (w) as a function of displacement (x), the slope of the straight line provides the force constant.

Spring 1

Experiment Run	m (kg)	w (N)	x (m)
1	0.020	0.196	0.006
2	0.040	0.392	0.010
3	0.050	0.490	0.014
4	0.075	0.735	0.021
5	0.100	0.980	0.028

Table I. Mass, weight and displacement for *Spring 1*

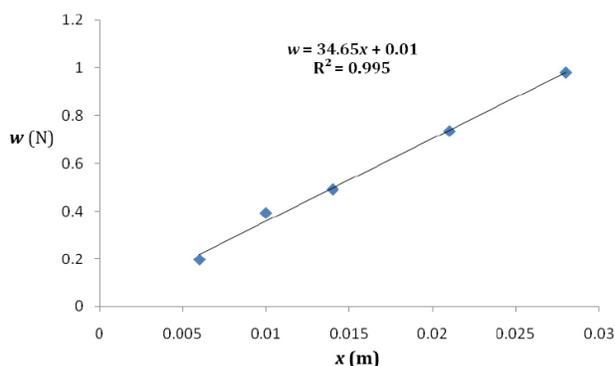


Figure 2. Weight as a function of displacement for *Spring 1*

Spring 2

Experiment Run	m (kg)	w (N)	x (m)
1	0.005	0.049	0.002
2	0.010	0.098	0.017
3	0.015	0.147	0.045
4	0.020	0.196	0.072
5	0.025	0.245	0.099

Table II. Mass, weight and displacement for *Spring 2*.

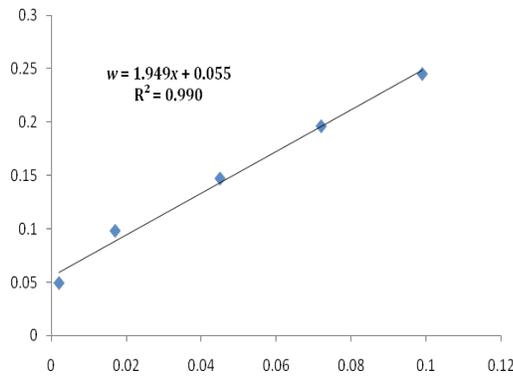


Figure 3

Weight as a function of displacement for *Spring 2*

Spring 3

Experiment Run	m (kg)	w (N)	x (m)
1	0.005	0.049	0.009
2	0.010	0.098	0.031
3	0.015	0.147	0.060
4	0.020	0.196	0.091
5	0.025	0.245	0.121

Table II. Mass, weight and displacement for *Spring 3*

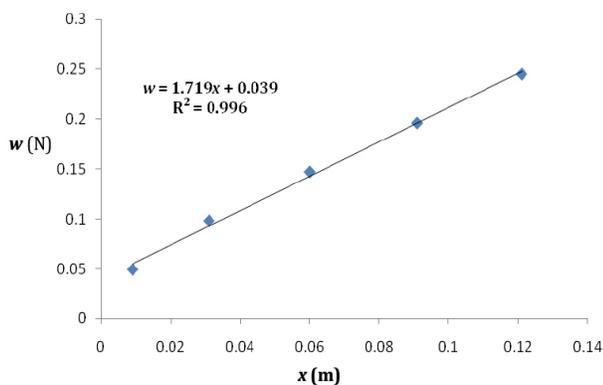


Figure 4.

Weight as a function of displacement for *Spring 3*

Discussion

Through an extrapolation of the linear fit we observed that the value of the intercept is small but non-zero, although we expected it

to be exactly zero according to Hooke's law. We attribute this to experimental error. For the three different springs the correlation coefficients were close to 1, $R^2 = 0.995, 0.990, 0.996$, respectively, which means that our experimental values fit very well to the straight-line relationship given by Eq. 2 (Figures 2 - 4). The force constant values for the three springs (labeled 1, 2 and 3) were 34.65 N/m, 1.949 N/m, and 1.719 N/m, respectively, which were in correspondence with their stiffness.

PART II: THE COEFFICIENT OF KINETIC FRICTION

The purpose of the second part for the physic project was to calculate the coefficient of kinetic friction (μ_k) between two surfaces: wood and lead.

Method: Apparatus

An inclined plane, 2 springs, a lead and a meter rule.

Experimental procedure

The inclined plane was set to an angle of 25° . The lead was tied to the spring which was then placed at the top of the inclined plane (Figure 5A). There are three forces acting on the lead. They are the gravitational force, the frictional force and the elastic force. The lead was given an initial velocity down the inclined plane and finally came to rest. The displacement from the initial position to the final was measured. This was done 25 times for spring 2 and 12 times for spring 3.

