SAFETY RISK ANALYSIS
E. CARU PROJECT

IGEM CIDEB 2014
http://2014hs.igem.org/Team:CIDEB-UANL_Mexico

Overview

IGEM CIDEB 2014 considers biosafety as important as every other points of the iGEM competition. Because of this reason, the team decided to perform a Safety Risk Assessment focused on the project and in the lab practices needed to accomplish it.

In this assessment, a description of our host organism is made, along with the genetic modifications that were applied to it, including preventive measures to avoid its dissemination and appropriate identification and containment measures, in the case it was released into the environment. Also the overall potential risks of the project were included, taking in consideration all of the possible risks of working in our laboratory, along with preventive measures to reduce risk to a minimum.

Organism's Description

Escherichia coli (*E. coli*) is a large and diverse genus of bacteria belonging to the *Enterobacteriaceae*. Although most strains of *E. coli* are relatively harmless, some can potentially affect humans and animals. Pathogenic kinds of *E. coli* can cause diarrhea, along with urinary tract infections, respiratory illness and pneumonia, among other symptoms. *E. coli* can be commonly found in the digestive tract of humans and many animals. It plays an important role in the decomposition and absorption of certain nutrients in the intestine that the body cannot break down by itself and to also prevent the digestive track to be colonized by other harmful bacteria.

E. coli are capable of both aerobic and anaerobic cellular respiration, which is a characteristic that allows them to live in both oxygen rich and oxygen poor environments, which has allowed them to thrive in a wide variety of ecosystems.

As a prokaryote, *E. coli* bacterium has no organelles, and its genetic information is not enclosed in a nucleus. *E. coli* protective layer consists on a cell wall and a capsule that protects it from the outside, potentially harmful environment. *E. coli* goes through binary fusion on a regular basis if given the right conditions, usually at 37° Celsius, and it is able to thrive and reproduce at a very fast rate.

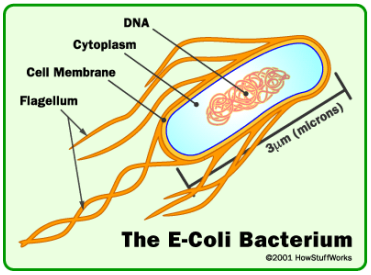

Figure 1: E. coli bacterium and its parts.

As previously mentioned, *E. coli* is one of the most diverse genera of bacteria, probably due to its adaptive abilities. Although there is a wide variety of different *E. coli* strains to choose from, not all of them have the same characteristics; some of them are pathogenic and are not safe to work with, which is the main reason why during the practices at the team's laboratory, the *E. coli*'s strain that was used is the K12 DH5-α strain, which is one of the safest strains to work with, and one of the most used in biotechnology research. The K12 DH5-α strain is characterized by its poor abilities to colonize plant and animal tissue, and a low

resistance to outside-lab environment, temperature fluctuation and different media composition causing the organism to die.

E. coli's K12 DH5-α inability to colonize intestinal tissue was experimented in 1978 in a work made by R. Curtiss "Biological containment and cloning vector transmissibility" showing that the K12 DH5-α strain is not likely to behave as a pathogen in mammal tissue. Due to these previous mentioned characteristics, it is classified as a Class 1 Containment under the European Federation of Biotechnology guidelines, and according to the United States Environmental Control Agency (EPA) *E. coli* K12 DH5-α strain opposes a very low risk for other organisms and under contained conditions of fermentation and are safe to work with.

Genetic modifications

In order to accomplish the iGEM CIDEB 2014 project's objective, *E. coli* went through some genetic modifications. The E. CARU project is divided into four different modules, each one of them adding a different characteristic to the bacterium. The four modules are:

1. Resistance
2. Capture
3. Aroma
4. Union

1. Resistance Module

Unmodified *E. coli* K-12 is able to tolerate added salt of up to 10% concentration (M. Don, 2008), however, E. CARU was tested with higher amounts than those mentioned (For further information, check the Capture module in the wiki).

