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Review Article

Identification of Bacterial Species by Combined Bioinformatic and Polymerase Chain Reaction

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ABSTRACT

The identification will be carried out by comparing a potential PCR product obtained from an unknown species with other PCR products specific to 5 known species, using the same five set of pairs of primers specific of these five species respectively. This implies that each PCR product obtained for each species has to be specific to each species and can be considered as a marker in this exercise. This specificity will be based on the uniqueness of the chosen template that is to be used for each PCR. In addition, since the PCR products will not be sequenced, they will be differentiated by their size, which will be identified by agarose gel electrophoresis. The PCR product can only be used as a marker that defines a species if the amplified sequence is unique to this species. Therefore, the first step in this exercise will be to identify a suitable sequence to amplify for each species. Following the identification of a suitable template, the size of the PCR will have to be defined; since the comparative analysis will be based not only on the presence of a product but also its size, all PCR products should have different sizes identifiable on agarose gel. Since the size of a PCR product is defined by the location of primers along the sequence, the second step in this exercise will be to design suitable primers. Finally, having defined specific template and primers for each species, PCR will be carried out using the DNA of unknown species as a template with all five sets of primers so that a successful PCR product and its size would identify the unknown species as one of the five species.

Key words: PCR, Primer, Marker.

INTRODUCTION (Dale J W et al., 2012)

To design suitable primers for identification, the following procedure will be followed:

1. Definition of the five marker organisms
2. Identification of the availability of their genome
3. Definition and design of primers

1) Definition of the five marker organisms

The five organisms to which the unknown organism will be compared are:

Pseudomonas fluorescens (1), E. Coli (2), Micrococcusluteus (3), Bacillus subtilis (4) and Bacillus cereus (5)

2) Identification of the availability of their genome

Since in this instance the PCR product has to be specific to the species, the gene sequence chosen as a template has to be unique to this species. Although the gene of a protein that would be unique to each species would be ideal, in practice such a gene is not always easy to identify; genes of proteins which are conserved and found in several species might be suitable as long as the degree of identity is low enough so that the PCR products can be specific of the species. In practice this means

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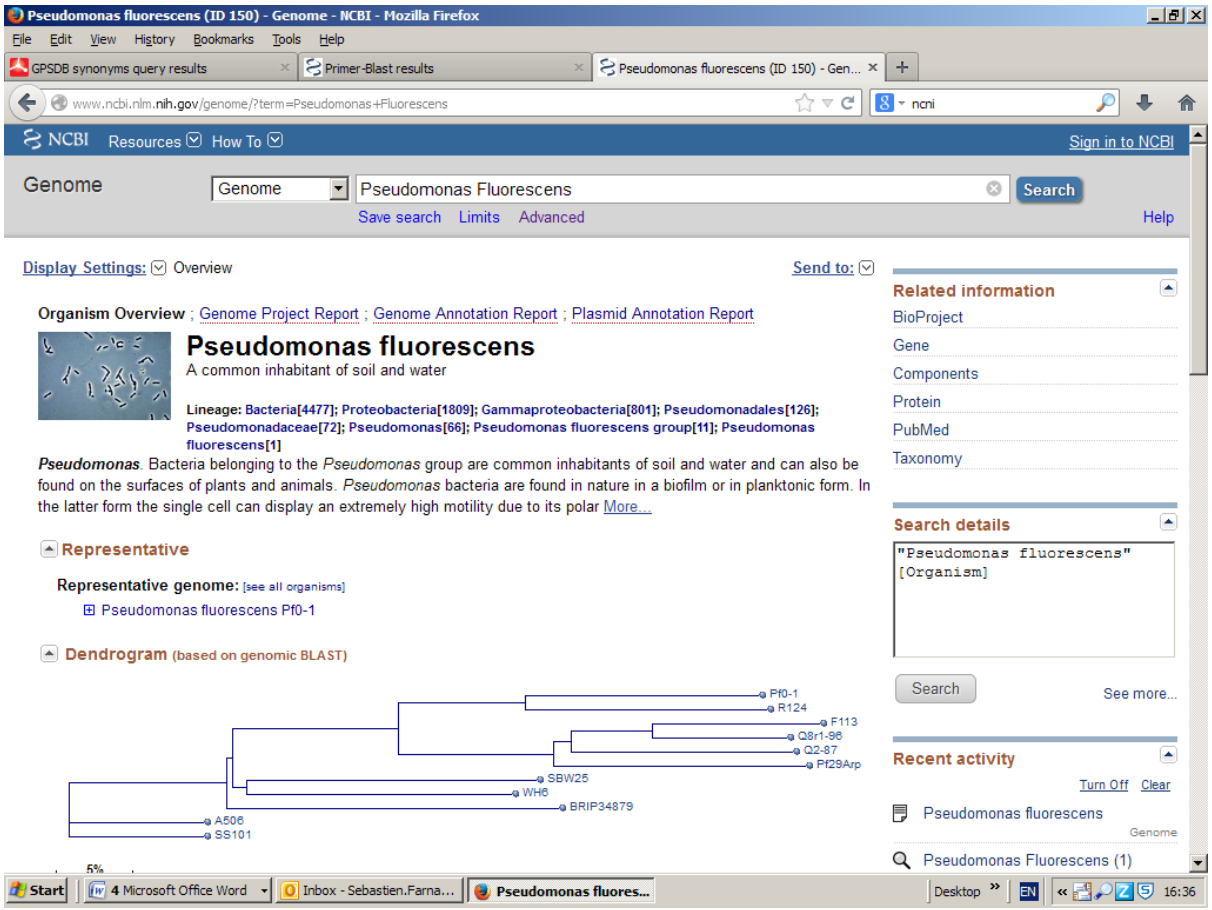
that the primers should be chosen within sequences, or part of sequences, unique to the species.

In order to check for the specificity of a sequence, it is essential that the whole genome of the species be known. To find out, go to NCBI website and check within the list of known genome that the genome of each species is available,

1. Enter the following address: ncbi.nlm.nih.gov
2. In the menu on the left, choose: ***Genomes & Maps***
3. Go down on this new page and choose the section entitled "***genome***"
4. At the top of this new page, there is a search window, into which you should type the name of one of your 5 micro-organism, for example "*Pseudomonas Fluorescens*". If the genome of this species is available, a new page will open that will give a summary of the genome.



Repeat the same procedure for each of the five species to check that their genome is available. You should keep a copy of each page to be included in the appendices of your report.

3) Definition and design of primers

The sequence of the primers will define the uniqueness of the product. It is not necessary for the whole sequence of the product to be unique, as long as the sequence of the five set of primers are different.

To design such primers, different methods can be used. The most laborious methods would be to choose a gene for each organism and to

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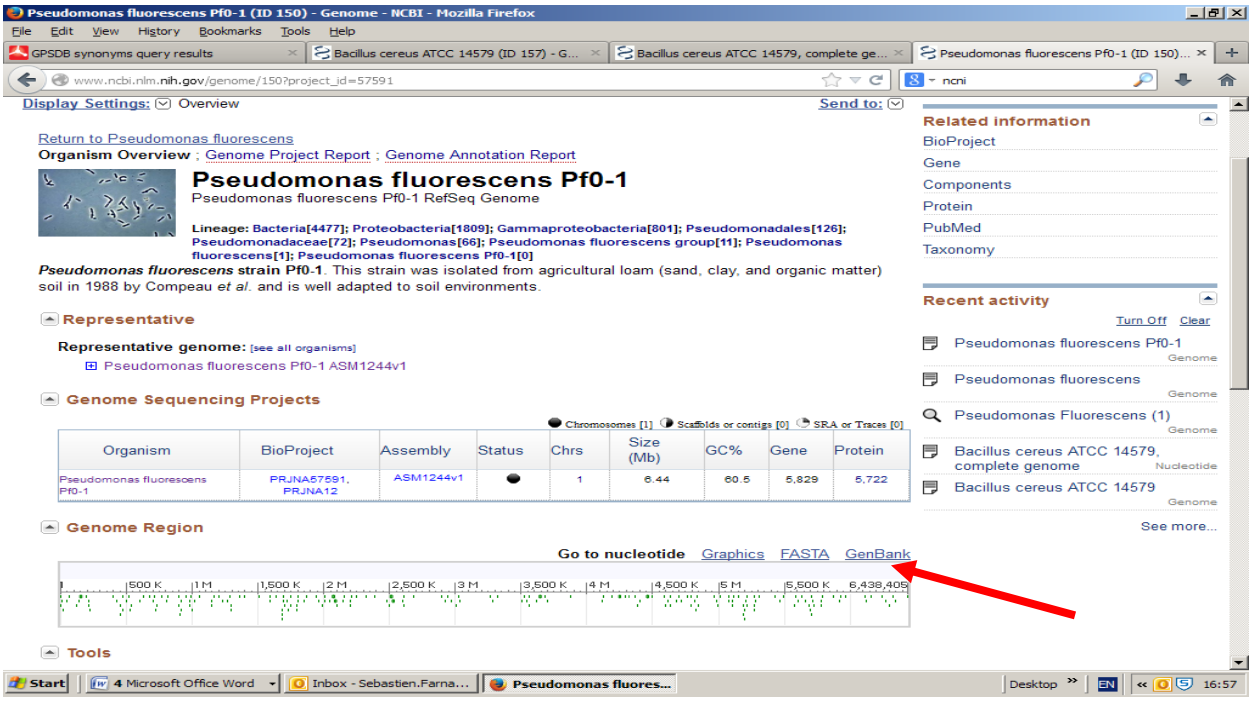
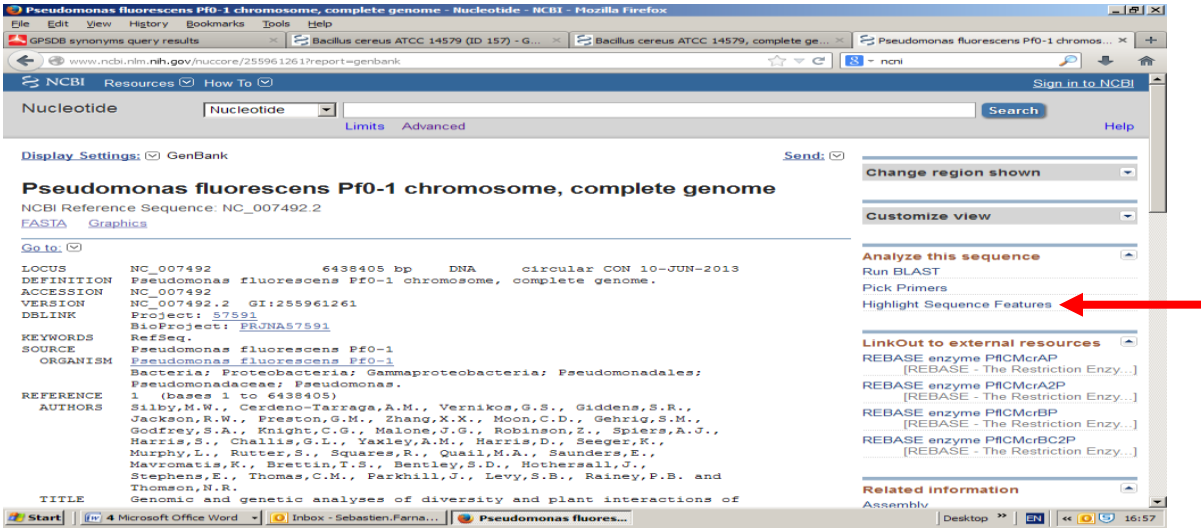
compare its sequence to homologous gene in the other genomes; the suitable gene or sequence would have to include part of the sequences unique to this single organism. Although this empirical method is still in practice and acceptable, some tools have been designed that do this work for you.