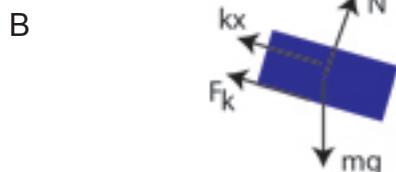
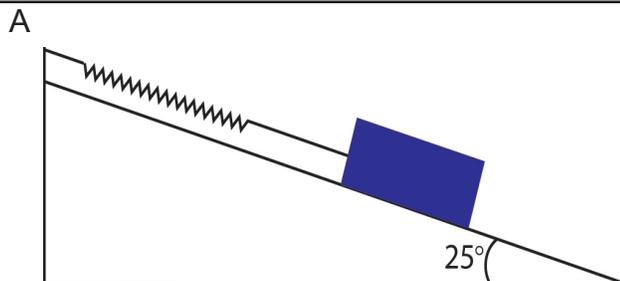


Figure 5. A body attached to a spring on the top of an inclined plane. A) A body is suspended from the spring. It is in equilibrium when the force exerted by the stretched spring and the friction force equal the component of the body's weight on the -x direction. B) Free-body diagram for the body.

By applying Newton's second law of motion to the body attached to the spring placed on the top of an inclined plane as shown in Figure 5A the coefficient of kinetic friction between two surfaces; wood and lead, can be determined. The forces acting on the body attached to the spring on the horizontal (parallel to the inclined plane -x direction) and the vertical (perpendicular to the inclined plane -y direction) directions are depicted in Figure 5B. The net force on the body in the -x direction is zero since the system is on rest when the maximum elongation of the spring is measured. In the -y direction there is no motion. Mathematically, this can be written as:

$$\sum_{i=1}^4 F_{xi} = w_x - F_{elastic} - F_k = 0,$$

$$mg \sin \alpha - kx - \mu_k N = 0, \quad (3)$$

$$\sum_{i=1}^2 F_{yi} = N - w_y = 0,$$

$$N - mg \cos \alpha = 0, \quad (4)$$

$F_k = \mu_k N$ where represents the friction force, and μ_k and N are the coefficient of kinetic friction and the normal force, respectively. w_x and w_y stand for the body's weight components on the -x and -y directions, respectively.

If Eq. 4 is solved for N and it is substituted into Eq. 3, μ_k turns out to be:

$$\mu_k = \frac{mg \sin \alpha - kx}{mg \cos \alpha}. \quad (5)$$

Data: Spring 2

$m = 0.343 \text{ kg}$

$k_2 = 1.949 \text{ N/m}$

inclined angle = 25°

Table 1 lists the values of the coefficient of kinetic friction for each of the trials measuring the elongation of the spring.

Experiment Run	x (m)	μ_k
1	0.40	0.21
2	0.27	0.29
3	0.34	0.25
4	0.29	0.28
5	0.35	0.24
6	0.43	0.19
7	0.30	0.27
8	0.31	0.28
9	0.48	0.16
10	0.40	0.21
11	0.27	0.29
12	0.28	0.29
13	0.32	0.26
14	0.31	0.28
15	0.43	0.19
16	0.40	0.21
17	0.43	0.19
18	0.43	0.19
19	0.49	0.15
20	0.27	0.29
21	0.31	0.28
22	0.54	0.12
23	0.33	0.25
24	0.53	0.13
25	0.43	0.19

Table 1.

Elongation and coefficient of kinetic friction for *Spring 2*.

This experiment led to an average coefficient of kinetic friction and standard deviation of

$$\overline{\mu_k} = \frac{\sum \mu_k}{25} = 0.22 \quad (2).$$

Data: Spring 3

$m = 0.343 \text{ kg}$

$k_s = 1.719 \text{ N/m}$

inclined angle = 25°

In Table 2 the elongations and their corresponding coefficient of kinetic friction are listed.

Experiment Run	x (m)	μ_k
1	0.39	0.25
2	0.39	0.20
3	0.47	0.20
4	0.48	0.25
5	0.38	0.27
6	0.35	0.24
7	0.40	0.22
8	0.43	0.23
9	0.41	0.23
10	0.42	0.23
11	0.33	0.28
12	0.44	0.22

Table 2.

Elongation and coefficient of kinetic friction for Spring 3.

Discussion

The project was performed with a careful observation and performance and therefore, there should be just minimal errors. Errors in this experiment could be attributed to the non-uniformly polished surfaces of the inclined plane and the object. The mean value of the coefficient of kinetic friction resulting from each spring, 2 and 3, experiment is $\overline{\mu_k} = 0.2(1)$, which is in a good correspondence with the accepted value described in the literature (*wood on metals* $\mu_k = 0.2 - 0.6$) (3).

CONCLUSIONS

This project allowed us to put in practice Newton's laws of motion, especially the second law. We determined the force constant through Hooke's law with a high degree of accuracy. The stiffer the spring is the bigger is its force constant. These experiments also helped us to understand the concept of the friction, especially the kinetic friction. We learned from this project that the coefficient of kinetic friction is universal and depends only on the surfaces that are in contact, no matter what force you apply.

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Construction of the disruption cassette for the deletion of the *EST3* gene in *C. parapsilosis*.

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Telomeres, the nucleoprotein structures located at the very ends of a chromosome, play an important role in preserving genome integrity. Telomeres are maintained by a specialized enzyme complex called telomerase. This article describes construction of a disruption cassette to be used in the study of a protein component of telomerase complex called Est3p.