In order to work with abnormal higher saline concentrations without killing the bacteria, IrrE, a gene that provides resistance to some adverse conditions for it, was introduced to *E. coli.*

The gene IrrE up regulates the production of several stress responsive proteins, protein kinases, metabolic proteins, and detoxification proteins. It also down-regulates glycerol degradation. With this global regulatory effect, *E. coli* becomes more salt tolerant (UCL, 2012).

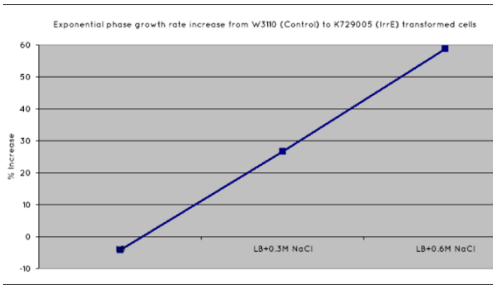

Figure 2: Exponential phase growth rate increase from W3110 (Control) to K729005 (IrrE) transformed cells. (UCL 2012)

The module's sequence is as follows:

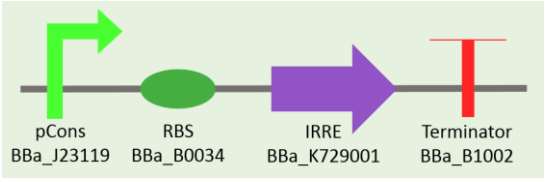

Figure 3: Resistance Module

The sequence begins with a constitutive promoter (BBa_J23119), followed by an RBS (BBa_B0034), the gene IrrE (BBa_K729001) and a terminator (BBa_B1002).

2. Capture Module

One of the most important genetic modifications in the project is the capture of sodium ions in order to desalinize water. This was made possible by taking advantage of NhaS, a putative gene which is characterized after its expression, "By its corresponding protein ability to bind and sequestering sodium ions." *(*Ivey, Krulwich, 1994*).*

The project's circuit sequence is:

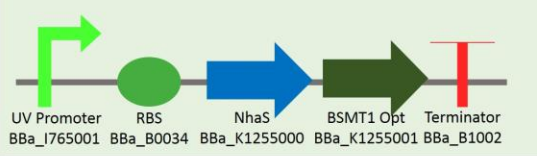

Figure 4: Project's sequence for Capture module

Since NhaS is putative, iGEM CIDEB 2014 decided to test the module with a red fluorescence protein (BBa_E1010), which is simpler than the original reporter idea for the module, and this allowed us to test one gene at a time in each module.

The sequence used for the NhaS experimentation is:

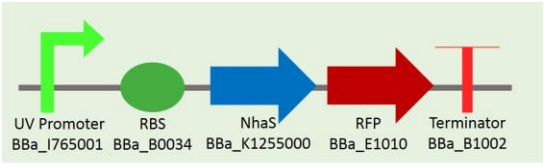

Figure 5: Practical sequence for Capture module

An UV Promoter (BBa_I765001) was chosen to begin the circuit in order to control the NhaS gene's expression in E. CARU. The promoter is followed by an RBS (BBa_B0034), the NhaS gene (BBa_K1255000), an RFP reporter (BBa_E1010) and a terminator (BBa_B1002).

Basically the same, just changing the RFP reporter for BSMT1 Opt (BBa_K1255001), which is the CDS that is able to produce a Wintergreen aroma. For further information look at the Aroma module in this document.

3. Aroma module

The use of reporters differing from the usual fluorescence proteins is one of the objectives iGEM CIDEB 2014 team is pursuing by using aromatic reporters, like banana or, in this case, wintergreen odor.

The aroma module is used in order to prove the effectiveness of BSMT1 Opt CDS (BBa_K1255001), for further use as an odor reporter for other teams and modules for this project.

