CHAPTER 3

EXPERIMENT

This chapter represents the part of the experiment consisting of a physical part and a biological part. The first experiment is the physical part. The physical part includes test of plasma machine, plasma power and plasma current measurement, obtaining plasma spectrum (plasma species), designing, constructing and installing the charge separation setup, and operation of the plasma jet bombardment of DNA samples. The biological part includes naked DNA preparation, naked DNA concentration measurement, gel electrophoresis for analyzing plasmid DNA form and finally electroporation for transferring plasmid DNA into *Escherichia coli (E.coli)*.

The research facilities available for this study are at Plasma-Bio & Green Energy Laboratory of Plasma and Beam Physics Research Facility (PBP) and Ion – beam building, Department of Physics and Materials Science, Faculty of Science**,** Chiang Mai University (CMU) and Microbiology laboratory, Department of Microbiology, Faculty of Science , Chiang Mai University (CMU).

3.1 Cold atmospheric pressure plasma jet system (CAPPJ)

The experimental facility is a cold atmospheric pressure plasma system (Atmospheric Pressure Plasma Jet: APPJ). The plasma generator is such a machine that can be operated at the atmospheric pressure. The generated plasma consists of high energy electrons and can be adapted and applied to clean surfaces of polymer or metal. Without a vacuum system, the facility is inexpensive, with less complexity scaled. Mixing different types of gases during operation can be done quickly.

In principle, plasmas produced using electricity further stimulates the molecules of gas or vapor for disintegration or ionization in the plasma state. The experimental setup of CAPPJ, as shown in Figure 3.1, was a commercial bio-plasma jet (Photo Bio Care Co. Ltd, Thailand) routinely used in clinic skin treatment.

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The plasma machine interface, as shown in Figure 3.2, can adjust intensity and frequency. Intensity represents a radio-frequency adjustment. Inside plasma output has an electrode. It can adjust intensity between levels 1 to 10. And another one, Frequency represents pulse of plasma jets. It can be adjusted between levels 10 to 110. The plasma was ignited in a standard way between coaxial central electrode needle and grounded outer electrode tube with an output diameter 2 millimeter. Helium (100%) gas was used in a flow rate of 0.6 slm (standard liter per minute) controlled by a Dwyer flowmeter. The plasma was powered by an RF supply unit, which included a pulse generator. This experiment set up used a stainless sample holder which could move a length of 30 centimeters and be adjusted in 3 axis movements (x-axis, y-axis and z-axis), as shown in Figure 3.3. For separation of charged species in the plasma jet to investigate effects of different physical agents, a pair of parallel electrodes (2 mm in width and 20 mm in length) made from aluminum was set with a gap of about 10 mm placed at 2 mm from the jet exist as shown Figure 3.4 and Figure 3.5. Dimensions of systems has width of the parallel electrode plates is 3 mm and gap of the parallel electrode plates is 6 mm and distance between the jet header exit and the sample stage is 8 mm, and diameter of the plasma jet exit is 1 mm. A voltage of 1 V was applied to the electrodes to produce an electrostatic field, through which the plasma jet passed. Then, we measured the power and current of the plasma down to sample holders. Finally, we measured plasma species by using optical emission spectroscopy (OES).

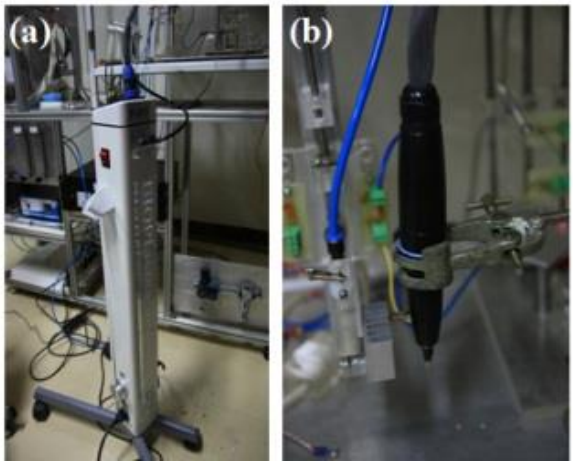

Figure.3.1. A photograph of the whole system (a) the standing column is the compact power supply, about 1.2 m high with wheels for easy move. (b) A photograph of the hand-held D-1 type jet, about 15 cm long. Now it was in the experimental fixture.