Preservation of genome integrity through multiple rounds of DNA replication is one of the major tasks facing all living cells. With the advent of knowledge of the mechanism of DNA replication came realization that this process contains an inherent paradox, the so-called DNA end-replication problem. It was found that DNA polymerases, the enzymes that play a major role in DNA replication, require a preexisting strand of nucleic acid, the so-called primer, to initiate synthesis of a new DNA molecule. Upon completion of DNA replication the primer is removed, leaving the very end of linear DNA molecule unreplacated (Fig 1).

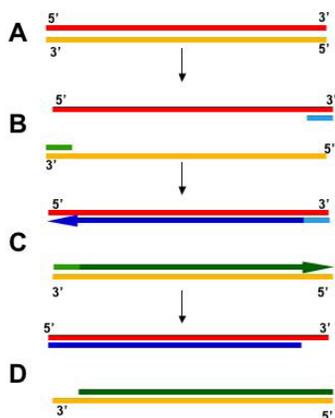


Figure 1. DNA replication

A. A double-stranded DNA molecule. B. Two strands of the DNA molecule are separated (DNA is denatured), and short RNA primers (blue and green lines) bind to 3'-end of each strand. C. Primers are elongated by DNA polymerase, complementary DNA strands are synthesized, DNA molecule is replicated. D. Primers are removed, one of the 3'-ends of each of the daughter molecules remains unreplacated

This condition, if not remedied, leads to chromosome shortening, and, eventually, loss of genetic information over subsequent rounds of cell division (6, 11). The solution to the end-replication problem was discovered in 1985, when C. Greider and E. Blackburn isolated an enzyme capable of nucleotide addition to the 3'-end of the linear DNA molecule (Fig 2). The activity isolated from the ciliate *Tetrahymena* was capable of elongating the very ends of chromosomes, or telomeres, and therefore was named telomerase (4). Since then telomerases have been identified in almost all eukaryotic organisms revealing the role of these proteins in preserving genome integrity of eukaryotic cells. Loss of telomerase leads to progressive telomere shortening, genome instability, cell senescence and, eventually, cell death (5).

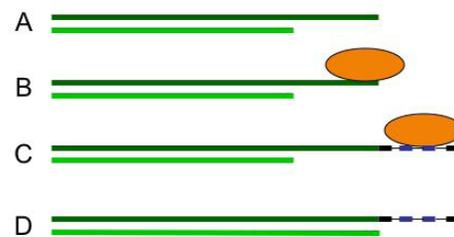


Figure 2. Action of telomerase.

A. Replicated DNA molecule with the 3' single-stranded overhang. B. Telomerase complex (orange oval) binds to the 3'-end overhang. C. Telomerase extends 3' single-stranded overhang (short black and blue lines represent nucleotides). D. DNA polymerase replicates 3' end of the single-stranded DNA. DNA molecule is now of the same length as a parental molecule.

The function of telomerase is not limited to telomere elongation: telomerase is also involved in

telomere capping/protection and regulation of gene expression. Telomerase activity itself is tightly regulated in all eukaryotic organisms. Both abnormal increase and decrease in the levels of telomerase activity have been linked to a number of adverse human conditions, such as dyskeratosis congenita, premature aging and cancer (3).

Telomerase activity is regulated on several different levels: transcriptional level, telomerase complex assembly level, cell cycle and recruitment to telomeres levels. Recently, it became clear that there exists one more level of telomerase activity regulation: a post-recruitment activation level. That means that recruiting, or bringing telomerase to telomeres is not enough to activate telomere elongation by this enzyme. It was proposed that enzymatic properties of telomerase are activated by telomerase complex components after telomerase was brought to telomeres. Research into this level of telomerase activity regulation has just begun, and many aspects of it still remain unclear (8).

The catalytic function of telomerase in yeasts is carried out by Est2p, a protein capable of nucleotide addition to the 3' end of the single-stranded DNA. For this activity Est2p needs a template, which is provided by specialized RNA called Tlc1. Tlc1 is associated with Est2 protein as its RNA subunit. Loss of either *EST2* or *TLC1* genes results in complete loss of telomerase functions both in vivo and in vitro. Two additional proteins, Est1p and Est3p, are physically associated with Est2p/Tlc1 in majority of yeast species. Both Est1p and Est3p were implicated in the regulation of telomerase activity. Loss of function of either of these proteins results in complete loss of telomerase function in cells. The nucleoprotein complex thus comprising Est1p, Est2p, Est3p and Tlc1 is usually referred to as the telomerase complex (5, 7).

In mammalian cells, a protein called TPP1 was found to have a structural and functional similarity to yeast protein Est3. These findings indicate that Est3-like proteins may be an important component of telomerase in a number of different species. EST1-like genes were also found in

mammalian cells, but there is no indication that their functions in higher eukaryotes are similar to the ones Est1p has in fungal cells (9, 11, 12).