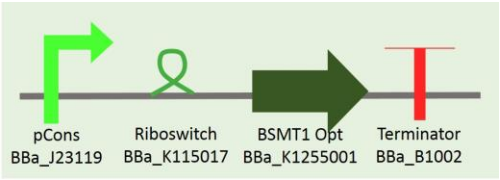

Figure 6: Aroma module

BSMT1 (Salicylic Acid Carboxyl Methyltransferase I) is formed as part of a different circuit, composed by a constitutive promoter (BBa_J23119), a riboswitch (RNA thermometer, BBa_K115017), a CDS that, when it is induced by salicylic acid, it releases an enzymatic product (methyl salicylate), responsible of wintergreen odor, and a terminator (BBa_B1002).

This sequence will help to test its effectiveness and future usage as an odor reporter, since other teams (MIT 2006)

have just analyzed it theoretically. IGEM CIDEB 2013 uses a riboswitch to induce the gene expression at high temperatures.

This piece (BSMT1 Opt) can replace RFP on capture module, or be added on union module; as wintergreen odor to demonstrate the presence of bacteria in silica beads or the capture of sodium ions on salty environments.

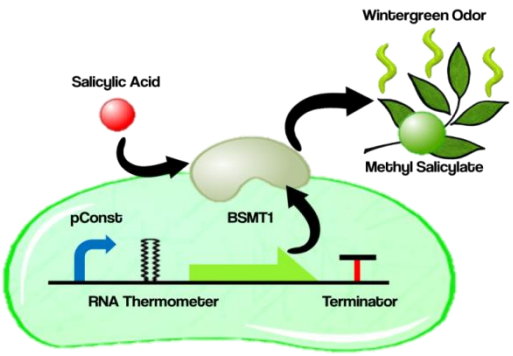

Figure 7: Aroma module's function

This module will be tested on a culture medium, and induced by salicylic acid to produce WG (WinterGreen) odor.

4. Union module

The main objective for iGEM CIDEB 2014 team is the construction of a biological circuit capable to capture sodium ions, and to remove them by using a silica-beads based bio-filter. In this module, the outer membrane of the bacteria is modified so it can bind silica or glass surfaces.

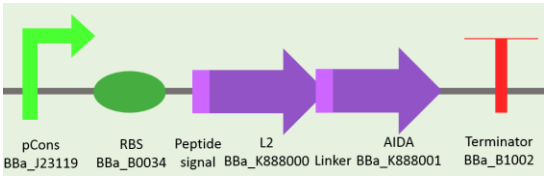

Figure 8: Union module

This device is composed by a constitutive promoter (BBa_J23119), a common RBS (BBa_B0034), a fusion protein that of a set which includes the CDS L2 with its peptide signal and AIDA, in order to make the protein for binding silica; a membrane protein L2 (BBa_K888000); AIDA (BBa_888001), and a terminator (BBa_B1002).

This module was available and proportionated by UANL iGEM 2013 team. A silica bio-filter will be used to remove *E. coli* from the water, but, in order to have also qualitative evidence of *E. coli's* attachment to silica beads, the aroma module's function as reporter will indicate its presence in the silica.

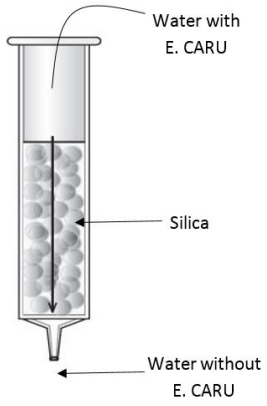

Figure 8: Biofilter with E. CARU

Potential Risks

The project uses *E. coli* K-12 as the host bacteria, causing it to be resistant to saline water, capture sodium ions, getting attached to silica surfaces and releasing the winter-green aroma as reporter.

Potential Risks with the bacteria are minimum because, as previously mentioned in the organism's description,

the strain used is a non-pathogenic type. *E. coli* K12 has no known survival mechanism in living tissues, nor any of the health affecting symptoms that some varieties have.

