Detection of a mutation in protooncogene *RET* causing multiple endocrine neoplasia, type 2A (MEN2A), using PCR and a restriction enzyme

(Practical activity in the Cybertory virtual laboratory)

Fundamentals:

Molecular genetics studies have evidenced that MEN2A neoplasia results from mutations if the RET protooncogene. A presymptomatic diagnosis is feasible by detecting such mutations in a sample of genomic DNA. The most common mutation (T>C, 80% of the MEN2A cases) produces a new target sequence for the *Hin* 6I restriction enzyme, a sequence missing in the normal gene. As a consequence, after using PCR to amplify the region that encompasses the point of mutation, the presence or absence of the mutation may be checked analysing the DNA fragments generated by *Hin* 6I.

How to start:

The virtual laboratory works inside a web page, so you should start by opening the web browser in your computer or tablet. Any modern browser should do, as long as JavaScript is enabled for it (we can recommend Firefox or Chrome).

Navigate to the Cybertory page at `http://biomodel.uah.es/en/lab/cybertory/`

First part of the assay:
PCR amplification of exon 11 in RET *protooncogene*

Objective:

Analysis of genetic material obtained from samples from several patients, using a pair of primers specific for a region in exon 11 which includes the polymorphic point of the T>C mutation responsible for MEN2A. Starting material is genomic DNA from the patients; PCR is used to amplify the region of interest. Resulting samples are stored to be used in the second part of the assay.

As a control to check specificity of results, additional reaction mixtures will be prepared, one omitting DNA, another omitting primers.

Virtual materials:

Software:
- "Cybertory" virtual molecular biology laboratory, `biomodel.uah.es/en/lab/cybertory/`

Virtual reagents:
- *Sample set for genetic diagnosis of MEN2A* (bought from CygnusLab™), which contains DNA from three patients to be diagnosed (Pt1, Pt2, Pt3)..
- CygnusLab™ 10x PCR Master Mix™ containing
  - 250 U/mL *CygnusTaq™* DNA polymerase
  - dATP, dGTP, dTTP, cCTP nucleotides, 2 mM each
  - 15 mM $MgCl_2$
  - 125 mM TAPS buffer, pH=8.5
- PCR primer pairs, specific for a region in exon 11 of the *RET* protooncogene
- Nuclease-free water

Virtual instrumentation:
- Variable volume micropipette, up to 100 µL
- Yellow pipette tips, usable from 1 to 100 µL
- CygnusLab PCtRonic™ thermocycler

Procedure:

Start by pressing the "Start Cybertory" button. After a few seconds, a notice will appear prompting you to place an order with samples and enzymes; please accept it.

1) Investigate the `Online catalog`.
2) Press the "`Place an order`" button.

doi:10.5281/zenodo.5168483

3) Select "`Sample set for RET protooncogene, 1st part of the experiment`" and the PCR401 primers.
4) At the bottom of the page you will need to provide some data. (This is a simulation of a real-life situation; it is not important what you type for first name and surname, but it is crucial that the password given is the one assigned to the institution).
5) Press the "`Send order`" button and then "`Confirm the order`". Wait until the screen is updated displaying the simulated laboratory (tubes, pipette, etc.).

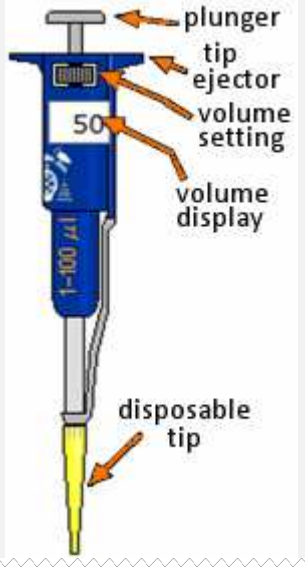
Using the virtual micropipette

To select the tube on which you want to act, you must click on it (not on the cap or on the label); the cap will open and the pipette will move to the tube. A click on the top plunger makes it slide, either expelling or aspirating the tip contents. The volume to be dispensed is set either clicking on the adjustment wheel (left half decreases, right half increases) or typing in the volume display slot (please do not press the Enter key).

The working range of this pipette is from 1 to 100 µL, in 1 µL steps.