Genomic analysis of fungus *C. parapsilosis* revealed the presence of the *EST3* gene, but not *EST1*. This discovery pointed to the possibility that telomerase regulation in *C. parapsilosis* may be closer to that in mammalian cells than in other fungi, and opened up a whole new avenue of investigation into telomerase regulation using this genetically tractable fungus. However, because investigations of telomerase regulation in *C. parapsilosis* (Cp) have only begun recently, the precise role of CpEst3 in telomerase regulation in this organism is not known yet. As a first step in this investigation, a *C. parapsilosis* Est3 deficient strain needs to be constructed and analyzed.

This work describes construction of a disruption cassette to be used for the deletion of *EST3* gene in *C. parapsilosis*. Construction of the plasmid will pave the way to the construction of the *C. parapsilosis* est3 mutant.

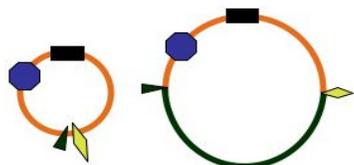
Materials and Methods.

pCD8 plasmid vector.

The disruption cassette used in this work was carried on a plasmid vector called pCD8, which contained a chloramphenicol resistance gene for the selection of transformed *E. coli* cells (2). The disruption cassette contained CaSAT1 gene (a nourseothricin resistance gene) under the constitutive actin promoter (CpACT1p). Expression of CaSAT insures transformant resistance to the antifungal drug nourseothricin, and thus allows for selection of transformants on media containing nourseothricin. The cassette also carried CaFLP gene under inducible maltose promoter (CpMAL2p). CaFLP gene encodes yeast recombinase protein called flippase. This protein is capable of binding to FRT DNA sequences and excising the DNA fragment located between two such sequences. In the disruption cassette, the two FRT sites surrounded the CaSAT1 gene sequence. Growth of transformant on maltose

containing medium would activate flippase expression and promote excision of antibiotic resistant gene from transformants genome. Loss of CaSAT gene will make transformants sensitive to nourseothricin, and thus allowed for the use of the same cassette for the disruption of the second allele of the gene (Fig. 3)

A



B

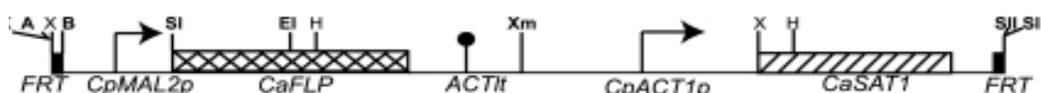


Figure 3. Disruption cassette and cloning into the plasmid vector.

A. Cloning of the disruption cassette into the plasmid vector. Orange circle is a plasmid vector. The blue octagon represents the site of the specific plasmid replication genes, the black bar represents the site of the antibiotic resistance gene, the green arrowhead indicates the Kpn1 restriction site, the yellow diamond indicates the Sac1 restriction site. The disruption cassette (green semicircle) is cloned into the plasmid vector (orange semicircle).

B. Disruption cassette. K and A are Kpn1 and Apa1 restriction sites upstream of the disruption cassette, SII and SI are SacII and SacI restriction sites downstream of the cassette. FRT is a sequence required for the action of CaFLP (flippase). CpMAL2p is a promoter for the flippase (CaFLP) gene, CpACTp is a promoter region for the CaSAT1 (nourseothricin resistant gene). Arrows denote the start of the transcription sites. Si, Ei, H, Xm, X indicate other restriction sites in the cassette (2).

Amplification of DNA sequence upstream and downstream of the EST3.

The 750 bp fragment upstream of EST3 gene sequence was amplified from *C. parapsilosis* genomic DNA using primers derived from the appropriate EST3 sequences. The upstream primer carried Kpn1 restriction site and downstream primer carried Apa1 restriction site. The PCR reaction was performed in accordance with manufacturer instructions. Briefly, PCR reaction contained ~ 400 ng of template genomic DNA from *C. parapsilosis*, 50 ng of each primer, 0.1 mM of the dNTP mix (dCTP, dATP, dGTP and dTTP) in 1 x reaction buffer supplemented with 2 mM MgCl₂ in 50 µl reaction. All ingredients were carefully mixed in 0.5 ml thin walled Eppendorf tubes, and 20 µl of mineral oil was added on top of the reaction mix. Tubes were placed in thermo-cycler machine, and the PCR cycles were set as fol-

lows: 5 minutes at 95°C to denature template DNA, followed by the thermocycle: 94°C for 1 minute, 55 °C for 30 minute, 72°C for 1 minute, for total of 30 cycles. After completion of 30 cycles, the product was allowed to anneal for additional 10 min at 72°C, and reaction was cooled down to 4 °C. The product of reaction was expected to be a 750 bp DNA fragment (Fig. 4 and Fig 6).