E. coli is absolutely safe, as P Kunhert states: "They are [E. coli K 12] classified as biologically safe vehicles for the propagation of many efficient gene cloning and expression vectors in all major national and international guidelines on biological safety for work with recombinant DNA technology" (1995).

K-12 strain is defective in its cell wall components relevant to the ability to reorganize and adhere to the mucosal surface of colonic cells (Curtiss 1978). It does not have the type of glycocalyx required for attachment to the mucosal surface of the human colon.

Performing genetic modifications in the project was not dangerous because the materials were handled carefully, in accordance with the "Laboratory Category One" guidelines published by the World Health Organization (WHO).

The modified *E. coli* parts are safe for the environment and for human use. Variables are controlled by the team, as in the case of Capture module (NhaS) which is controlled by a UV promoter.

Similarly, the Aroma module is controlled by a Riboswitch regulated by temperature in order to decide when and how is going to be activated, besides adding salicylic acid, which is only harmful in high doses for humans, and in the project it is used in very small amounts (2mm) to activate it.

The resistance module is also safe, since it gives the bacteria the ability to resist adverse conditions that would normally kill it, like high temperatures, high saline levels and high UV radiation, and nevertheless the bacteria would die in a normal environment.

The Union module gives E. CARU the ability to bind glass and silica surfaces, which we will use in beads form with the presentation that is used commercially. No direct risks are related to the module itself, but Silica gel beads may contain toxic and potentially carcinogenic cobalt (II) chloride, which is added as a moisture indicator in commercially available product. This point is analyzed further in the "Union as a safety measurement" section.

Lab methods

IGEM CIDEB 2014 had to take in consideration some aspects about biosafety when working in the project. At all times an instructor was present as a supervisor during the practices.

The team followed what the WHO's category 1 laboratory guidelines indicated. The use of lab coat, long jeans, closed shoes and, if needed, tied hair was performed at all times while working on the lab. When managing dangerous substances gloves and/or special glasses were used depending on the situation.

When working with the trans-illuminator, the team took care of not looking directly the UV rays, instead a plastic UV protective shield and glasses were used while working with UV.

The laboratory was kept perfectly clean and dry, especially the place in which the instruments were located nearby electrical outputs. Glassware were reviewed and checked for cracks, before each usage; especially before applying under vacuum or pressure on them.

The generated waste was always placed in a properly labeled waste container, placed handily to avoid spills.

Hazardous substances

While working in the E.CARU project, specifically in the laboratory, the team was exposed to different substances that, if managed incorrectly, could cause harmful effects.

The main substances used in the project were the following (click for further information):

- Ethidium bromide
- NaOH
- Acetic acid
- Methyl salicylate
- UV radiation
- Tris HCl
- EDTA (Ethylenediamineetetraacetic acid)
- DSS (Dodecyl-sodium sulfate)
- Potassium acetate
- Agarose
- Calcium Chloride ($CaCl_2$)
- Ethanol (Ethyl Alcohol)
- "Lysogenic Broth" (LB) broth and agar
- Buffers
- RNase
- Restriction enzymes
- Saline water
- Milli-Q water

Union as a safety measurement

The Union module in the project has a genetic device that gives the bacteria the ability to anchor itself to silica surfaces. The team used this ability to use the bacteria as a biological filter. This biological filter would clean the water free of *Escherichia coli* to meet a solution for salty water.

Why silica pearls? Because silica pearls are very common to find, and even though that they are known for being harmful for humans if ingested, it's not the silica gel that is toxic, what is toxic are the substances that the silica pearl can absorb (silica gel is known for its characteristic of having a great ability for absorbing humidity). Silica is, for example, used as a safe food additive, this means no permit is needed when added.

If people are not comfortable with using silica gel pearls for the biological filter, glass pearls can also be used, as the genetic device permits anchorage to glass surfaces, and glass has the advantage of not releasing any kind of substance if introduced in water, even for long periods of time, for the bacterium to attach. Also, for more efficiency in the de-

contamination, glass recipients could be used, that way it would be easier for the anchorage of the bacterium.