Figure.3.2.The CAPPJ apparatus used in the experiment. A photograph of the control panel's interface is at the top systems. It allows adjusting intensity and frequency of the plasma output.

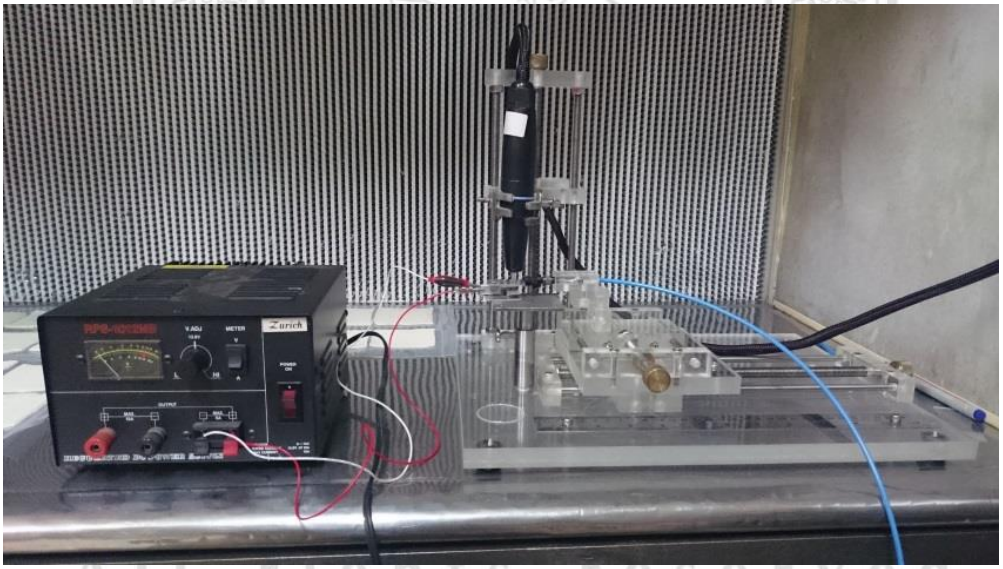

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Figure.3.3. Photograph of experimental set up consisting of CAPPJ, spectrum receiver, DC- voltage power supply and stainless sample holder.

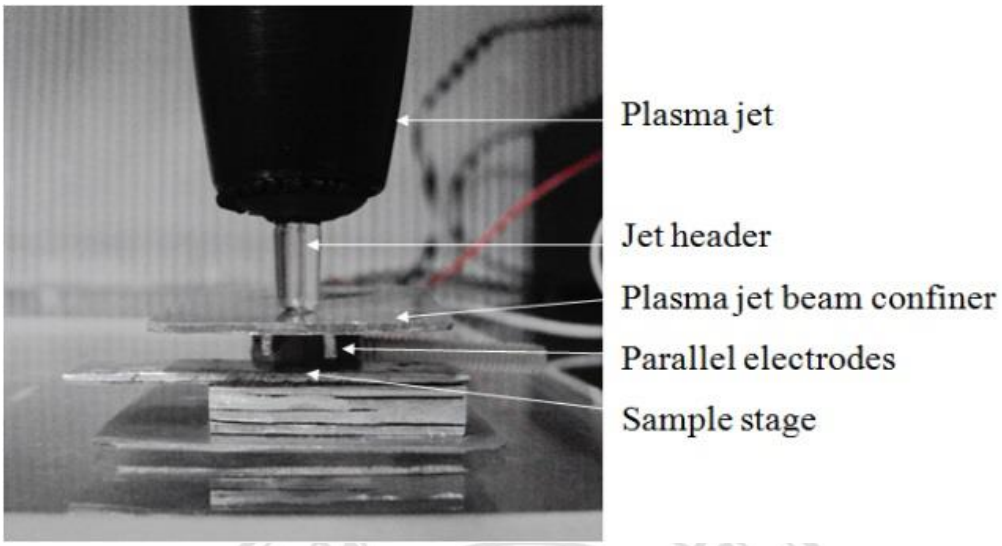

Figure.3.4. Photograph (front view) of the plasma jet charge separation setup using a traversal electrical field.