To avoid contamination, you should change tips every time you change samples. The tip is expelled by clicking on the ejector button or on the trash bin.

You get a new tip by clicking on the tip box.



Possible problems:

Should you need, due to any mistake, to start anew with fresh tubes, click on the image of the container to the left of the tip box.

After clicking on a tube, the pipette, etc., please allow some time for anything to happen. Do not be impatient.

If you click on the pipette plunger and the hand cursor does not disappear, move the pointer away from the pipette.

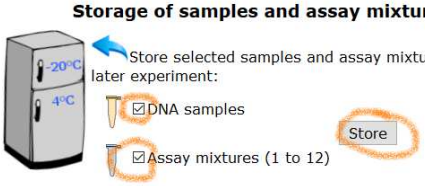
You can better see the liquid inside a tube by having the pipette leave the tube; to do so, click again on the tube.

6) Prepare reaction mixtures, different in each tube, for the PCR assay. To do so, add into the tubes the amounts indicated in this table:

	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**
DNA sample	25 µL from Pt1	25 µL from Pt2	25 µL from Pt3	–	25 µL from Pt3
Water, sterile				25 µL	2 µL
Primer mixture "PCR401"	2 µL	2 µL	2 µL	2 µL	–
PCR mix 10×	3 µL	3 µL	3 µL	3 µL	3 µL

Tube 4 serves as control lacking DNA.
Tube 5 serves as control lacking primers.

7) Start the restriction reaction by pressing the "`incubate`" button (located at the bottom centre, under the timer). After the required virtual time is up, the reaction will be complete. Depending on how the laboratory is configured, a warning may be displayed informing of what has been added to each tube; please check that it matches what you intended.
8) To preserve for the 2nd part of the experiment the DNA samples and the reaction mixtures resulting from PCR, click on the refrigerator, tick both checkboxes and click on "`Store`".



Note:
to preserve samples between the 1st and 2nd parts of the experiment, you must not close the browser tab or window.

doi:10.5281/zenodo.5168483

Second part of the assay: restriction enzyme digestion of the PCR products, to detect the mutation

Objective:

Once PCR has provided sufficient amount of DNA to be observed in an electrophoretic gel, the presence or absence of the mutation may be ascertained by observing the different DNA fragments generated by the restriction enzyme.

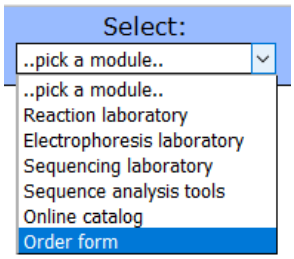
As a control to check specificity of results, an additional reaction mixture will be prepared omitting the enzyme. Controls from the first part of the experiment will also be subjected to the enzyme.

Virtual materials:

Software:
- "Cybertory" virtual molecular biology laboratory, `biomodel.uah.es/en/lab/cybertory/`

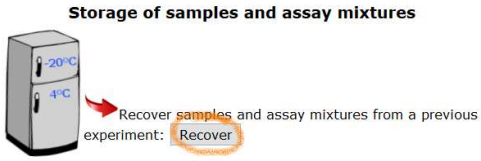
Virtual reagents:
- Restriction enzyme *Hin* 6I (bought from CygnusLab™, as detailed further ahead)
- Samples prepared in the first part of the assay, from the genomic DNA of the 3 patients, and strored in the refrigerator.
- Ladder2™ DNA molecular mass marker (from CygnusLab™)
- Universal restriction buffer 10x (from CygnusLab™)
- Nuclease-free water
- Agarose
- 0.5x TBE electrophoresis buffer (Tris/Borate/EDTA)
- Ethidium bromide
- Gel loading dye (containing bromophenol blue, xylene cyanol and glycerol)

Virtual instrumentation:
- Variable volume micropipette, up to 100 µL
- Yellow pipette tips, usable from 1 to 100 µL
- CygnusLab TempeMatic™ incubator with thermal regulation
- CygnusLab GelMatic™Plus tank for horizontal agarose gel electrophoresis
- CygnusLab 1000ZX™ DC power source (from 10 to 1000 V)
- Ultraviolet transilluminator (attached to the electrophoresis tank)
- Protective screen ad goggles against UV light



Procedure:

1) Using the main Cybertory menu (top-right of the window) to return to the "`Order form`".
2) Select "`Sample set for RET protooncogene, 2nd part of the experiment`" and the *Hin* 6I enzyme. (Actually, we will not use these samples but those we have prepared using PCR in the 1st part of the experiment)
3) Enter data and password, press the "`Send order`" button and then "`Confirm the order`". Wait until the screen is updated displaying the simulated laboratory (tubes, pipette, etc.).
4) To work with those samples prepared in the 1st part, click on the refrigerator and then on "`Recover`".