The functioning of the biological filter is very simple: due to the reason that after the first three modules of the project, salt water would still have the genetically modified bacteria, which will have sodium ions within its cellular membrane, a filtration process will be carried out by silica pearls, which will result in desalinized water without the before mentioned bacteria.

For the filtration process to happen, no modification was used besides the genetic information that gives *Escherichia coli* the ability to adhere to the silica gel or to glass surfaces. Thus, when bacteria have already captured the desired amount of sodium ions, by adding silica or glass beads, the bacteria will attach in these and this will help remove the GMO (Genetically Modified Organism) in the water by an easy filtration of the water to be free of silica or glass beads containing the bacteria.

The risks considered in the use of silica beads are minimal since "The silica gel is inert and considered a non-toxic product," according to CITUC (UC Toxicological Information Centre for its acronym in Spanish). Actually, if the pearls are not correctly removed, there is only one risk: choking hazard, which is prevented by the safety guidelines that are followed in the laboratory and warns that under no circumstance water should be ingested with or without the silica beads before being tested and approved.

The biological filter, besides being the main purpose of our project, is one of the team's safety measurements, because it prevents the bacterium from contaminating and reproducing in water by being removed.

Method to shut off E.CARU

When treating with GMOs there's no such thing as "overprotection". iGEM CIDEB 2014 takes safety seriously and decided that the project should include at least one method to shut off *E. coli* in case of an unexpected emergency situation, at least in a theoretical way.

The method proposed is simple, increasing the intensity of the UV light already used in the Project to turn off the organism.

First of all, Ultraviolet or UV light is light with wavelengths from 100 to 400 nm. According to Meulemans in 1986, its spectrum is divided into 4 different categories, as the following table shows:

Type of UV	Wavelength
Vacuum UV	100 to 200 nm
UV-C	200 to 280 nm
UV-B	280 to 315 nm
UV-A	315 to 400 nm

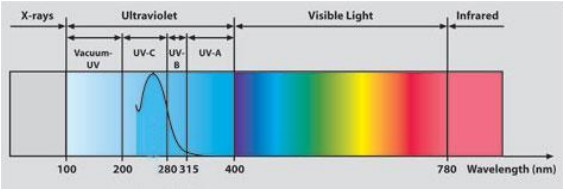

Figure 9: Different types of UV rays in the light spectrum.

EPA (United States Environmental Protection Agency) states that disinfection by UV primarily occurs due to the germicidal action of UV-B and UV-C light on microorganisms. And that the germicidal action of UV-A light is small relative to UV-B and UV-C light. So, in order for UV-A light to cause a "shut off" effect in the organism, very long exposure times are necessary.

What UV light does to microorganisms is that, once absorbed, it modifies and damages irreparably the DNA of the organism on the matters of question, in this case *E. coli K12*, by causing a photochemical damage. Therefore, RNA and DNA, molecules in charge of storing and carrying genetic information, can't function properly, causing it to lose the ability to reproduce.

As Wolfe in 1990 states, and with the information from EPA, 254 nm is the most potent wavelength that can cause damage in DNA. 254 nm belongs to the UV-C light category and the team is planning to use it that way to deactivate E. CARU in extreme situations.

The UV light already used in the project is a promoter of the Capture module. This promoter is activated by 360nm, so it belongs in the UV-A category, and as it was mentioned before, this does not cause damage to the bacteria in short periods of time.

According to our project, UV is one of the easiest methods to shut off our GMO.

Conclusions

After doing an analysis of the possible risk related to our project and the safety measures that should be taken into consideration, it can be concluded that the project itself do not represents a considerable risk for human health, due to the poor resistance of the used strain of bacteria, even with the IrrE resistance module, the bacteria is still too weak to thrive in a non-controlled environment. Even though the bacteria cannot live outside the lab, all of the safety related issues to bio-contention of the organisms were all taken into consideration to avoid any possible contamination outside the laboratory. As previously mentioned, the bacteria does not oppose a significant threat for safety, but some substances used in the lab can be a major health concern if not managed properly. Because of this, the laboratory rules were made according to the WHO level 1 laboratory guidelines, and supervision was always present to avoid threats for the team.