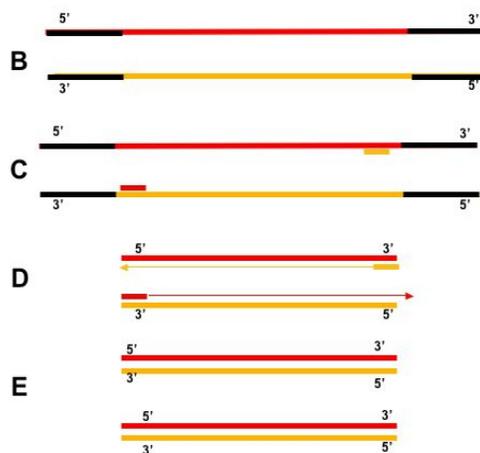


Figure 4. PCR cartoon

A. Double-stranded DNA molecule. Red and yellow strands depict the gene of interest.

B. Double-stranded DNA is denatures.

C. PCR primers (yellow and red bands) bind (anneal) to the respective DNA strands.

D. Primers are elongate in DNA replication reaction.

E. Multiple copies of the desired DNA fragment are produced.

Agarose gel electrophoresis.

Molecular weight of different DNA fragments is compared by separation in the agarose gel under electric field. This process is known as agarose gel electrophoresis. Agarose is a polysaccharide that when melted and allowed to solidify after forms a mesh. DNA molecules are negatively charged and move in the electric field in the direction of the positive electrode. DNA molecules applied into the gel are forced to move through the mesh under the force of the electric field. Since the mesh of the agarose gel retards larger molecules more than the smaller ones, DNA fragments are separated in the agarose gel according to their molecular weight. A DNA marker, which is a commercially available mix of DNA fragments of the known size, is usually loaded next to an experimental sample. Comparing position on the gel of the experimental sample band with the position of the makers' band allows estimation of the molecular weight of the sample DNA fragment (Fig. 5).

Purification and restriction digest of the PCR product.

To purify 750 bp DNA fragment from the PCR mix, 50 µl of reaction was loaded on 0.7% agarose gel and electrophoresed for 2 hours at 100 volts. 750 bp DNA fragment was cut out from the gel and purified from agarose. The fragment was further digested with Kpn1 and Apa1 restriction enzymes. First, the DNA fragment was digested with Apa1 at room temperature overnight. Protein components of the reaction were extracted by mixing reaction with phenol-chloroform followed by ethanol precipitation with 2 volumes of 100% ethanol and 1/10 volume of 5 M Na Acetate solution, pH 5.2. Precipitated DNA was dried and re-suspended in small amount of water. 1 µl of DNA was loaded on agarose gel to verify amount and molecular weight of a fragment (Fig 6).

Ligation

The 800 bp fragment of EST3 was cloned in

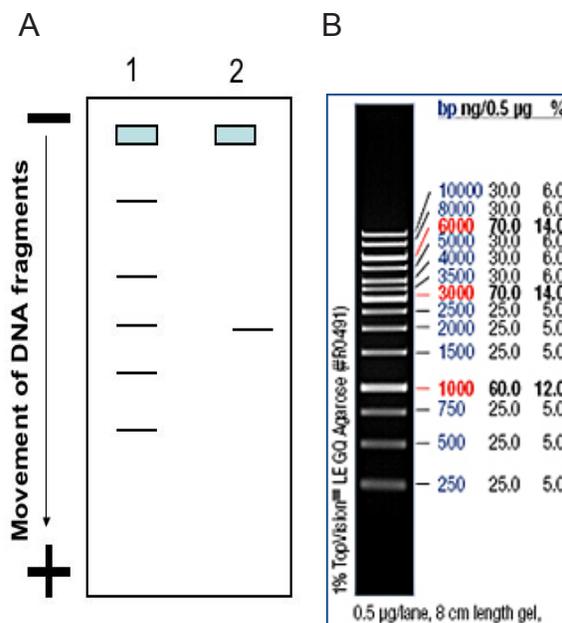


Figure 5. Agarose gel electrophoresis

A. cartoon of the gel electrophoresis. Lane 1 is a DNA marker, lane 2 is a DNA sample.

B. Photograph of the commercially available 1 kb DNA marker after DNA fragments were resolved by gel electrophoresis. Colored numbers on the left indicate molecular weight of the bands in base pairs (bp).

http://fermentas.com/templates/files/tiny_mce/media_pdf/la-baid_generuler_L3G.pdf

pCD8 plasmid earlier, and the new plasmid was named pCD8CpEST3-3'. This plasmid was digested with Kpn1 and Apa1 enzymes, the same enzymes that were used to digest 750 bp PCR product, thus creating compatible DNA ends in both vector (pCD8CpEST3-3') and insert (700 bp fragment of EST3 gene amplified by PCR) (Fig 6). Ligation reaction was performed in accordance with manufacturer instructions (New England Biolabs, Ipswich, MA USA), Briefly, vector and the insert DNA were mixed in 3:1 molar ratio in reaction supplemented with 1 x manufacturers' buffer, 1 mM of ATP and 1 µl of T4 DNA ligase, and incubated at 16°C overnight. It was expected that this reaction would result in insert DNA fragment forming sugar-phosphate bond with the vector fragment of DNA (Fig 6). To verify formation of the plasmid, T4 DNA ligase in reaction was inactivated by incubating the reaction mix for 10 min at 65°C, and 1/10 of the reaction (1.5 µl) was used to transform competent *E. coli* cells.