5) Prepare reaction mixtures using the contents of tubes 1 to 5, to perform digestion with the *Hin* 6I enzyme, for example like this:

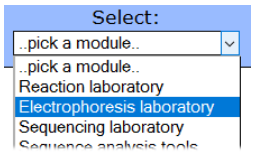
	Tube 7	Tube 8	Tube 9	Tube 10	Tube 11	Tube 12
PCR product	10 µL from tube 1	10 µL from tube 2	10 µL from tube 3	10 µL from tube 4	10 µL from tube 5	10 µL from tube 2
10× buffer	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL
Water, sterile	16 µL	16 µL	16 µL	16 µL	16 µL	17 µL
Hin 6I	1 µL	1 µL	1 µL	1 µL	1 µL	–

Tube12 serves as a control, lacking enzyme.

doi:10.5281/zenodo.5168483

6) Start the restriction reaction by pressing the "`incubate`" button (located at the bottom centre, under the timer). After the required virtual time is up, the reaction will be complete. Depending on how the laboratory is configured, a warning may be displayed informing of what has been added to each tube; please check that it matches what you intended.

7) In the main Cybertory menu, select the "`Electrophoresis laboratory`" module. Choosing in the tank a `5% polyacrylamide` gel is recommended, but the assay may be repeated with a 2% `agarose` gel. Press the "`autoload`" button, so the 12 samples prepared in the previous PCR will be transferred to wells in the virtual gel; well number 13 will be automatically loaded with a mixture of size standards (the Ladder2™ DNA).



After loading, the wells are coloured blue due to the loading dyes commonly added to samples (bromophenol blue and xylene cyanol), which makes it easier to see initially the samples that are being loaded and, afterwards, the electrophoresis front. The loading solution also includes some dense compound (like glycerol, sucrose or ficoll) which makes the samples sink to the bottom of the well. Agarose gels like this one are regularly set horizontally.

"Autoloading" is a unique feature of the CygnusLab™ gels; in real life, samples must be laboriously transferred manually from reaction tubes to gel wells using a micropipette.

8) In the power source, adjust voltage and virtual time (200 V during one hour if the `5% polyacrylamide` gel was chosen, or 300 V during 1.5 hours for a 2% `agarose` gel). Switch the current on to start the electrophoresis. During its progress you may switch the UV light on (please make sure you have put on your protecting goggles or face mask) and switch the virtual room lights off in order to watch the DNA moving forward and the bands separating.

In any electrophoresis, if the available power source does not indicate current intensity (milliamperes), it is important that you make sure the current is indeed circulating. Observe how bubbles are released from both electrodes, as a result of electrolysis of the buffer. You will also see that the blue bands of both dyes move along the gel (as well as do the DNA bands, but you only see these if you switch the UV light on).

If higher voltages are used, samples will progress quicker along the gel, but high voltages produce high intensity current that will heat up the gel; this may affect the sample integrity (for instance, denaturing DNA) and may also melt the agarose. Unless real life gels, the CygnusLab™ gels never melt, so the only inconvenience of using very high voltage is that samples may be lost off the bottom of the gel before you have time to react.

9) Once the electrophoresis is finished, draw a sketch of the position of bands (you may capture an image using the digital camera attached to the electrophoresis tank) and interpret your results:

- Explain the difference observed between tubes 1 and 7 (prepared with DNA from the same patient). Similarly, differences in the pairs 2 and 8, 3 and 9.
- Interpret the identity of each DNA band observed in the gel under UV light.
- Which patient(s) present the mutation?
- Are they homozygotic or heterozygotic for the mutation?
- What information can be obtained from the controls (tubes 4, 5, 10, 11 and 12)?
- Why is it important that water and all materials used have been previously sterilised?

doi:10.5281/zenodo.5168483