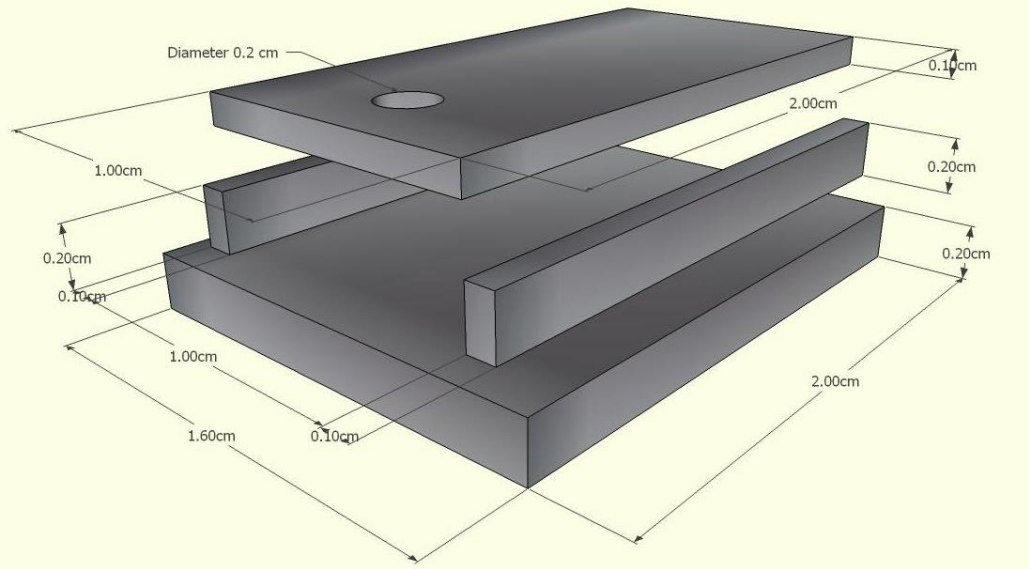

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Figure.3.5. Schematic of aluminum sample holder, which can load sample by inserting into between gaps of electrodes.

3.2. Measurement of the Cold Atmospheric Pressure Plasma generating energy and generating power

The plasma generating power was measured during the experiment by probes of Tektrinix P 6015A, as shown in Figure 3.6, and visualized on an oscilloscope. The sinusoidal shaped pulse was generated peak to peak with a repetition rate of 16.67 kHz. The CAPPJ plasma-generating power was measured using the V-Q lissajous figure

method (e.g. [14]). As shown in Figure 3.7, a voltage divider of V1 and V2 was used to measure the voltage of modulator which reduced the voltage drop in the ratio of 1/1000 and was connected to an oscilloscope. Charge was calculated from the capacitor voltage V_x and V_y which were read from the oscilloscope areas of the V-Q lissajous figure. The power to generate the plasma was calculated by multiplying the frequency used and the high voltage. The CAAPJ current was measured by using a multimeter set up under the sample holder as shown in Figure 3.7.

The V-Q lissajous graph is ellipse which has an area representing to plasma generating energy. The oscilloscope screen showed x-y axis display. It looked like an ellipse. The area inside the ellipse display can be used to calculate energy (Joules) and multiply with frequency of generator (Hertz) to equalize to the power of generated plasma (Watts).

Figure.3.6. Photograph of probes (Tektrinix P 6015A), which reduced the voltage drop in the ratio of 1/1000.