References

- ACETIC ACID (80%, 99.5%, Glacial). *Hazard Communication Sheet*. Retrieved May 12, 2014, from http://www.riskassessmentservices.co.uk/HazCom/Acetic%20Acid.pdf
- Agaroses Manual. *Condalab*. Retrieved May 12, 2014, from http://www.condalab.com/pdf/agarose_manual_09.pdf
- "A toxic death for ethidium bromide". (n.d.). *Laboratory News.* Retrieved May 13, 2014, from http://www.labnews.co.uk/features/a-toxic-death-for-ethidium-bromide/
- Boric Acid. *R.E.D. Facts*. Retrieved May 11, 2014, from

http://www.epa.gov/oppsrrd1/REDs/factsheets/0024fact.pdf

- Calcium Chloride - MSDS. (n.d.). *Calcium Chloride - MSDS*. Retrieved May 13, 2014, from http://www.calciumchloride.co.uk/calcium_chloride_msds.html
- CHEMWATCH. Sodium Dodecyl Sulfate. *Santa Cruz Biotechnology, Inc*. Retrieved May 12, 2014, from http://datasheets.scbt.com/sc-264510.pdf
- Curtiss, R, 1978, Biological containment and cloning vector transmissibility, J. *Infectious Dis.* 137:668-675
- Don Sarah (2008). *Optimal Conditions for the Growth of E Coli*. http://es.scribd.com/doc/11337868/Optimal-Conditions-for-the-Growth-of-E-Coli Retrieved May 11, 2014.
- EDETIC ACID (EDTA). *Summary Risk Assessment Report*. Retrieved May 12, 2014, from http://echa.europa.eu/documents/10162/5ed7db13-e932-4999-8514-378ce88ca51f
- Environmental Health and Safety (2007-09-10). "Silica Gel" http://hazard.com/msds/mf/baker/baker/files/s1610.htm. Retrieved 05-29-2014.
- Environmental Protection Agency. Sodium Hydroxide. *R.E.ED. Facts*. Retrieved May 1, 2014, from http://www.epa.gov/oppsrrd1/REDs/factsheets/4065fact.pdf
- *Escherichia coli K-12 Derivatives Final Risk Assessment.* EPA. Retrieved May 6, 2014, from *http://epa.gov/biotech_rule/pubs/fra/fra004.htm*
- ETHANOL. *Hazard Communication Sheet*. Retrieved May 12, 2014, from http://www.riskassessmentservices.co.uk/HazCom/Ethanol.pdf
- Ethylenediaminetetraacetic acid. *Summary of Initial Risk Assessment Report*. Retrieved May 12, 2014, from http://www.safe.nite.go.jp/english/risk/pdf/03_summary/047sum.pdf
- Fotadar U, Zaveloff P, Terracio L (2005). *"Growth of Escherichia coli at elevated temperatures"*. J. Basic Microbiology. **45** (5): 403–4.doi:10.1002/jobm.200410542. PMID 16187264
- EPA (2006). Ultraviolet disinfection guidance manual for the final long term 2 enhanced surface water treatment rule. Retrieved May 29th from http://www.epa.gov/ogwdw/disinfection/lt2/pdfs/guide_lt2_uvguidance.pdf
- EPA Guidance Manual (1999). *Alternative Disinfectants and Oxidants.* Chapter 8. Retrieved May 29th 2014.
- *General Information Escherichia coli (E. coli).* (2012, August 3). Centers for Disease Control and Prevention. Retrieved May 6, 2014, from *http://www.cdc.gov/ecoli/general/index.html*
- UANL Mexico (2012). Recovery module. Retrieved on March 28th, from: http://2012.igem.org/Team:UANL_Mty-Mexico/Project/recovery
- Helmestine Anne Marie, 2014. *What happens if you eat silica gel beads?* From http://chemistry.about.com/od/medicalhealth/f/What-Happens-If-You-Eat-Silica-Gel-Beads.htm Retrieved May 16, 2014.
- *IGEM biosafety 2013* from: http://www.biofaction.com/wp-content/uploads/2012/04/igem-biosafety-2013.pdf Retrieved May 16, 2014.
- IVEY Mark, KRULWICH Terry. (1994) *Sodium ion binding proteins*. Retrieved April 1st 2014 from http://www.google.com.mx/patents/US5346815
- Jin Huh (2008). Part BBa_k112808. From http://2012.igem.org/Team:METU/KillSwitch Overview Retrieved May 16, 2014.
- Jones, T. F. (2007). Investigation of Foodborne and Waterborne Disease Outbreaks. In P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry & M. A. Pfaller (Eds.), *Manual of Clinical Microbiology* (9th ed., pp. 152-169). Washignton, DC: ASM press.
- Kunhert, P, J Nicolet, and J Frey. "Applied and Environmental Microbiology." *Rapid and accurate identification of Escherichia Coli k-12 strands*. Ed. P Kunhert. N.P., 31 Aug. 1995. Web. 13 May 2014. <http://aem.asm.org/content/61/11/4135.full.pdf>.
- McWilliams, M. Luria Broth and Luria Agar Media and Their Uses Protocol. American Society for Microbiology: Microbe Library. Retrieved May 12, 2014, from http://www.microbelibrary.org/component/resource/laboratory-test/3031-luria-broth-lb-and-luria-agar-la-media-and-their-uses-protocol