Such primers can be designed using software available on the same NCBI website. As an example, the procedure for the 1st microorganism, *Pseudomonas fluorescens* is described below.

From the Genome summary page you will have obtained in the previous section, scroll down the page to the last figure, and click on "GENBANK"; this will lead you to the page that describes the details of the genomic sequence.



On this new page that describes the genomic sequence, choose in the menu on the right, "***Pick primers***"



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A new page entitled "*Primers BLAST*" will open. It is in this page that all instructions that will define the primers and therefore the PCR product should be entered.

To keep all the results consistent and comparable, the size of the PCR products will be different for each species but with a given size for each species so that different groups will be able to compare their results.

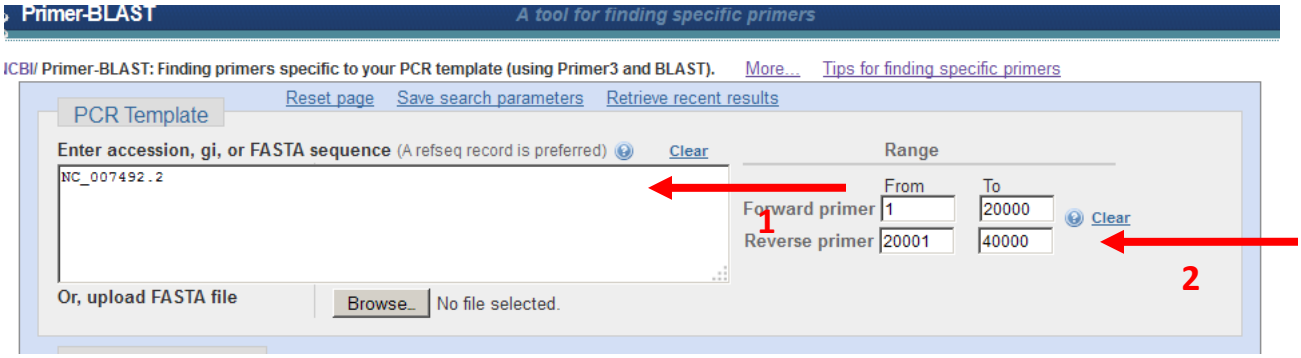
The size of the PCR products should be as follows:

Species	PCR product (bp)
Pseudomonas fluorescens (1)	1000
E. Coli (2)	800
Micrococcus luteus (3)	600
Bacillus subtilis (4)	400
Bacillus cereus (5)	200

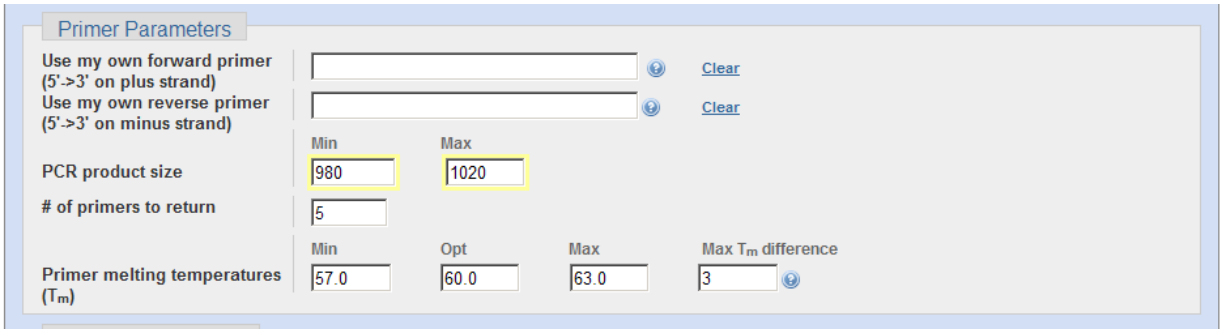
Because this page was accessed from the page of entire genome of the organism you chose, the whole genome is chosen as the sequence from which primer should be chosen (top window below 1).

SPECIFIC PARAMETERS HAVE TO BE DEFINED TO DESIGN YOUR PRIMERS

The range of the sequence within which the origin and the end of each primer (forward and reverse) should be chosen; in this case because the template is an entire genome, its size is over the limit permitted (50000bp). The size you will choose is arbitrary, so make sure that you choose a length long enough to increase the chance to find some unique sequence. In this case, values have been chosen to be from 1 to 20000 for the forward primer and form 20001 to 40000 (2).



Regarding the primers parameter, most of the default can be used, apart from their size which will be according to the allocated size of each product in the table above. In this case, to expect a PCR product of around 1kb, the size is chosen as min 980bb, and max 1020bp.



The final important parameter to define is what is the sequence or group of sequences that you want to compare your primers sequence with, to ensure that your primers are unique and will not initiate the amplification of other sequences. In this case, the default is "Genome (Chromosome from all other

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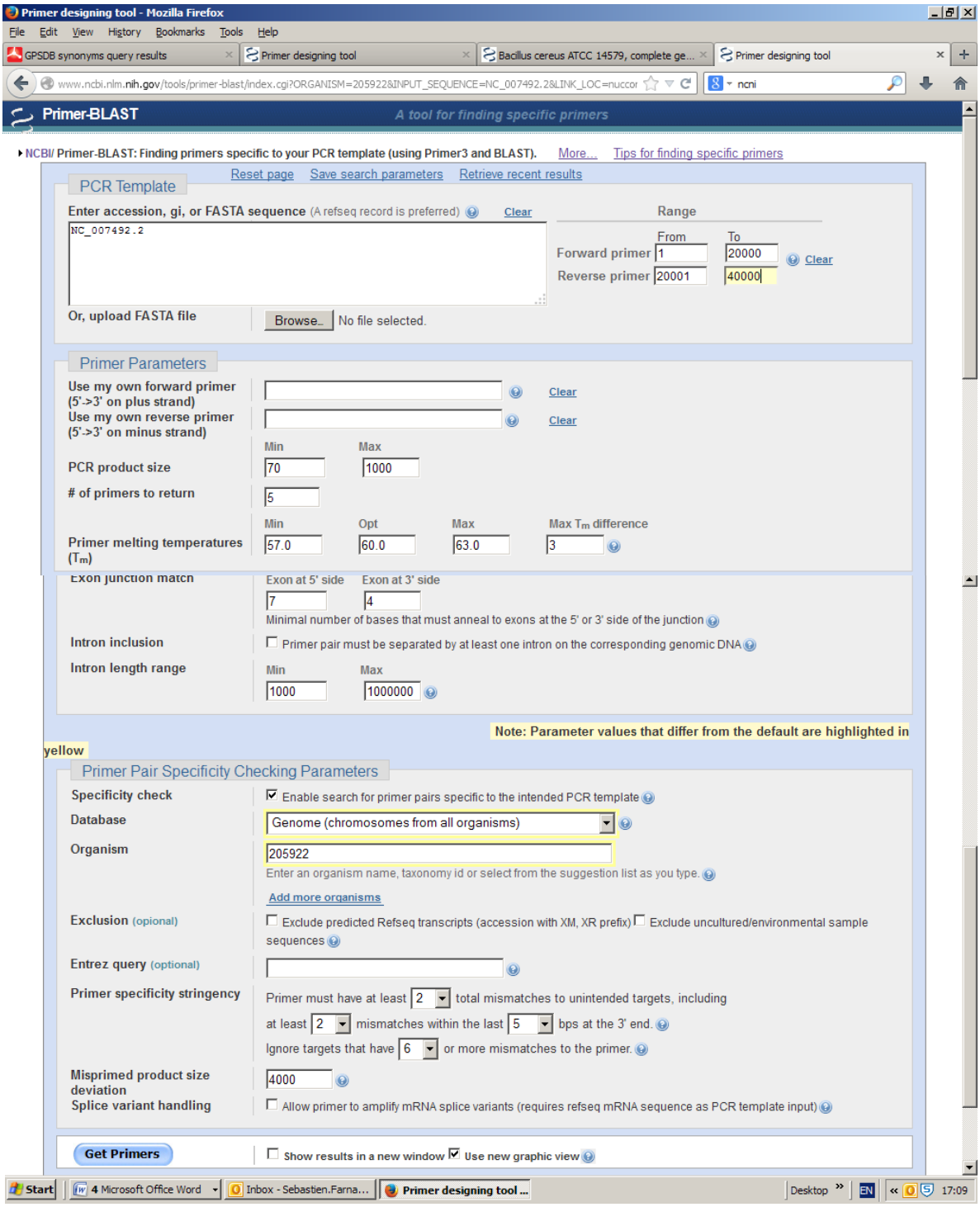
organisms) which includes 226900 genomes… This means that the sequence of your primers will be checked for similarity with all known sequences within known genomes.