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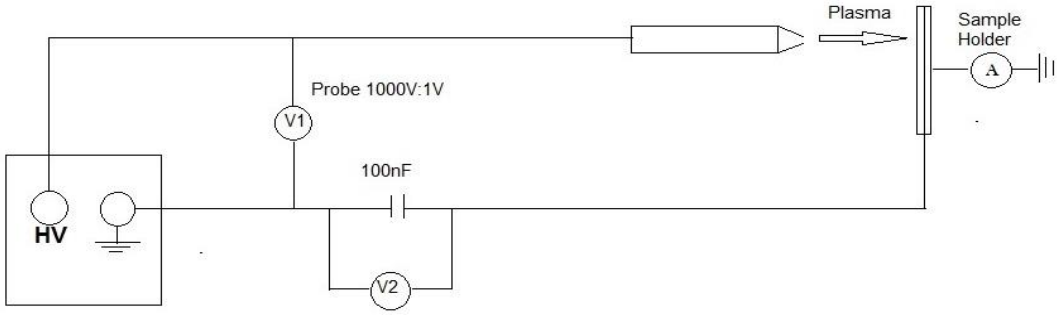

Figure.3.7. Schematic circuits of the V-Q lissajous figure method for measurement of the plasma power.

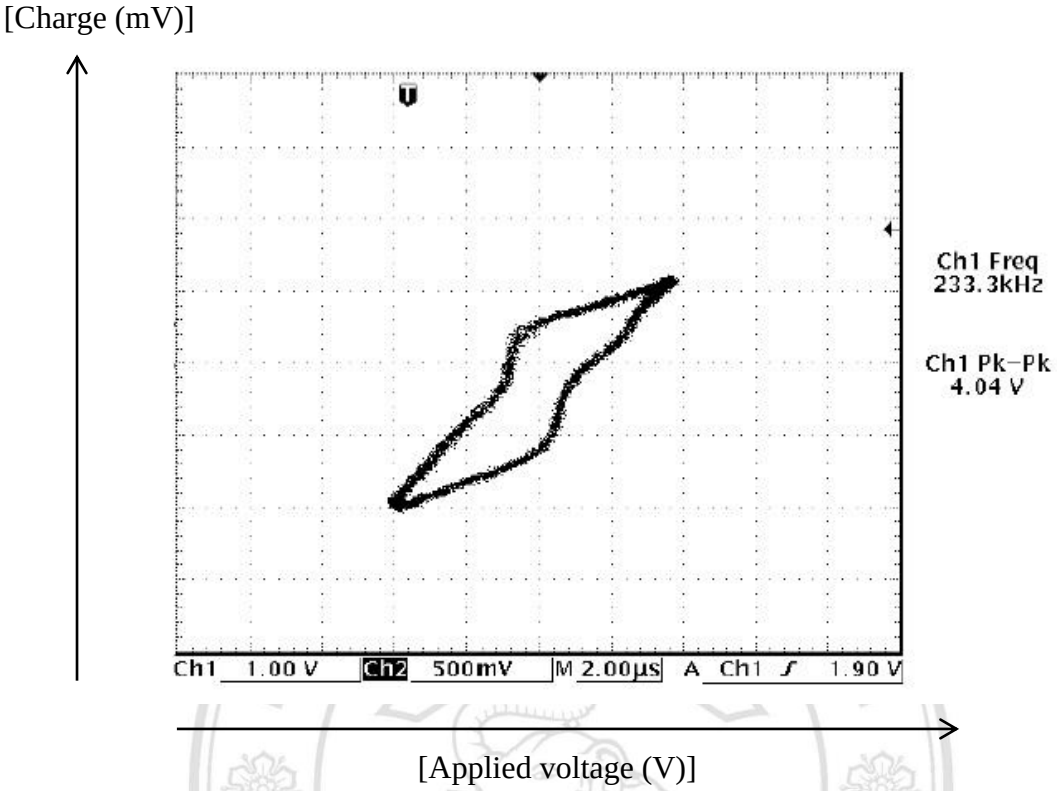

Figure.3.8. Picture of oscilloscope screen, x-axis represented applied voltage and y-axis represented charge.