Transformation

Transformation of the competent *E. coli* cells was performed as follows. 100 μ l of the competent cells were incubated on ice with 1.5 μ l of transformation reaction for 30 minutes, heat-shocked for 45 seconds at 42°C, and chilled on ice for 2 minutes. 900 μ l of nutrient medium SOB was added to transformation reaction and the mixture was incubated for 1 hour in 37°C water bath shaker. After incubation, transformation mixture was plated on LB agar plates supplemented with 20 μ g/ml of antibiotic chloramphenicol and incubate at 37°C overnight.

Verification of the cloning.

To verify that the ligation reaction was successful and that 750 bp fragment was actually cloned into the vector, plasmid DNA was isolated from *E. coli* and subjected to restriction analysis. To generate more copies of plasmid, *E. coli* transformants were first allowed to grow in nutrient medium supplemented with chloramphenicol. One colony of chloramphenicol-resistant transformants was inoculated into 6 ml of LB media supplemented with 20 μ g/ml of chloramphenicol and grown 37°C overnight. Cell culture was collected and used to purify plasmid DNA. Purified DNA was digested by Kpn1 and SacII enzymes (Fig. 8).

Results

Plasmid vector.

Plasmids are double-stranded circular extra-chromosomal DNA molecules that are naturally found in some bacterial and fungal cells. It was found that plasmid DNA is replicated within the cell it inhabits by the cell's DNA-replication machinery, and that plasmid can be transferred from one cell to another through a process called transformation. With the advent of molecular biology, plasmids became one of the widely used tools for cloning DNA. The word "cloning" is used here to indicate that more of the same DNA sequences, or clones, are generated. Plasmids commonly used for cloning are artificially created, and are called cloning vectors. A vector

carries DNA sequences necessary for its replication in a cell, a selection gene that carries a characteristic useful for selection of transformed cells, and cloning sites, which are specific DNA sequences recognized by restriction enzymes, in which foreign DNA of interest can be inserted (Fig 3B). A chimeric plasmid, or a plasmid consisting of part of the original genome combined with the pieces of the foreign DNA, can be introduced into receptive cells, also called competent cells, within which it would be replicated as well as the original native plasmid. Thus more copies/clones of the plasmid containing DNA of interest are produced in cells. Plasmid DNA can be isolated from cells, and DNA of interest can be cut out, or excised, from the plasmid (Fig 3). Thus, many more copies of DNA of interest are obtained. This DNA can be used for many different molecular biology applications, such as sequencing, deletion of a gene from the cell genome, and so on. The plasmid constructed in this work was intended for the deletion of the ESR3 gene from fungi *C. parapsilosis*. It contained the plasmid vector and the EST3 disruption cassette. Generally, a disruption cassette is a fragment of DNA that carries a selection gene flanked by sequences homologous to the 5'-end and 3'-end sequences of the gene of interest (Fig. 3). A disruption cassette is first cloned into the plasmid vector and introduced into the competent *E. coli* cells to produce more copies of a plasmid. Plasmid DNA is then purified from cells, and disruption cassette is excised from the vector. Introduction of a disruption cassette into a fungal cell triggers homologous recombination between the cassette and the gene of interest. Recombination event between the gene of interest and disruption cassette results in the fungal gene replacement by a selection gene carried on the disruption cassette.

A plasmid vector for this work called pCD8 was provided as a gift from Geraldine Butler, UCD School of Biomolecular and Biomedical Sciences, Conway Institute, University College Dublin, Ireland. The plasmid carried a chloramphenicol resistance gene for selection of transformed

in *E. coli* cells (see below) and nourseothricin resistance gene for selection of *Candida* transformants. The plasmid vector features are described in details Materials and Methods section of the paper (Fig 3). The purpose of this work was to insert short DNA fragments homologous to the upstream and downstream regions of EST3 gene into the cassette, so the resulting DNA fragment was capable of recombination with the EST3 gene in the *C. parapsilosis*. The downstream 800 bp fragment of EST3 was already cloned into the vector creating a plasmid pCD8-3'. Insertion of the 750 bp DNA fragment corresponding to the 5' region of EST3 into the pCD8-3' is described below.

PCR.

PCR or Polymerase Chain Reaction is a method of artificial amplification of comparatively short DNA fragments. The advantage of this method is that it allows for amplification of just one specific DNA sequence out of a whole genome in a cell-free environment. Setting up a PCR reaction requires knowledge of the DNA sequence on both sides of the region to be amplified. Once the sequence is known, a set of short single-stranded primers homologous to the upstream (5'-end) and downstream (3'-end) region of the target sequence are designed. The primers are used in DNA replication reaction similar to the one occurring in cells in S-phase of the cell cycle. It requires presence of target DNA, primers, nucleotides and DNA polymerase. A template for the PCR reaction is usually a total genomic DNA, which contains a target sequence. The first step in the reaction is a denaturation or separation of strands in double-stranded template DNA. In the second step of the reaction, primers get annealed, or bind, to the target DNA within the template, and in the third step of reaction the primers are elongated. The process is repeated several more times, or cycles, to yield multiple copies of a target DNA (Fig. 4). PCR reaction was analyzed by gel electrophoresis and the band corresponding to 750 bp was cut out from agarose gel and purified. To visualize DNA fragments in an agarose gel, the gel is usually stained with

Ethidium Bromide. The image of the DNA band can be seen inside the gel upon irradiation of the gel with UV light. However, irradiation of the gel with UV causes DNA-agarose binding, or cross-linking, thus impeding the DNA recovery from the gel. To prevent cross-linking of DNA to agarose gel, we did not take the photograph of this gel electrophoresis until after the PCR band was cut out of the gel. DNA was purified from the gel (see materials and methods). and 1 μ l of the purified DNA was loaded on agarose gel and subjected to gel electrophoresis (Fig. 6).