The final page should be as below:



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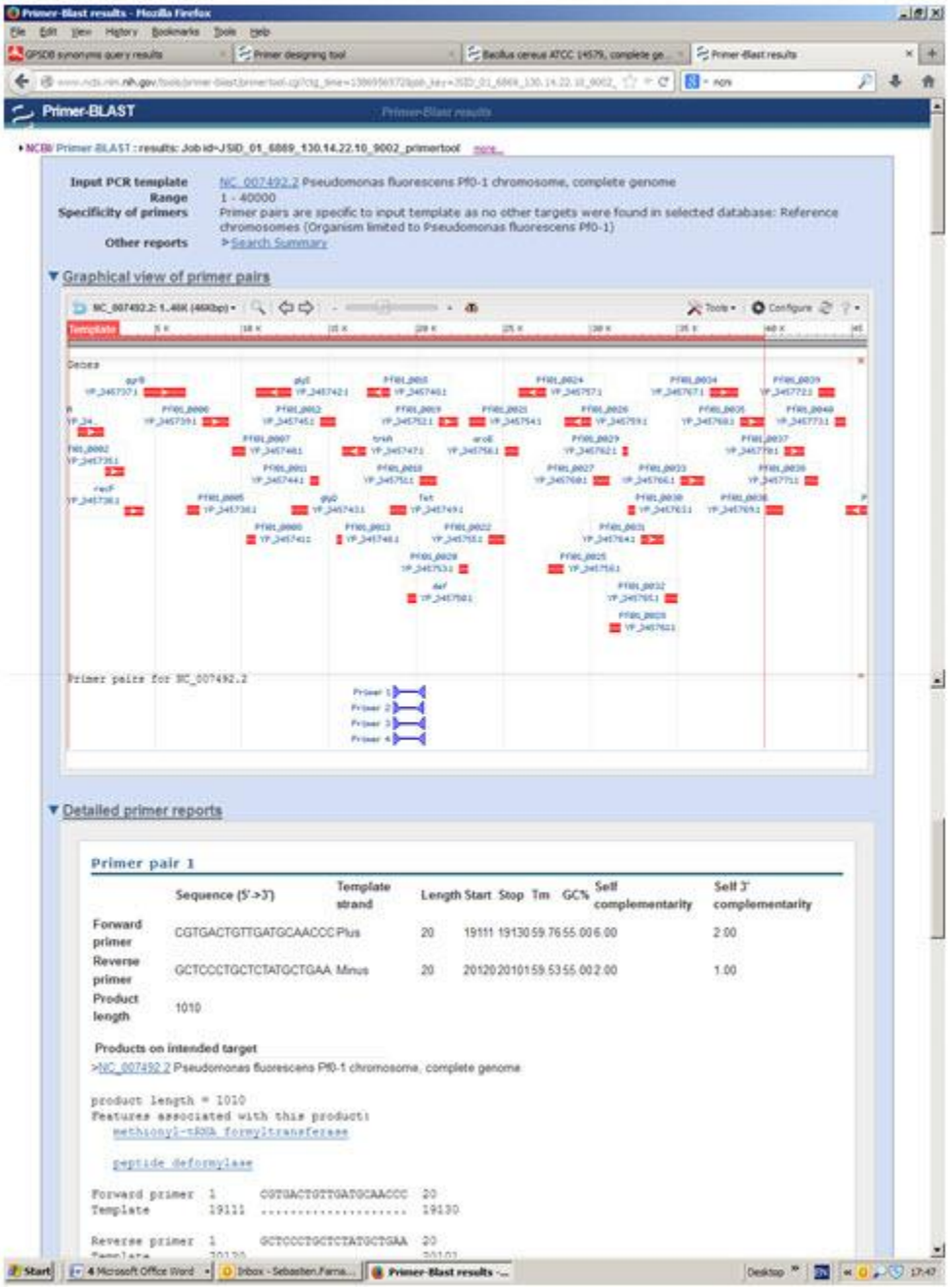
The next step is to click the final instruction at the bottom "***Get Primers***" which will generate 5 sets of primers.

After a short while, a new page will appear (see below), which describe a number of possibilities for different sets of primers that correspond to your parameters, and in particular 5 pairs of which a detailed description is given for the size of the product, the gene amplified, and the position of each set of primers:



Of the five pairs of primers obtained for each of the five organisms, you will need to choose only one pair. This procedure should be repeated for each of the five organisms.

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At the end of this session, you should have five pairs of primers, each defining a PCR product of a given length matching the table above, and using different genes as template. The sequence of your primers will be collected from you and ordered so that you will be using them during the last week of the project.

These primers will be used at two different stages of the project:

1. In the first instance, each primer should be used with the matching organism as a template to confirm that the template can be amplified and give the right size. These will constitute positive controls. It should be noted that if given enough time, negative controls should also be performed to confirm the specificity of the primers: this could be done by setting up 4 PCR for each set of primers with the non-matching template; it would be expected that no product should be obtained.
2. Following successful positive control, the final PCR that will allow the identification of the unknown organism can be performed: in this case, a multiplex reaction containing the five sets of primers should be used with the unknown template. It is expected that a PCR product matching any of the five products will identify the unknown organism as the matching microorganism.

CHECKING PRIMER SPECIFICITY

Aim and objectives

In this session, you will use several bioinformatic tools to confirm the uniqueness of your set of primers. To this aim, you will compare the sequence of your selected primers with relevant databases to confirm their suitability. For this you will need to investigate the specificity of the sequence of the pair of primers and the size of the PCR product they should generate.

1) Searching for sequence similarities

Your selected primers should be used as "queries" to carry out similarity searches within databases. In other words, your sequence of choice can be compared to each sequence contained within a database to check for similarities.

In the first instance, for each of the five species, each primer of the chosen pair should be checked for uniqueness within the genome of the respective microorganism.

As an example, the procedure is shown below for Pseudomonas fluorescens.

Based on your previous search, the sequence of your forward primer is for the PCR product marker of this species which is as follows:

5' CGTGACTGTTGATGCACCC 3'

This sequence can used for homology/identity search against any database using the programme BLAST on the NCBI website.

1) Enter the following address: ncbi.nlm.nih.gov
2) In the menu on the left, choose: Genomes & Maps
3) Go down on this new page and choose the section entitled "genome"
4) At the top of this new page, there is a search window, into which you should type the name of one of your 5 micro-organism, for example "Pseudomonas Fluorescens". If the genome of this species is available, a new page will open that will give a summary of the genome.
5) From the Genome summary page you will have obtained in the previous section, scroll down the page to the last figure, and click on "GENBANK"; this will lead you to the page that describes the details of the genomic sequence.
6) On the Genbank page, click on the instruction "RUN BLAST", which is the first instruction in the menu on the right column of the page
7) In the new page you will have to define your search (see illustration below)

a. Your primer sequence should be typed within the window of the query (top section)
b. The database you want to use to search for similarities should be defined too; to start with

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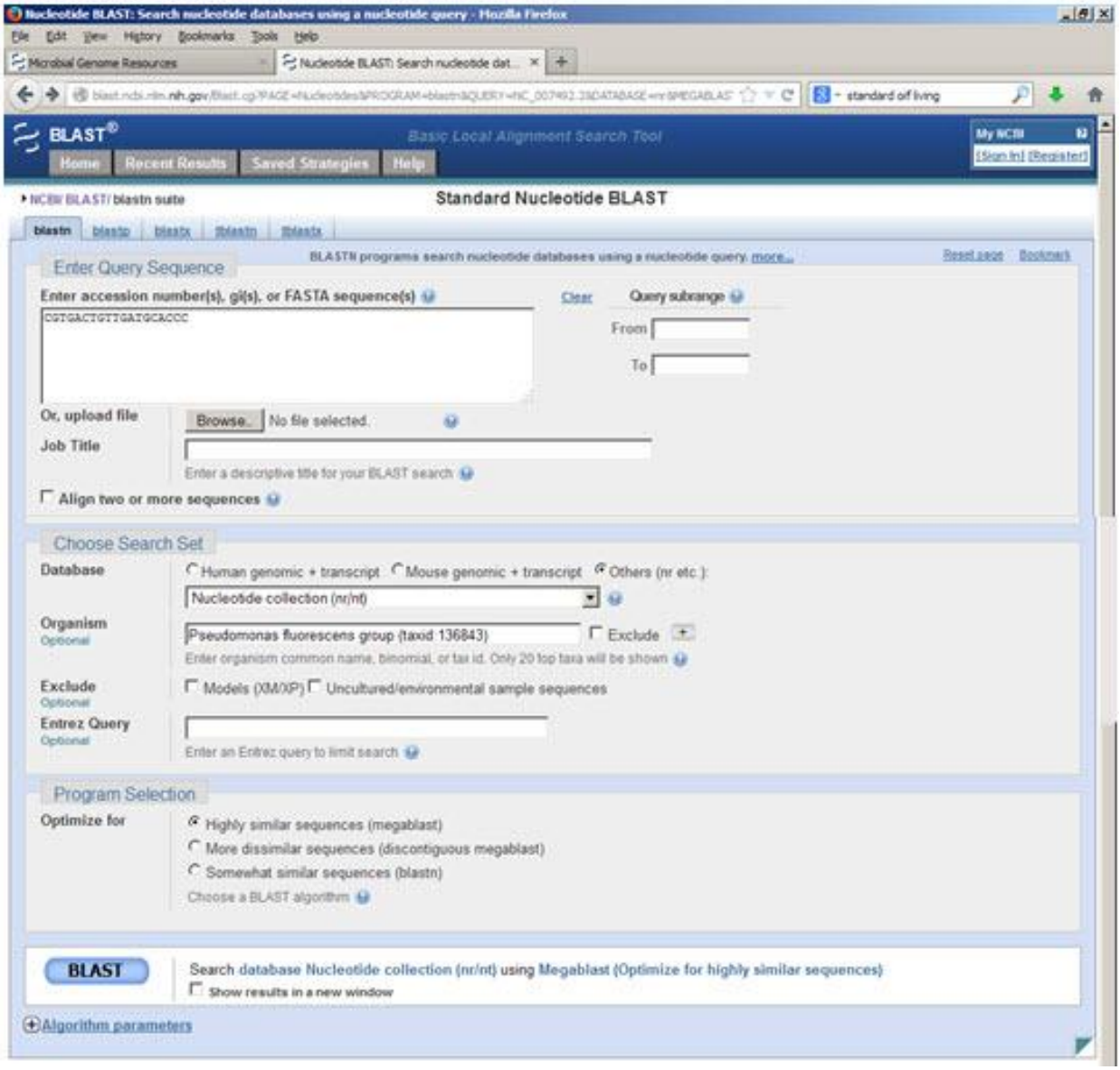
you will search the corresponding species genome, P. fluorescens which you will have to enter in the "Organism" window of the "Choose Search Set" section. When you start typing "Pseudomonas fluorescens", a list of choices will come up from which you can choose Pseudomonas fluorescens group (taxid:136843) which is representative of all strains.