In the calculation, the graph was taken from the oscilloscope and printed on paper to find the area inside the graph. The area inside graph was divided into small geometric shape such as triangle, rectangle, trapezoid and etc, and the all areas were summed together. From figure.3.8, x-axis converts ratio of the oscilloscope with the actual measured value per channel by multiplying 1000 in volt (V) and y-axis converts ratio of the oscilloscope with the actual measured value per channel in millivolt (mV).

The sum of areas was multiplied by ratio of x- axis and ratio of y-axis. The result was plasma generating energy, in Joule (J). The plasma generating power could be calculated from plasma generating energy multiplied with frequency of generator, in Watts (W).

3.3 Optical Emission Spectroscopy

Optical emission spectroscopy was used to measure the optical emission of the CAPPJ. The experimental setup of CAPPJ, as shown in Figure 3.8, was the optical emission spectrometer (AveSpec – 2048). An optical fiber was inserted through a small

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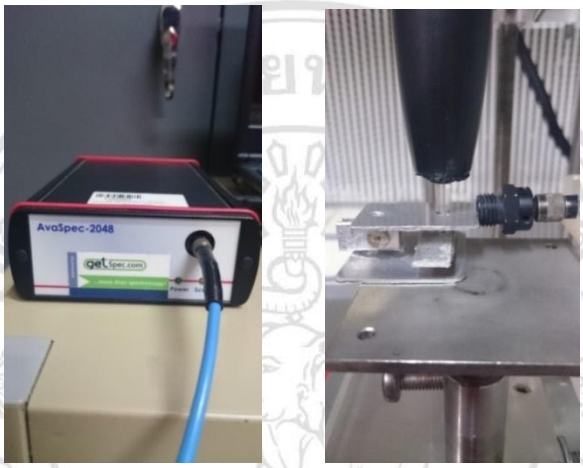
hole (with a diameter of 2 millimeter). The distance between the head detector with CAPPJ was almost 1 cm in order to avoid any effect on the discharge, as shown in Figure 3.9 which shows the typical emission spectra (200-800 nm) under three different conditions, namely, the original plasma jet, the unbent part of the plasma directly under the jet exit when the plasma jet was bent away by the traversal electrical field, and the bent plasma jet by the electrical field. The spectroscopy was analyzed by AvaSoft 7.4 promgram (Avantes, Inc.).



(a) (b)

Figure.3.9. A photograhp of spectum analyzer (a) and Input spectum receivor set up near parallel aluminum plate (b)

3.4. Biological preparation

3.4.1 Naked DNA extraction

Naked DNA was extracted from *Eschericia coli* (*E. coli*) which had 2 types of DNA consisting of chromosomal DNA and plasmids DNA inside. We needed only plasmid DNA. An initial sample of DNA plasmid puff, pGFP (plasmid green fluorescent protein, 3344 base pairs), was purchased from Clonetech. The plasmid DNA was replicated following transformation into *Eschericia coli* (*E. coli*) and subsequently extracted and purified using a QIAGEN® Plasmid Purification kit according to the manufacturer's protocol. The plasmid DNA was prepared by stocking in -20 °C. In next step, plasmid DNA concentration was measured.