Restriction digest

Restriction digest is a process of cutting DNA using special enzymes called restriction enzymes. Restriction enzymes are specific, e.g. they cleave double-stranded DNA within a specific DNA content. Thus the restriction digest could be used to analyze DNA sequence. Each restriction enzyme has a name derived from the name of the organism in which the enzyme was first discovered. For example, EcoRI stands for *E. coli* Restriction enzyme I. The upstream restriction sites in the pCD8 -3' plasmid were Kpn1 and Apa1. Kpn1 and Apa1 sites were also created in the 750 bp PCR product that by including specific nucleotide sequences to the primers. As a result, the 750 bp fragment synthesized in the PCR reaction was both similar to the upstream DNA sequence of the *C. parapsilosis* EST3 and contained restriction sites for these two enzymes. Both the PCR fragment (insert) and the pCD8 -3' plasmid (vector) were digested with these Kpn1 and Apa1 enzymes (see Materials and Methods and Fig. 7).

Ligation.

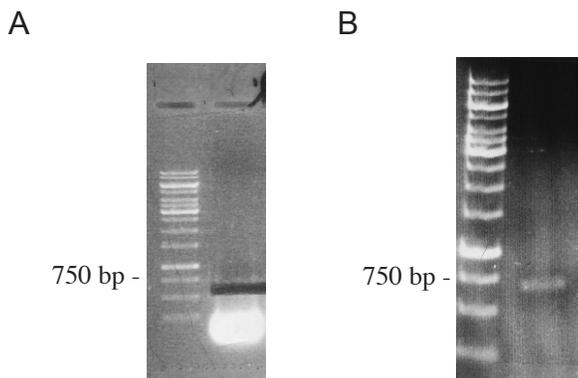
Digestion of two DNA fragments with the same enzymes created compatible or "matching" DNA ends that can be joined together, or ligated, by the DNA ligase enzyme. DNA ligase is an enzyme which catalyze reaction between phosphate group of one nucleotide and sugar of the other, thus creating a sugar-phosphate bond. In ligation reaction, Kpn1 site of the insert was ligated to the Kpn1 site of the vector,

and Apa1 site of the insert was ligated with the Apa1 site of the vector, thus creating a circular DNA molecule (Fig. 7).

Transformation.

Number of DNA molecules in which insert is successfully ligated into vector is rather small, so it is not usually possible to analyze results of ligation without first amplifying amount of the reaction product. This is done by introducing ligation product into bacterial cell, letting cell multiply and replicate plasmid DNA, and then isolating plasmid DNA from bacterial cells.

Transformation is a process of introducing of a plasmid or linear DNA into bacterial or fungal cells. There are some bacterial species that are



capable of accepting foreign DNA into their

Figure 6. PCR results

A. PCR reaction was loaded on the agarose gel and subjected to electrophoresis. DNA band corresponding to 750 bp was cut out of the gel and purified. B. 1 μ l of the purified DNA was loaded on agarose gel and subjected to electrophoresis.

cells naturally. In laboratory conditions, cells are specifically treated to make them receptive to the uptake of foreign DNA. These cells are called competent cells. In this experiment, competent *E. coli* cells were used into which 1/10 of ligation reaction was introduced after the ligase itself was heat inactivated. The pCD8 plasmid vector carried a chloramphenicol-resistant gene, so ensure that only plasmid carrying cell will grow, the growth medium was

supplemented with antibiotic chloramphenicol (See Materials and Methods). Plasmid DNA was purified from overnight culture of transformed cells.

Analysis of the plasmid.

Analysis of the new plasmid DNA was performed by plasmid digestion with Kpn1 and Apa1. Parental plasmids pCD8-3' and pCD8 were also digested with the same enzymes for comparison. It was expected that Kpn1 – Sac II digest of the new plasmids would produce 3.8 kb and 5.75 kb fragments, pCD8-3' digest would produce 3.8 and 5 kb fragments, and pCD8 digest would produce 3.8 and 4.2 kb.