The default choice can be used for other parameters, and your page should look like the example below:



The next step is to click the instruction "BLAST" so that the search can be carried out. This will take a few seconds, after which, the result of the search will appear on your screen (see below).

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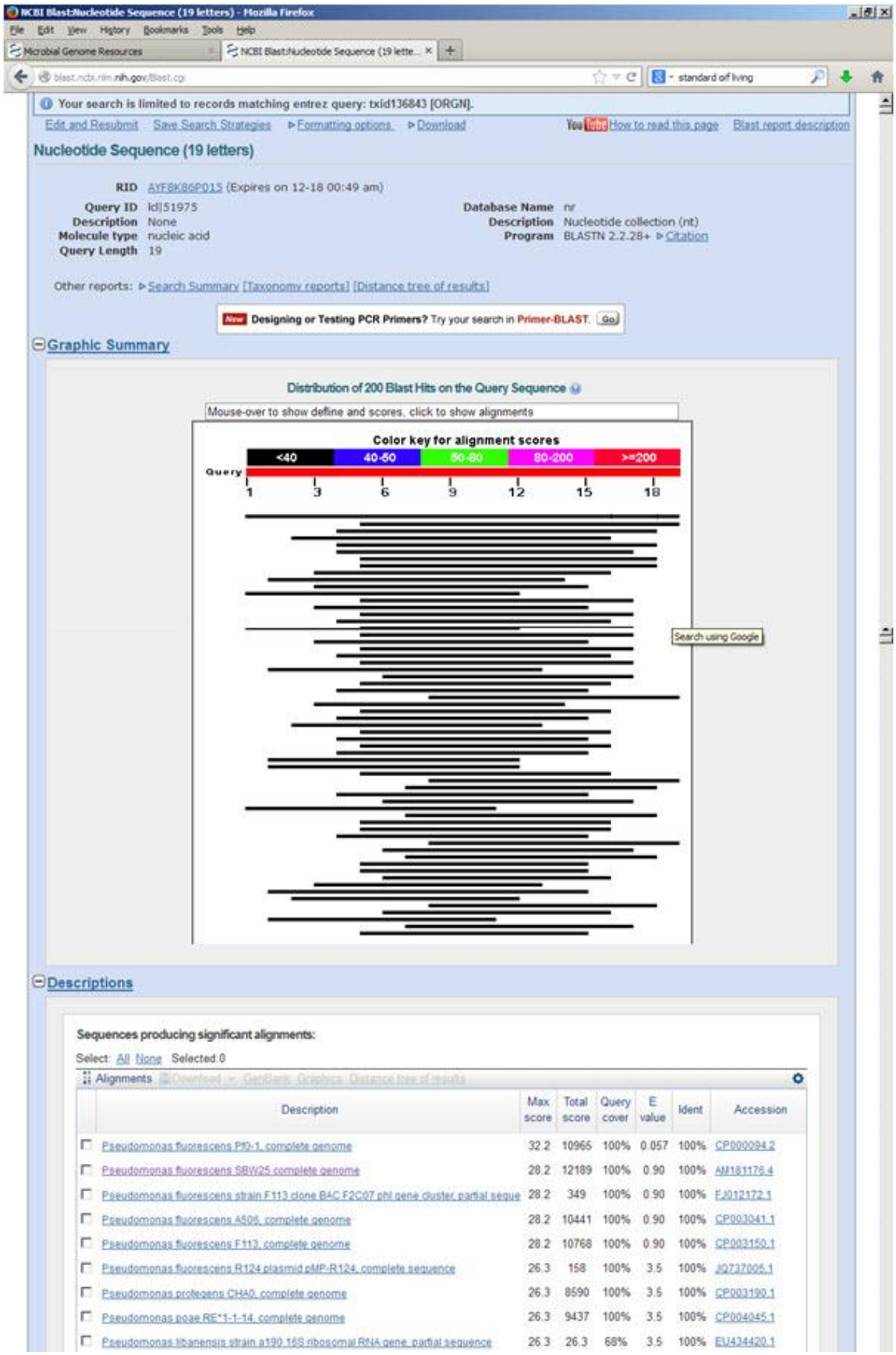

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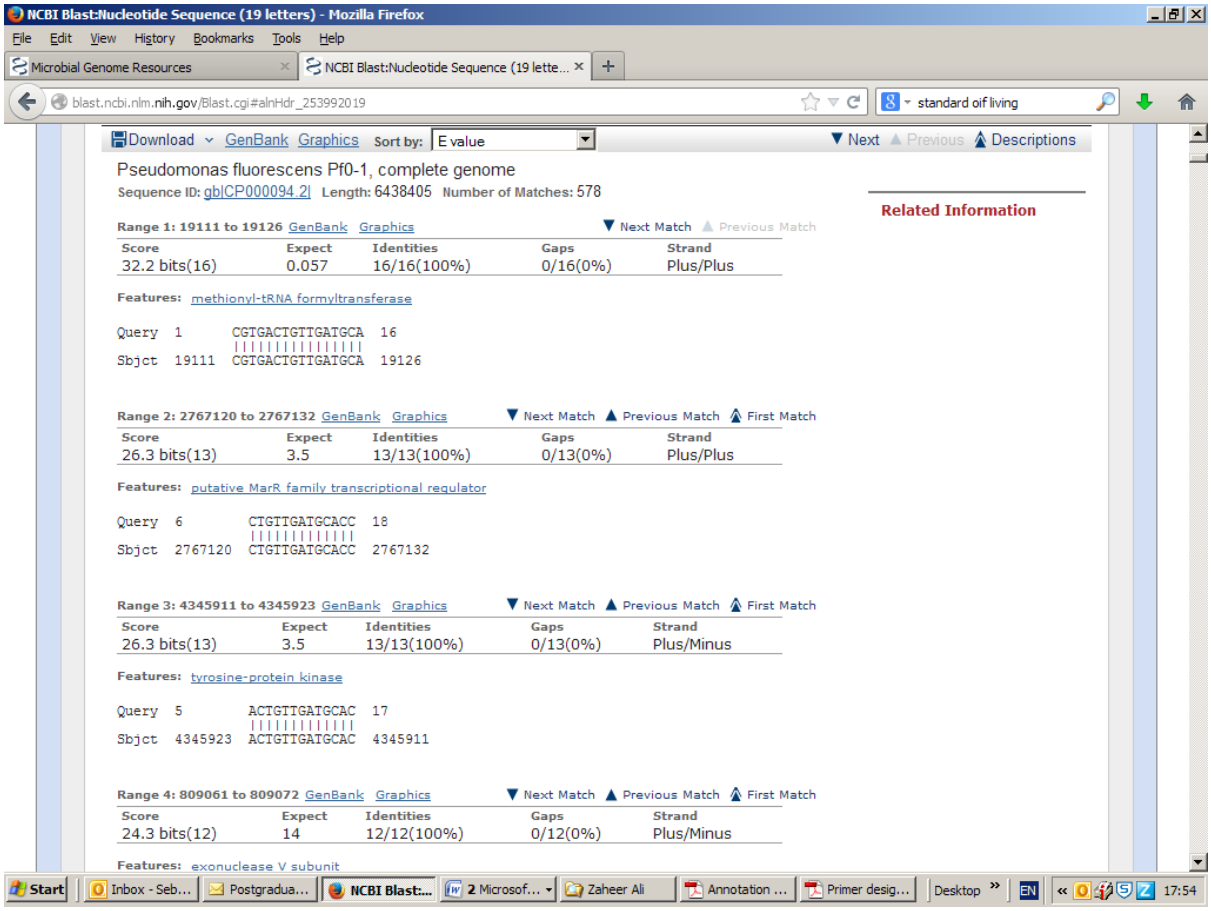
This page includes all the results of the search; it is interactive and allows you to access each result and see a detailed analysis of the sequence similarity using sequence alignment.

If you click on the first result, the bottom page comes up, and shows the details of the highest similarity score:



In this example, the highest similarity is the gene from which the primer sequence is extracted; in addition, any other further sequence similarities does not cover the whole primer sequence which indicates that the chosen sequence is unique.

This exercise should be repeated for all primers and also using other databases, to show that the primers are unique to a single genome.

The significant part of these results, that confirm the suitability of the primers, should be included in your reports.