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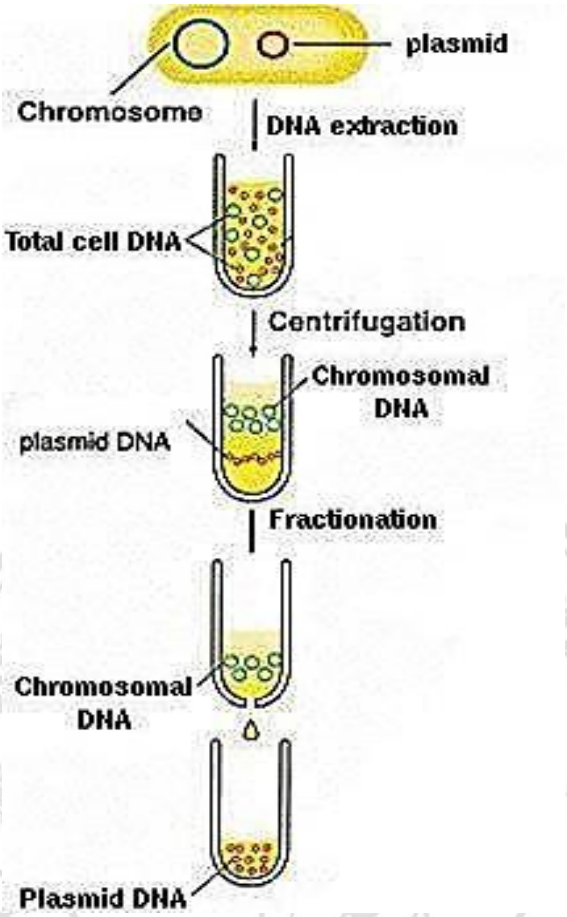

Figure.3.10. Plasmid DNA prepared steps.

3.4.2. Measurement of naked DNA concentration

Naked DNA concentration was measured by using a Bio-drop spectrophotometer (BioDrop TOUCH, Biodrop LTD) as shown in Figure 3.11. Nucleic acids can be quantified at 260 nm because it is well established that solutions of DNA and RNA in 10 mm pathlength cuvettes with an optical density (absorbance) of 1.0 have concentrations of 50µg/ml and 40 µg per ml respectively. Oligonucleotides typically have a factor of 33µg/ml, although this does vary with base composition. The BioDrop Touch is designed for use with the BioDrop CUVETTES. Moreover, the BioDrop Touch can be used by dropping DNA droplet on a direct micro-volume sample port with a pathlength of 0.5 mm which can be selected from the drop-down menu if the pathlength list. Pathlength factors are pre-programmed in the software for quick calculations to be a number and graph data, respectively.

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Figure.3.11. Photograph of BioDrop TOUCH, the gray circle is a direct micro-volume sample port

Figure.3.12. Dropping area for measuring naked DNA concentration is stand on middle of Bio-drop spectrophotometer, which is a small shallow pit.

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3.4.3 Competent cell preparation

Not all bacteria are capable of taking up exogenous DNA from their environment. The practical approach to acquire competent cells is to make the bacterial cells artificially competent using chemicals or electrical pulses.

This experiment used electrical pulses technique as known in another name are Electroporation. This technique used electrical field to pushing plasmid DNA into competent cell. As shown before, this experiment used *Eschericia coli* (*E. coli*) to be a sample. The competent cell preparation had many methods. This experiment started by

growing 5ml in 2 hours culture of cells in 100 ml LB media in a 200ml conical flask. In the next step, this culture was separated back into 1.5 ml of LB media into 1.5 ml eppendorf tubes. Then, 1.5 ml eppemdorf tube was soaked in ice. All subsequent steps should be carried out at 4^{o}C and the cells should be kept on ice wherever possible so that they are cold when cells are aliquoted into them later as shown in figure 3.13.

Cool water was used for washing and shocking cell for preparing other cell acceptation. 1.5 ml eppendorf was centrifuged by using a centrifuge machine (LMS, Laboratory & Medical Supply, Inc as shown in figure 3.14) at 5000 rpm (Revolutions per minute) for 15 minutes. Then, separate the cells from just water and add cool water into eppendorf and smoothly shake it for about 10 minute, and then centrifuge it agian. Lastly, competent baterial cells were kept in a refrigerator at -20°C.