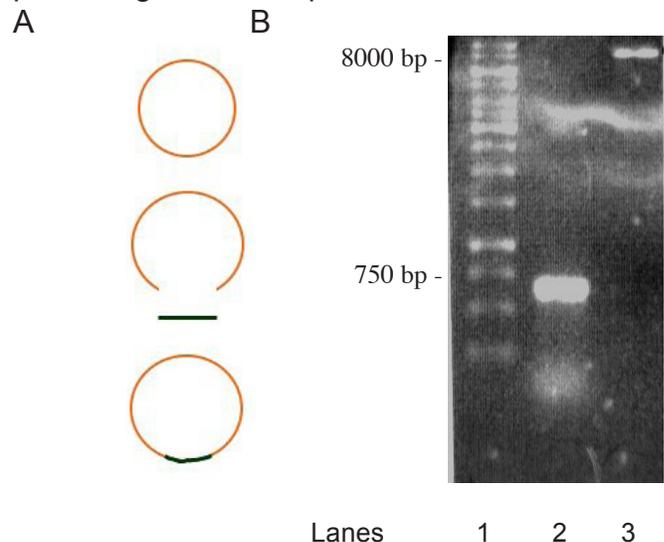


Figure 7. Ligation of Kpn1/Apa1 digested PCR fragment into Kpn1/Apa1 digested pCD8EST3-3'.

A. Cartoon of the ligation. Orange line – vector, green line – insert.

B. Agarose gel electrophoresis of the Kpn1/Apa1 digested insert and vector.

Lane 1 DNA molecular weight marker; lane 2 Kpn1/Apa1 digested PCR fragment; lane 3 - Kpn1/Apa1 digested pCD8EST3-3' Molecular weight of the PCR product is ~ 750 bp, molecular weight of pCD8EST3-3' is 8800 bp

fragments. To compare molecular weight of the restriction fragment, they were separated by gel electrophoresis in agarose gel. Agarose gel was stained with Ethidium Bromide and photographed in UV-light (Fig. 8). As expected, the Kpn1 – SacII of a new plasmid was 750 bp larger than the Kpn1 – SacII fragment of pCD8CpEST3-3'. The new plasmid was named pCD8CpEST3 5'-3'.

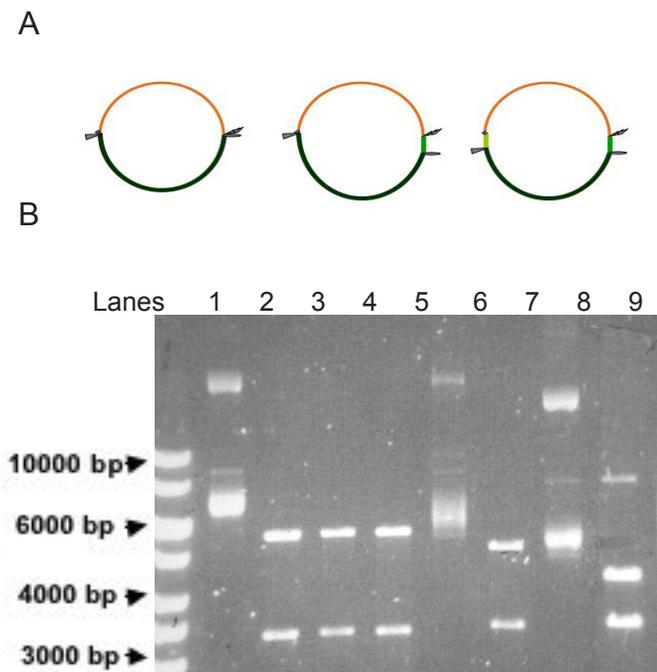


Figure 8. Restriction analysis of the plasmid

A. From left to right: pCD8; pCD8 EST2-3'; pCD8EST3 3'-5'. Grey figures indicate restriction sites. Counterclockwise from the left: rhomboid – Kpn1; arrow – Apa1; oval – SacI; jagged arrow – SacII.

B. Lane 1 – DNA molecular weight marker; lane 2 – pCD8EST3 3'-5' undigested; lane 3-5 – Kpn1/SacII digest of pCD8EST3 3'-5'; lane 6 – pCD8EST3- 3' undigested; lane 7 – Kpn1/SacII digest of pCD8EST3 3'; lane 8 – pCD8EST3 undigested; lane 9 – Kpn1/SacII digest of pCD8EST3.

Discussion.

We described cloning of the DNA t into the plasmid vector. The new plasmid, pCD8CpEST3 5'-3' now contains a disruption cassette that has upstream and downstream regions similar to those of *C. parapsilosis* EST3 gene. The plasmid will be used to delete EST3 gene from *C. parapsilosis* genome. Further steps will include amplification of plasmid DNA, purification of plasmid DNA from bacterial cells, excising the disruption cassette from the plasmid and using it for transformation of fungal cells.

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Front page photographs:

Morphology of M-Ras KO astrocytes. (A) Immunofluorescence analysis of WT (/_/) and M-Ras KO (/_/) astrocytes. Astrocytes are stained with a Cy3-conjugated anti-GFAP antibody (red), and the actin cytoskeleton at the cell periphery was detected with an antiphospho-ERM antibody (green). Two representative astrocytes are shown for each genotype. Magnification, X60.

Courtesy of Nelson Nunez-Rodriguez

Nuñez Rodriguez N., Lee I.N., Banno A., Qiao H.F., Qiao R.F., Yao Z., Hoang T., Kimmelman A.C., and A.M. Chan. 2006
Mol Cell Biol. 26:7145-54.