- Material Safety Data Sheet. *United Biochemicals*. Retrieved May 12, 2014, from http://www.unitedbiochemicals.com/msds/tris_hcl.pdf
  Methyl salicylate overdose: Medline plus Medical Encyclopedia. (n.d.). *U.S National Library of Medicine*. Retrieved May 13, 2014, from http://www.nlm.nih.gov/medlineplus/ency/article/002683.htm
- MIT IGEM Team. (2006). MIT 2006. Retrieved on March 31th, 2014. From: http://2006.igem.org/wiki/index.php/MIT_2006
- *Normas de Seguridad Laboratorio* From http://www.uv.es/gammmm/Subsitio%20Operaciones/7%20normas%20de%20seguridad_archivos/NORMAS%20SEGURIDAD%20LABORATO RIO.pdf Retrieved May 16, 2014.
- Pérez, E. (n.d.). Boric acid poisoning: Medline Plus Medical Encyclopedia. *U.S National Library of Medicine*. Retrieved May 12, 2014, from http://www.nlm.nih.gov/medlineplus/ency/article/002485.html
- Ribonuclease A. (n.d.). - *Worthington Enzyme Manual*. Retrieved May 12, 2014, from http://www.worthington-biochem.com/RNASE/
- Ribonuclease A. (n.d.). *RCSB PDB-101*. Retrieved May 12, 2014, from http://www.rcsb.org/pdb/101/motm.do?momID=105
- "Silica Gel." *Centro De Información Toxicológica. CITUC*. Red Salud UC Facultad De Medicina. Web. http://www.cituc.cl/temas/ficha1.php?id=22 Retrieved 05-30-2014.
- The known health effects of UV. (n.d.). *WHO*. Retrieved May 13, 2014, from http://www.who.int/uv/faq/uvhealtfac/en/
- Type 1 Water, Milli-Q and Ultrapure Water System. (n.d.). Retrieved May 12, 2014, from http://www.millipore.com/lab_water/clw4/type1
- UCL iGEM Team. (2012). *IrrE module*. http://2012.igem.org/Team:University_College_London/Module_5. Retrieved March 31, 2014.
- Zubieta, Chole et al. (2003). Structural Basis for Substrate Recognition in the Salicylic Acid Carboxyl Methyltransferase Family. Manuscript submitted for publication. Retrieved from www.plantcell.org; American Society of Plant Biologists.