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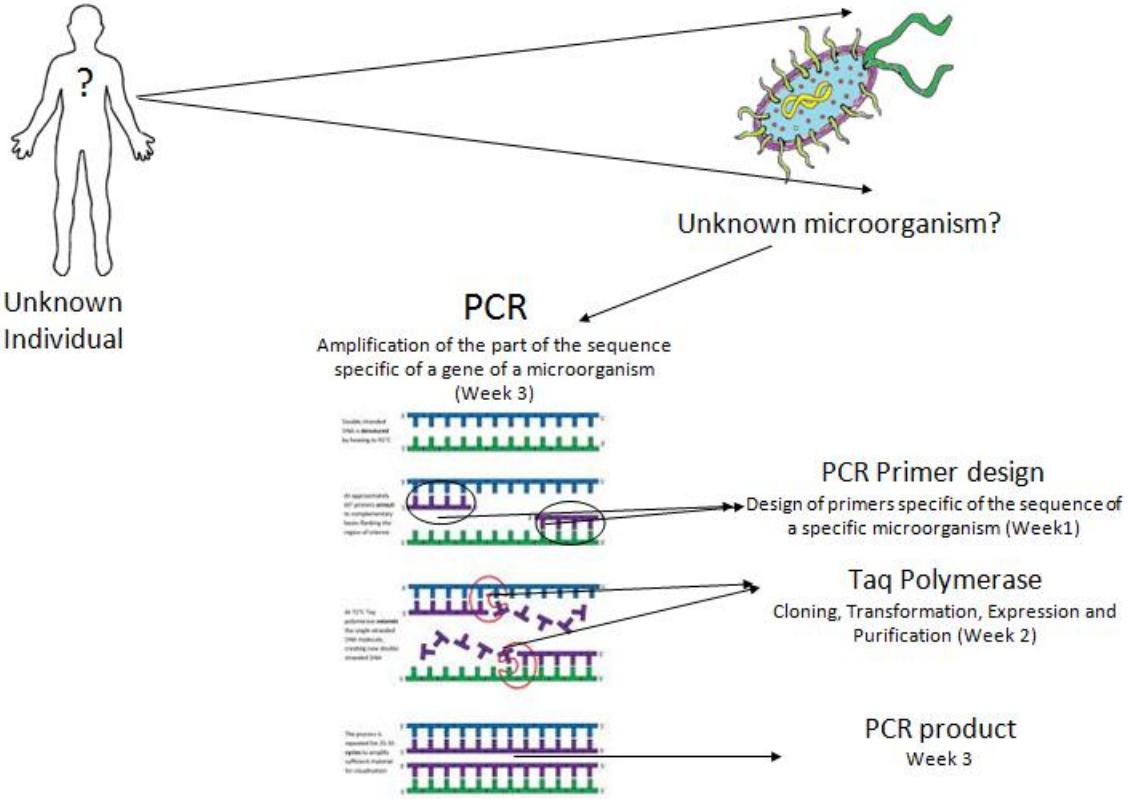
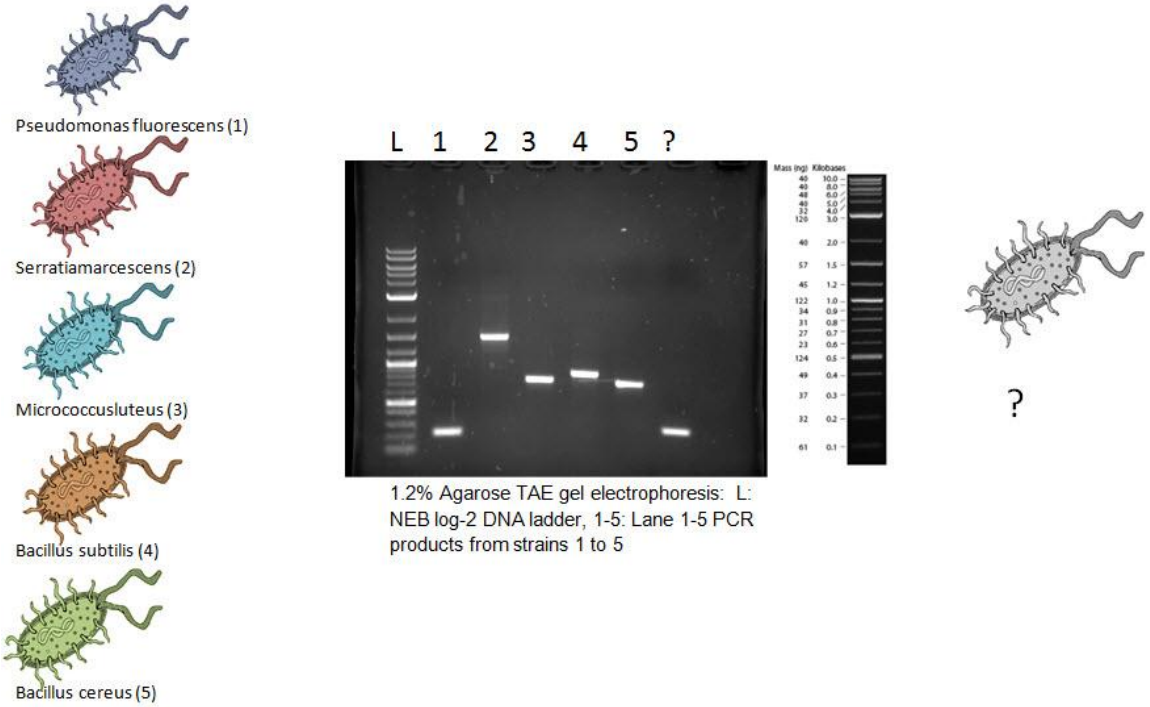
Appendix 1: Identification of an individual by identifying a carried microorganism



Appendix 2: Microorganism identification by PCR product length comparison



1.2% Agarose TAE gel electrophoresis: L: NEB log-2 DNA ladder, 1-5: Lane 1-5 PCR products from strains 1 to 5

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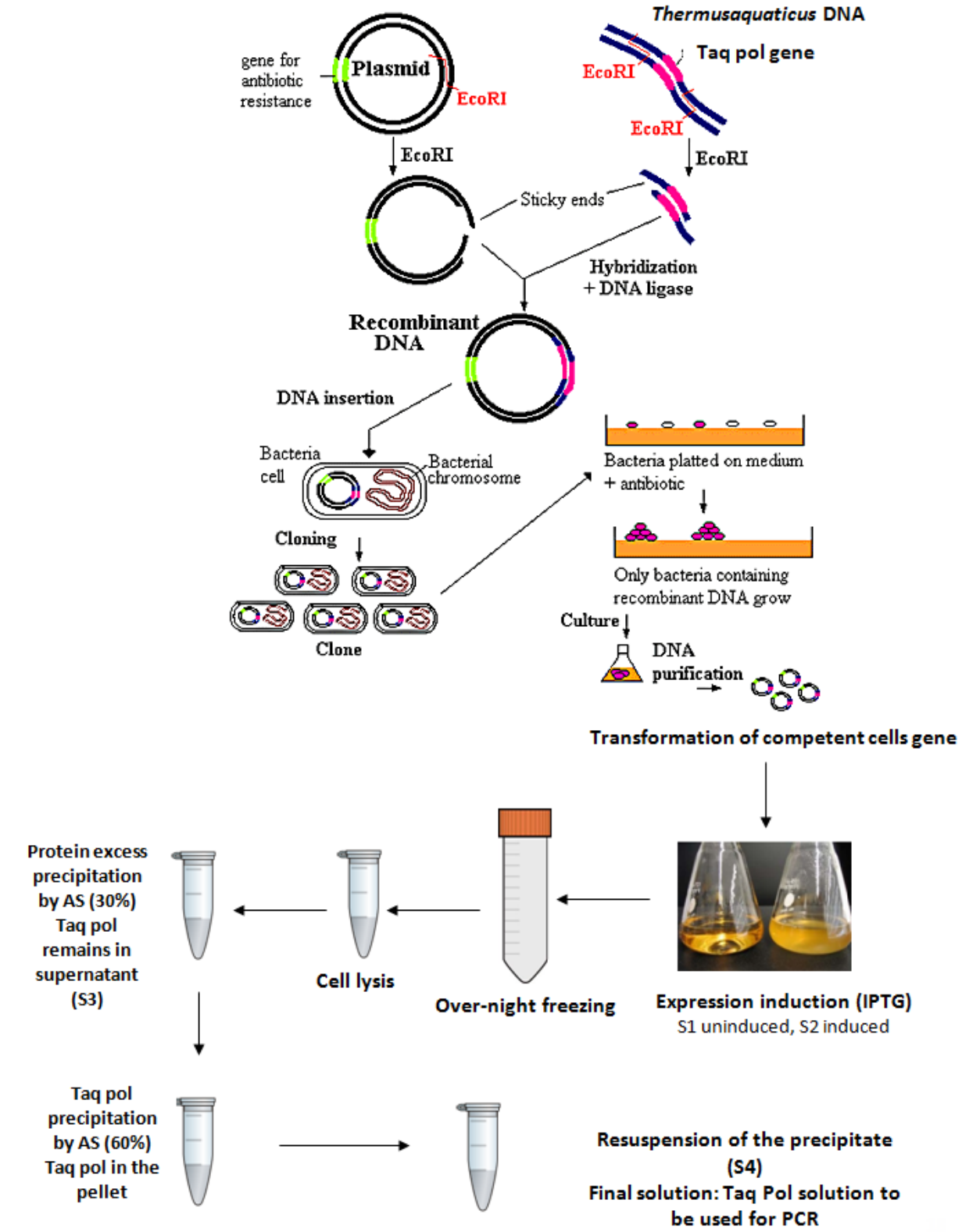
Taq Polymerase Cloning , Expression and Purification



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PCR REACTIONS

Technical considerations

All PCRs will be carried out using colonies, without purifying DNA, so you will be using colonies provided for the control PCRs, and the colonies from the plates grown in week 2 for the identification on the last day. To obtain the result necessary to identify the unknown organism, you will first need to check all the elements used in the final reaction are suitable for the success of the reaction.

The first set of reactions, named **PCR-1** is therefore the **set of control reactions** that will be using the set of primers you designed with the corresponding organism. In the second part of the practical, a second set of reactions named **PCR-2** will be used **to identify the unknown organism,** using the five set of primers. As an additional control, you will also amplify a sequence from one of the known organisms, for which a pair of primers has been previously designed and shown to work. This will be your positive control to confirm that all elements of the PCR reactions, are optimized and suitable for such a reaction.