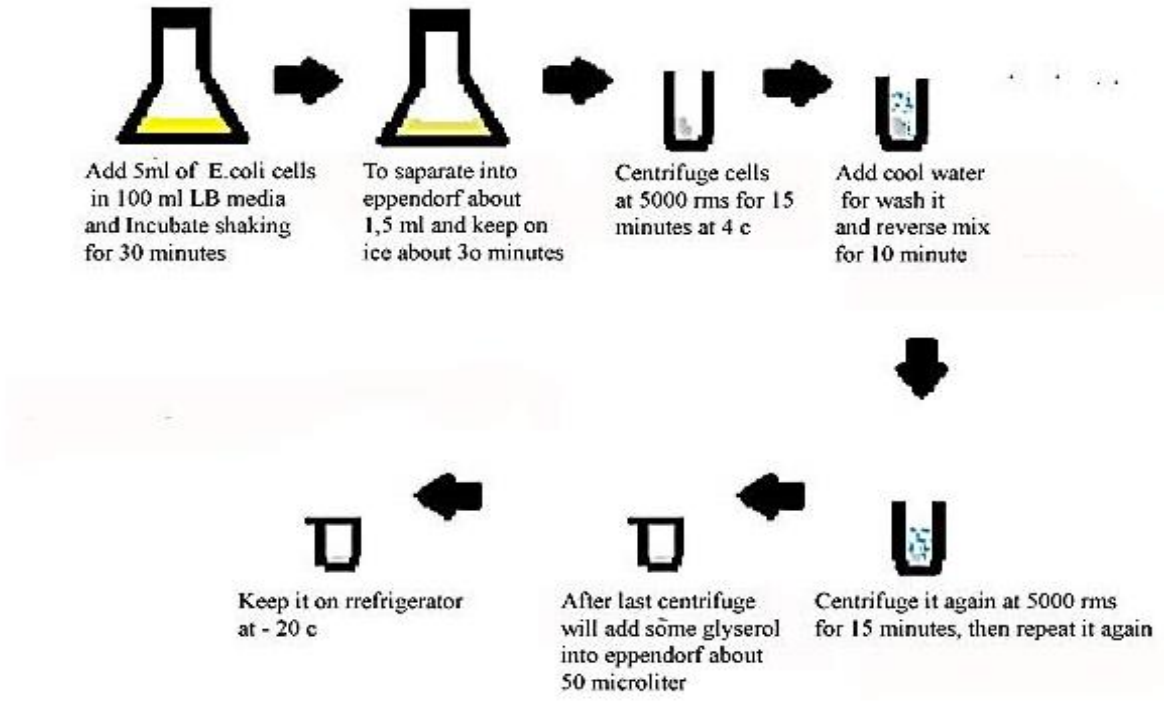

Figure.3.13. Step of competent bacterial cell preparation.

Figure.3.14. Photograph of centrifuge mechanic, which is MCF-1350 LMS mini-centrifuge from LMS, Laboratory & Medical Supply,Inc.

3.5 Plasma treatment of plasmid DNA

This part of the experiment is plasma treatment of plasmid DNA. This part has 3 parts, consisting of preparing plasmid DNA on slide which is a sample dish, using the cold atmospheric pressure plasma jet to treat plasmid DNA and harvesting treated naked DNA into Eppendorf for analyzing by gel electrophoresis lastly.

Firstly, a petri slide was deposited with plasmid DNA samples, which were pre-dried in a biological hood for 30 min. This work used 9 treating conditions and therefore the same sample was prepared on 18 slides. The slides were marked with condition as shown in Figure 3.15. A treatment part was stricken by adhesive tape and was marked for the target area by drawing a 1 mm circle. Then, plasmid DNA was dropped on that area. The slide was kept in a biological hood about 15 minutes for waiting plasmid DNA dried.