Solutions

Electrophoresis Buffer	**TAE**: 40 mM Tris 20mM acetic acid; 1mM EDTA pH 7.8
Sample loading buffer	6g glycerol, 5ml 0.2M EDTA, 0.8ml saturated bromophenol blue dye
1.0% Agarose gel	0.5g agarose in 50ml of buffer TAE, microwave to melt. Cool to 50°C. Add 50 µl ethidium bromide to give 0.5 µg/ml
PCR buffer (1x final)	10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 @ 25°C

METHODS

PCR-1: Control reactions (Patelia EM et al., 2013)

Primers stocks:

The primers that have been designed have been synthesized for you to stock concentrations of 100 µM. (µmol/l)

Primers working concentration:

Your working concentration should be 0.5 µM in your PCR tube; since the dilution factor from your stock solution is 200X, you should prepare for each primer a more diluted stock solution. A new stock solution of 10 µM seems suitable since to reach the desired concentration in your PCR reaction (which is 0.5 µM) you will only need to dilute your primers 20X.

Prepare a new stock solution of a volume of 500µl, and a final concentration of 5 µM:

Volume of initial Primer solution at 100 µM =

Volume of water added…….. =

Total volume ……..= 500µl

Check the values with a demonstrator before you make your solution. Record your values

Preparing the PCR components:

Template preparation:

You are provided with 5 plates containing colonies of the five control organisms that you used to design your primers. You will use this as the source of DNA in the PCR controls. In this method you do not need to extract DNA, you will use the whole cells directly transferred from a colony. To facilitate access to the chromosome, the bacteria cells will be lysed by boiling the cells:

- pick up a single colony with a provided loop
- twirl the loop in 100µl of water in a fresh Eppendorf tube to resuspend the bacteria
- boil the solution for 5 min in a heating block at 100°C

Let the solution cool on the bench for a few minutes at room temperature, and centrifuge the tube for 30 sec to eliminate the debris. Your chromosomal DNA is now in solution, and can be used as a template for the PCRs.

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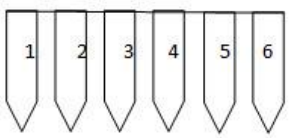
You are given 6 PCR tubes to set up your reactions in. Carefully number the first and last tubes 1 and 6 and mark your initials so you can identify them.



Tube 1 to 5 will contain the PCR reaction of the respective microorganisms (see list below) that will be using your primers and the DNA template from the corresponding organism. Tube 6 will contain the positive control PCR that will contain the provided primers and microorganism template.

List of markers microorganisms:

Pseudomonas fluorescens (1), E. Coli (2), Micrococcus luteus (3), Bacillus subtilis (4) and Bacillus cereus (5)

Preparing the PCR mixture:

The components should be added carefully IN THE ORDER STATED. Tick the box in each column after you have added each solution to keep a clear record of what you have added!

Since all PCR use the same reaction except for the template and the primers, a master-mix that contains all other components (except the Taq Polymerase, can be prepared for all 6 reactions.

Once the master-mix has been aliquoted into all 6 labeled tubes, the specific template and primers can be added individually to each reaction.

Although the master mix will be used for 6 reactions only, it is preferable to prepare a volume in excess of what is necessary; the master mix will therefore be prepared for 7 potential reactions of 50 µl each; the total volume of the master mix in each solution can be calculated since the volume of each component is known:

V(mast-mix) = [PCR (total vol)] – [(Primers vol) + Template vol (lysed cells)]

V(mast-mix) = 50 µl – [(2.5 µl x2) + (15 µl)] = 30 µl/ reaction

Total V(mast mix) = 7 x 30 = 210 µl

Master mix:

PCR buffer 10X: …….5 µl x 7 = 35 µl

dNTPs 10X: ……..5 µl x. 7 = 35 µl

Water: 210 – (35 + 35) = 140 µl

PLEASE NOTE THAT THE PRIMERS ARE DIFFERENT IN EACH REACTION!!!!

PCRs mixtures

Components	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
Master mix	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
Forward **Primer**	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Reverse **Primer**	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Lysed cells	19 µl	19 µl	19 µl	19 µl	19 µl	19 µl
Total volume	49 µl	49 µl	49 µl	49 µl	49 µl	49 µl

When you add the water containing the lysed cells to the tube, MIX it carefully by pipetting it up and down.

When all your tubes are ready, the Taq Polymerase can be added; 1µl to each tube. To avoid starting any unspecific reactions, once the Taq Polymerase is added to the tube, the tubes should be kept on ice until they are placed in the PCR machine.

Ensure your PCR reactions are well mixed, this can be done by flicking the tube and spinning (**just a quick pulse**) the liquid down – **please note you MUST use special adaptors to centrifuge PCR tubes**. The Technical Staff will facilitate this by asking you to bring your tubes to them. The samples will then be run for you in the thermal cycler.

PCR programme:

Denaturation 94° C 2mins
Denaturation 94° C 20sec
Annealing 50°C 20sec } (x 35)
Elongation 72° C 40sec
Final elongation 74°C 4mins
Storage 4° C

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Agarose gel electrophoresis of PCR (1) products

SAFETY: Ethidium bromide is extremely TOXIC. It is a MUTAGEN.

*Gloves must be worn when pouring agarose, applying samples and during viewing.

**Risk of Electric shock from Powerpacks*

*Cover the electrophoresis tank with the lid before switching on.

*Switch off the power before removing the lid from the electrophoresis tank.

The gel can be prepared while the PCRs are taking place.

Method

1. Place the sample comb in the gel casting tray and set up the electrophoresis system.

Pour in the gel to a depth of ~5mm and allow 20 min to set. Remove the comb.

Add electrophoresis buffer (TAE) to cover the gel by approximately 0.5cm.

2. To each of your 6 PCR tubes, add 5 µl of DNA loading buffer and mix carefully by pipetting up and down.

3. Add 10 µl of each sample (Tubes 1-6) to individual wells. Record the positions of the samples in the lanes.

4. To one lane on the gel add 5 µl of marker. This will allow you to determine the size of your DNA fragments. Note down the size of your fragments.

5. Run the gel at 100Volts for approximately 40 minutes or until the blue dye runs half way down the gel. The dye runs at approx.600bp.

SAFETY: *UV light can damage eyes and skin*. Wear plastic glasses or a facemask.

*Gloves must be worn when handling the agarose gel.

6. The gel will be visualised under UV light, using a Gel-doc. You will be able to see your gel on the screen and to save a picture for your report. From this picture you will be able to determine the size of each PCR product and confirm whether the primers are suitable.

NOTE:

The left-over in your 6 PCR reactions should be kept for the following day!!!

PCR-2: Identification of the unknown organism

This set of reactions is similar to the previous PCR-1 set. The differences are the following:

1) The DNA template is unknown and will be obtained from the plates provided to you.

2) Instead of using a single pair of primers, all 5 pairs of primers (you designed) will be used together in this multiplex reaction.

Template preparation:

You are using the unknown organism which you will extract from one of the plates provided to you. This template will be used for the multiplex PCR and for the positive control. As in the previous section, to facilitate access to the chromosome, the bacterial cells will be lysed by boiling the cells:

- pick up a single colony with a provided loop
- twirl the loop in 100µl of water in a fresh Eppendorf tube to resuspend the bacteria
- boil the solution for 5 min in a heating block at 100°C

Let the solution cool on the bench for a few minutes at room temperature, and centrifuge the tube for 30s to eliminate the debris. Your chromosomal DNA is now in solution, and can be used as a template for the PCRs.

Preparing the PCR mixture:

The components should be added carefully IN THE ORDER STATED. Tick the box in each column after you have added each solution to keep a clear record of what you have added!

Since both PCRs use the same reaction, except for the primers, a master-mix that contains all other components (except the Taq Polymerase) can be prepared for both reactions.

Although the master mix will be used for 2 reactions only, it is preferable to prepare a volume in excess of what is necessary; the master mix will therefore be prepared for 3

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potential reactions of 50 µl each; the total volume of the master mix in each solution can be calculated since the volume of each component is known:

Master mix:
PCR buffer 10X: ……..5 µl x 3 = 15 µl
dNTPs 10X: ……5 µl x 3 = 15 µl
Lysed unknown organism: 14.5 µl x 3 = 43.5 µl
Total volume:……= 73.5 µl

Primers preparation:
The multiplex should contain the five pairs of primers; therefore the previous working concentration cannot be used **WHY?**

A new preparation will be used

PLEASE NOTE THAT THE PRIMERS ARE DIFFERENT IN EACH REACTION!!!!

PCRs mixtures

Components	Tube 1	Tube 2
Master mix	24.5 µl	24.5 µl
Forward **Primer**	5x 2.5 µl	2.5 µl
Reverse **Primer**	5x 2.5 µl	25 µl
H_2O	0	20
Total volume	49.5 µl	49.5 µl

After you add the primers, MIX the solution carefully by pipetting it up and down.
Tube 1 will contain a PCR reaction that will be using your 5 sets of primers and the DNA template from the unknown corresponding organism. Tube 2 will contain a PCR reaction that will be using the same DNA template but a set of control primers that will be provided.
The final step is to add Taq polymerase to each tube; 1µl to each tube. To avoid starting any unspecific reactions, once the Taq Polymerase is added to the tube, the tubes should be kept on ice until they are placed in the PCR machine.

Ensure your PCR reactions are well mixed, this can be done by flicking the tube and spinning (**just a quick pulse**) the liquid down – **please note you MUST use special adaptors to centrifuge PCR tubes**. The Technical Staff will facilitate this by asking you to bring your tubes to them. The samples will then be run for you in the thermal cycler.
The programme used in the thermocycler is the same as the one previously used unless mentioned otherwise. While the PCR is being completed, the agarose gel that you will use to analyse your PCR can be prepared following the same protocol as the previous one (see below).

Agarose gel electrophoresis of PCR products

SAFETY: Ethidium bromide is extremely TOXIC.
It is a MUTAGEN.
Gloves must be worn when pouring agarose, applying samples and during viewing.
Risk of Electric shock from Powerpacks
Cover the electrophoresis tank with the lid before switching on.
Switch off the power before removing the lid from the electrophoresis tank.

Method
1. Place the sample comb in the gel casting tray and set up the electrophoresis system. Pour in the gel to a depth of ~5mm and allow 20 min to set. Remove the comb. Add electrophoresis buffer (TAE) to cover the gel by approximately 0.5cm.
2. To each PCR tube add 5 µl of DNA loading buffer and mix carefully by pipetting up and down.
3. Add 10 µl of each sample (Tubes 1-2) to individual wells. Record the positions of the samples in the lanes.
4. To one lane on the gel add 5 µl of marker. This will allow you to determine the size of your DNA fragments. Note down the size of your fragments.

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5. Run the gel at 120Volts for approximately 40 minutes or until the blue dye runs half way down the gel. The dye runs at approx.600bp.
SAFETY: UV light can damage eyes and skin. Wear plastic glasses or a facemask.
*Gloves must be worn when handling the agarose gel.
6. View the gel under UV light. Save a picture for your report.

Comparison of Commercial Taq vs Your Taq
This set of reactions is similar to the previous PCR-2 set in that you will be running a multiplex reaction with DNA template that is unknown (obtained from the plates provided to you). The difference is that:
You will be performing a comparison of the Taq polymerase you purified with commercial Taq polymerase.

Template preparation:
You are using an unknown organism which you will extract from one of the plates provided to you. This template will be used for the multiplex PCR and for the positive control. As in the previous section, to facilitate access to the chromosome, the bacterial cells will be lysed by boiling the cells:
● pick up a single colony with a provided loop
●twirl the loop in 100µl of water in a fresh Eppendorf tube to resuspend the bacteria
●boil the solution for 5 min in a heating block at 100°C
Let the solution cool on the bench for a few minutes at room temperature, and centrifuge the tube for 30s to eliminate the debris. Your chromosomal DNA is now in solution, and can be used as a template for the PCRs.

Preparing the PCR mixture:
The components should be added carefully IN THE ORDER STATED. Tick the box in each column after you have added each solution to keep a clear record of what you have added!

Since both PCRs use the same reaction, except for the primers, a master-mix that contains all other components (except the Taq Polymerase) can be prepared for both reactions. Remember you need to set this up to test both Taq polymerases.
Although the master mix will be used for 4 reactions only, it is preferable to prepare a volume in excess of what is necessary; the master mix will therefore be prepared for 5 potential reactions of 50 µl each; the total volume of the master mix in each solution can be calculated since the volume of each component is known:

Master mix:
PCR buffer 10X: ……..5 µl x 5 = 25 µl
dNTPs 10X: ……5 µl x 5 = 25 µl
Lysed unknown organism: 14.5 µl x 5 = 72.5 µl
Total volume:……= 122.5 µl

Primers preparation:
The multiplex should contain the five pairs of primers; therefore the previous working concentration cannot be used **WHY?**
A new preparation will be used

PLEASE NOTE THAT THE PRIMERS ARE DIFFERENT IN EACH REACTION!!!!

PCRs mixtures

Components	Tube 1 (Comm Taq)	Tube 2 (Comm Taq)	Tube 3 (Your Taq)	Tube 4 (Your Taq)
Master mix	24.5 µl	24.5 µl	24.5 µl	24.5 µl
Forward **Primer**	5x 2.5 µl	2.5 µl	5x 2.5 µl	2.5 µl
Reverse **Primer**	5x 2.5 µl	25 µl	5x 2.5 µl	25 µl
H2O	0	20	0	20
Total volume	49.5 µl	49.5 µl	49.5 µl	49.5 µl

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After you add the primers, MIX the solution carefully by pipetting it up and down.
Tube 1 will contain a PCR reaction that will be using your 5 sets of primers and the DNA template from the unknown corresponding organism. Tube 2 will contain a PCR reaction that will be using the same DNA template but a set of control primers that will be provided.
The final step is to add Taq polymerase to each tube – ensure you add the correct Taq! To Tubes 1 and 2 add 1µl of Commercial (Comm) Taq. To Tubes 3 and 4 add 1µl of Your Taq (that you purified last week).
To avoid starting any unspecific reactions, once the Taq Polymerase is added to the tube, the tubes should be kept on ice until they are placed in the PCR machine.

Ensure your PCR reactions are well mixed, this can be done by flicking the tube and spinning (**just a quick pulse**) the liquid down – **please note you MUST use special adaptors to centrifuge PCR tubes**. The Technical Staff will facilitate this by asking you to bring your tubes to them. The samples will then be run for you in the thermal cycler.

The programme used in the thermocycler is the same as the one previously used unless mentioned otherwise. While the PCR is being completed, the agarose gel that you will use to analyse your PCR can be prepared following the same protocol as the previous one (see below).

Agarose gel electrophoresis of PCR products

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Method
1. Place the sample comb in the gel casting tray and set up the electrophoresis system.
Pour in the gel to a depth of ~5mm and allow 20 min to set. Remove the comb.
Add electrophoresis buffer (**TAE**) to cover the gel by approximately 0.5cm.
2. To each PCR tube add 5 µl of DNA loading buffer and mix carefully by pipetting up and down.
3. Add 10 µl of each sample (Tubes 1-2) to individual wells. Record the positions of the samples in the lanes.
4. To one lane on the gel add 5 µl of marker. This will allow you to determine the size of your DNA fragments. Note down the size of your fragments.
5. Run the gel at 120Volts for approximately 40 minutes or until the blue dye runs half way down the gel. The dye runs at approx.600bp.
SAFETY: UV light *can damage eyes and skin*. Wear plastic glasses or a facemask.
Gloves must be worn when handling the agarose gel.
6. View the gel under UV light. Save a picture for your report.

Taq Polymerase Purification
Engelke et. al. (1990) which was modified by Dr. Baron of Harvard Medical School.

Solutions
1000X IPTG
0.4M IPTG (100 mg/ml IPTG)

Buffer A
50 mM Tris 7.9
50 mM dextrose
1 mM EDTA
up to 500 ml with water
*Check pH and bring to 7.9 as the dextrose will reduce the pH.
Store at room temperature. For some steps add Lysozyme to a final concentration of 4 mg/ml.

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Buffer B
10 mM Tris 7.9
50 mM KCl
1 mM EDTA
0.5% Tween 20
0.5% NP-40
Store at room temperature and add PMSF to a final concentration of 1 mM just prior to use.

Buffer C
50 mM Tris 8.0
50 mM KCl
1 mM EDTA
50% glycerol
0.5% Tween 20
0.5% NP-40
1 mM DTT
1 mM PMSF
up to 1 litre with water

LB Media
10 g/l Bactopeptone
5 g/l Yeast extract
5 g/l NaCl

Expression and Purification of Taq Polymerase
In this practical you will express and purify the thermostable DNA polymerase from *Thermophilus aqauticus,* generally known as Taq polymerase. This polymerase will then be used in a PCR reaction to amplify your target gene from *Saccharomycetes cerevisiae* (Baker's yeast).
You are given cells that have already been transformed so they contain an expression plasmid that contains the gene encoding the Taq polymerase. You will grow these cells in a selective media containing the antibiotic ampicillin. The presence of the antibiotic means that only cells containing the plasmid, which encodes both the Taq polymerase and the amplicillin resistance gene will grow.
The expression of the Taq gene is under the control of the *lac* promoter system. This allows the gene to be expressed only in the presence of galactose. You will be using the non-hydrolysable galactose analogue IPTG to induce the cells to express protein. This cannot be metabolised by the cells, so once you add it to the cells they will continuously to express your protein.

During the protein expression / purification you will take 4 samples to run on SDS-PAGE to show the steps in making your protin.
Calculate the appropriate amounts of Bactopeptone (**10g/l**), Yeast Extract (**5g/l**) and NaCl (**5g/l**) to make 50 ml of LB media. Weigh out and add 50 ml of water. pH your medium to ensure it is at pH7.
Inoculate a 1 ml overnight culture of LB-amp with a single colony from an *E.coli* Taq plate using a pipette tip.
Grow overnight at 37 ºC – This is done in a shaking incubator.
Add ampicillin to a final concentration of 100mg/litre to your 50 ml flask of sterile LB
(Stock is 100 mg/ml, show your calculation)

Grow culture in 37 ºC shaker for approximately 3 hours.
Remove a 125 µl sample and place in a 1.5 ml centrifuge tube. Spin at 13k rpm for 1 minute. Carefully remove the supernatant and add 20 µl H_20 and 20 µl SDS loading buffer. Incubate this at 95 ºC for 4 min to make a sample for SDS-PAGE – LABEL CAREFULLY '**uninduced**'

To your 50ml flask, add IPTG to a final concentration of 0.4 mM and culture for 3 hours.

Remove a 125µl sample and place in a 1.5 ml centrifuge tube. Spin at 13k rpm for 1 minute. Carefully remove the supernatant and add 20 µl H_20 and 20 µl SDS loading buffer. Incubate this at 95 ºC for 4 min to make a sample for SDS-PAGE – LABEL CAREFULLY '**induced**"
Pour the rest of your cells into 50ml Falcon tube and centrifuge for 10 mins to pellet cells

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YOUR TUBE MUST BE BALANCED BY ANOTHER TUBE!

Carefully pour off the supernatant and remove any remaining liquid with a pipettor

LABEL YOUR 50ml TUBE AND FREEZE OVERNIGHT

Place your 2 samples for SDS-PAGE in the fridge overnight for storage.

Place 0.5 ml of **Buffer A with lysozyme** into a 2ml centrifuge tube and chill on ice.

When it is cold, use your chilled 0.5 ml of Buffer A with lysozyme (ice cold) to resuspend the pellet by carefully pipetting up and down. *Keep the tip in the liquid at all times to prevent bubbles*.

Transfer the resuspended cells back to the 2ml centrifuge tube

Incubate at room temperature for 30 mins to allow the lysozyme to digest the cell wall of the *E.coli*.

Add 0.5 ml Buffer B, mix and incubate at 80 °C for 15min.

Chill on ice for 10 minutes, then spin at 13k rpm in a microfuge for 15 minutes.

YOUR TUBE MUST BE BALANCED BY ANOTHER TUBE!

Using a pipettor, carefully transfer the supernatant to a fresh 1.5ml tube and keep on ice.

Take a 20 µl sample from this tube into a fresh 1.5ml centrifuge tube and add 20 µl of SDS-loading buffer. Heat at 95 ºC for 4 min. - LABEL CAREFULLY '**heat-treated**'

Measure the volume by placing an empty 1.5ml tube on the balance and zeroing it. Then place your tube with the sample on. The weight gives you the volume.

Add pulverized ammonium sulphate slowly (add a bit, mix to dissolve, add a bit more mix, so on) to a final concentration of 0.164 g/ml (30% saturation). Incubate on ice for 30 minutes.

Pellet the precipitate by spinning in a microfuge at 13k rpm for 15 minutes.

Transfer the supernatant to a fresh tube on ice, add ammonium sulphate to a final concentration of 0.181 g/ml (60% saturation) using the same method as before **but**. *divide the weight by 1.2 to allow for the increase in density caused by the ammonium sulphate already present*

Mix and ensure fully dissolved as before. Incubate for 15 minutes on ice.

Pellet the precipitate in the microfuge at 13k rpm for 15 minutes. Carefully remove and discard **all** the supernatant.

Resuspend in 0.5 ml Buffer C.

Take a 20 µl sample into a fresh 1.5ml centrifuge tube and add 20 µl of SDS-loading buffer. Heat at 95 ºC for 4 min. - LABEL CAREFULLY '**final protein**'

Carefully label your final Taq polymerase and store in the freezer for use next week.

Transformation

The process of transformation allows DNA to be absorbed by bacterial cells.

In this case the process you are using is chemical transformation. This uses treatment of the bacterial cells with divalent metal ions to make cells 'competent' to absorb DNA.

Competent cells are able to absorb DNA. This is done in the process of transformation.

To do this the cells are incubated with the plasmid DNA on ice. This allows the DNA to associate with the surface of the cells. The cells are then heat shocked at 42 ºC. This causes the DNA to be absorbed through the cell membrane into the cell. The cells are then chilled on ice to stabilize the cell membrane. Finally the cells are grown for 1 hour to allow the cells that have absorbed plasmid DNA to express the antibiotic resistance gene. The cells are then plated on selective media containing an antibiotic that will kill cells that have not been transformed and contain the plasmid.

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You are given an aliquot of competent cells (100 µl), you will incubate this with plasmid DNA, which will allow expression of a gene to make protein, such as the Taq expression plasmid you are using to make Taq polymerase.
You do not have sufficient time to do the transformation and express the protein from your own transformed cells, but you will do the transformation and expression in parallel.
Take your aliquot of 100µl competent cells on ice and add 1 µl of (100pg/µl) plasmid DNA

THIS MUST BE KEPT ON ICE
Incubate on ice for 15 mins
Heat shock at 42 ºC for 90 seconds.
Incubate on ice for 2 minutes.
Add 900 µl of LB
Incubate at 37 ºC for 1 hour.
Ensure the cells are mixed by gently pipetting up and down.
Pipette 100 µl of cells onto a LB-amp plate and spread across the surface of the plate.
Spin the cells briefly in a microfuge for 30 seconds, this will pellet all the remaining cells.
Carefully pour off the supernatant, leaving a small drop behind.
Resuspend the pellet by gently pipetting the remaining solution up and down.
Pipette the remaining solution onto the second plate and spread across the surface.
Incubate the plates overnight at 37 ºC.
After incubating the plates overnight the cells that are transformed will grow and turn into colonies.
By counting the number of colonies that have grown you can calculate the transformation efficiency.
You will need to take pictures of both the plates and count the number of cells on each plate.

SDS-PolyAcrylamide Gel Electrophoresis
SDS-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) allows proteins to be separated according to their size. Proteins are unfolded in the SDS-loading buffer which uses the detergent Sodium Dodecyl Sulphate (SDS) to bind to the proteins, keeping them unfolded and giving them a uniform mass to charge ratio when the gel is run.
Using SDS-PAGE you will be able to show the proteins that are present in your samples. This will allow you to show the purification of the Taq polymerase through the steps of expression and purification.
You will need to cast an SDS-PAGE gel. Each gel will allow you to run a total of 10 samples, so **gels can be shared between two.**

SDS-PAGE (BioRad)
Equipment & Materials:
Bio-Rad Mini-PROTEAN 3 Cell
Acrylamide/bis-acrylamide (30% w/v/0.8% w/v)
1.5M Tris/HCl, pH 8.8 (resolving gel stock)
0.5M Tris/HCl, pH 6.8 (stacking gel stock)
TEMED
SDS (10% w/v)
Ammonium persulphate (APS; 25% w/v)
Running buffer [0.025M Tris/0.192M glycine/0.1% (w/v) SDS, pH8.3]
Pre-stained molecular markers (PageRuler)
Coomassie Blue Stain Solution (0.25% Comassie Blue, 10% MeOH, 8% Acetic Acid)
Samples

Experiment Procedures:

Preparation of Sodium Dodecyl Sulphate-Polyacrylamide (SDS-PAGE) gel

Gel Cassette Sandwich Preparation:
1: Place the Casting Frame upright with the pressure cams in the open position and facing forward on a flat surface.
2: Select a Spacer Plate and place a Short Plate on top of it.
C: Slide the two glass into the Casting Frame, keeping the Short Plate facing the front of the frame.

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3: When the glass plates are in place, engage the pressure cams to secure the glass cassette sandwich in the Casting Frame. Check that both plates are flush at the bottom.
4: Engage the spring loaded lever and place the gel cassette assembly on the gray casting stand gasket. Insure the horizontal ribs on the back of the Casting Frame are flush against the face of the Casting Stand and the glass plates are perpendicular to the level surface. The lever pushes the Spacer Place down against the gray rubber gasket.
5: Repeat steps A-E for a second gel.

Add water into the Gel Cassette Sandwich and ensure no leaking occurs!

6: Place a comb completely into the assembled gel cassette. Mark the glass plate 1 cm below the comb teeth. This is the level to which the resolving gel is poured. **Remove the comb.**
7: Prepare the resolving gel solution in a tube by mixing all reagents except TEMED and APS as listed in Table 1.
8: Add TEMED and APS to the solution and pour to the mark using a disposable plastic pipette. Pour the solution smoothly to prevent it from mixing with air.
9: Immediately overlay the solution with water. Add water slowly and evenly to prevent mixing.
10: Allow the gel to polymerize for 30-45 mins.
11. While you are waiting for polymerisation of the gel. Prepare the stacking gel solution. Combine all reagents except TEMED and APS as listed in Table 1.
12: After 30-45 mins of polymerisation, rinse the gel surface completely with distilled water.
13: Before casting the stacking gel, insert a piece of filter paper to dry the area in between the glass plates above the resolving gel. Take care not to touch the surface of the gel.
14: Insert the comb between the spacers starting at the top of the Spacer Plate, making sure that tabs at the ends of each comb are guided between the spacers.
15: Add TEMED and APS to the stacking gel solution and pour the solution between the glass plates. Continue to pour until the top of the short plate is reached.
16: Allow the stacking gel to polymerize.

Table Preparation of resolving and stacking gels (x 1) for SDS-PAGE (13.5%)

Solution	Resolving gel (7.5 ml)	Stacking gel (5 ml)
Acrylamide/bis-acrylamide	3.38	1
1.5M Tris/HCl (pH8.8)	3	
0.5M Tris/HCl (pH6.8)		0.630
dH_2O	1.062	3.32
10% SDS	0.038	0.025
Ammonium persulphate (25%)	0.015	0.010
TEMED	0.005	0.005

ELECTROPHORESIS MODULE ASSEMBLY

1: Gently remove the comb and fill the wells with running buffer.
2: Remove the Gel Cassette Assemblies from the Casting Stand. Rotate the cams of the Casting Frames inward to release the Gel Cassette Sandwich.
3: Place a Gel Cassette Sandwich into the slots at the bottom of each side of the Electrode Assembly. Be sure the Short Plate of the Gel Cassette Sandwich faces inward toward the notches of the U-shaped gaskets.
4: Lift the Gel Cassette Sandwich into place against the green gaskets and slide into the Clamping Frame.
5: Press down on the Electrode Assembly while closing the two cam levers of the Clamping Frame to form the Inner Chamber and to insure a proper seal of the short plate against the notch on the U-shaped gasket. Short plate must align with notch in gasket.

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6: Lower the Inner Chamber Assembly into the Mini Tank. Fill the inner chamber with ~125ml of running buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the Gel Cassettes. Do not overfill the Inner Chamber Assembly.
7: Add ~200ml of running buffer to the Mini Tank (lower buffer chamber).

Sample Loading:
8: Heat all samples in a hotblock (set to 100°C) for 2 mins and then cool them down to R.T..
9: Load each of the samples into the wells using a gel loading tip. Load samples slowly to allow them to settle evenly on the bottom of the well. Be careful not to puncture the bottom of the well with the tip.
10: Lane 1 - load 5µl of the pre-stained molecular markers (PageRuler) to check migration of proteins.
11: Lane 2 –
12: Lane 3 –
13: Lane 4 –
14: Lane 5 –

Gel Electrophoresis:
12: Place the Lid on the Mini Tank. Make sure to align the colour coded banana plugs and jacks. The correct orientation is made by matching the jacks on the lid with the banana plugs on the electrode assembly. A stop on the lid prevents incorrect orientation.
13: Insert the electrical leads into a suitable power supply with the proper polarity.
14: Apply power to the Mini-PROTEAN 3 cell and begin electrophoresis. 200 volts constant is set for SDS-PAGE gel and run time is about 35 mins, until the bromophenol blue dye-front has migrated 0.5-1 cm to the bottom of gel.

Stain and De-stain gel(s):
15: After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
16: Remove the tank lid and carefully lift out the Inner Chamber Assembly. Pour off and discard the running buffer before opening the cams to avoid spilling the buffer.
17: Open the cams of the Champing Frame. Pull the Electrode Assembly out of the Clamping Frame and remove the Gel Cassette Sandwiches.
18: Remove the gels from the Gel Cassette Sandwich by gently separating the two plates of the gel cassette. The green, wedge-shaped, plastic Gel Releaser may be used to help pry the glass plates apart.
19: Run the sharp edge of the Gel Releaser or a razor blade alone each spacer to separate the gel from the spacer.
20: The gel is then submerged with 20-30ml Instant-blue stain solution for 15 mins with gently shaking.
21: Rinse the gel with water until the protein bands are seen clearly.
22: Use camera or mobile phone to take photo of the gel.

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