The cold atmospheric pressure plasma jet was set up on the holder as shown in Figure 3.4. The aluminum stand has a gap about 1 mm between electrodes and stand for inserting a petri slide. The cold atmospheric pressure plasma jet treatment of the plasmid DNA was operated in dry atmosphere at room temperature. Each treatment

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took 5 seconds. Two different treatment environments for the sample were adopted: dry and wet. The difference was only that in the wet treatment little water was slightly sprayed on the pre-dried DNA samples before the plasma jet was ignited

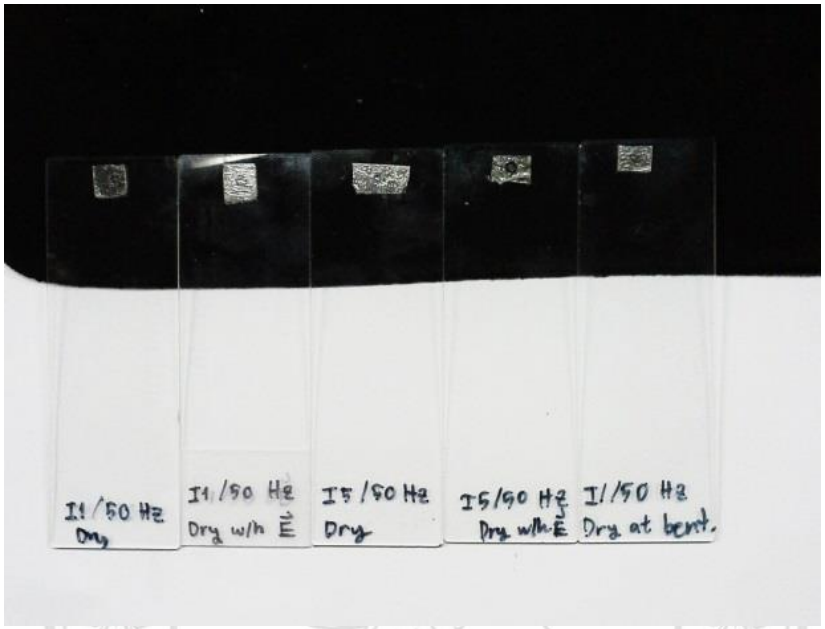

Figure.3.15. Photograph of the slides, which were marked with the treatment condition of varied intensity level and frequency of plasma machine and dry (D) and wet (W) condition.

There were 18 sample conditions including 9 dry conditions and 9 wet conditions. The plasma conditions had intensity 1 with frequency 10 Hertz, intensity 1 with frequency 50 Hertz, intensity 1 with frequency 110 Hertz, intensity 5 with frequency 10 Hertz , intensity 5 with frequency 50 Hertz , intensity 5 with frequency 110 Hertz , intensity 10 with frequency 10 Hertz , intensity 10 with frequency 50 Hz and intensity 10 with frequency 110 Hertz.

After the plasma jet treatment, plasmid DNA was harvested into Eppendorf by using dionized water sucking plasmid DNA from slides so the plasmid DNA was pGFP encoded with *GFP* gene which contained the green fluorescent protein, a protein composed of 238 amino acid residues (26.9 kDa) that could exhibit bright green fluorescence when exposed to UV light if the DNA was inside bacterial (*E.coli)* cell.

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3.6. Gel electrophoresis for analyzing plasmid DNA form

Electrophoresis is a simple technique to analyze DNA forms. After plasma jet treatment, the DNA samples were suspended in high purity water then loaded into a 1.4 % agarose gel (1.4 g of agarose dissolved in 100 ml of 0.5×TBE (tris-borate-EDTA) buffer, which contained Tris base, Boric acid, Ethylene-diamine tetra acetic acid), mixed with SYBR® green I dye and Gel Red.

The electrophoresis machine is Mupid-EXU Gel Electrophoresis System, Advance, Inc, As shown in Figure 3.16. Electrophoresis tank was filled with 0.5xTBE buffer covering a gel holder in middle of the tank. The system could adjust input power and set time for analyzing.

Gel Red was used for staining DNA in agarose gels. Gel Red™ (GelRed™ 10,000X, Biotium, Inc.) is an ultra- sensitive, extremely stable and environmentally safe fluorescent nucleic acid dye designed to replace the highly toxic ethidium bromide (EB). The power-source of the electrophoresis was set at 135 V and the gel, placed in a tank filled with 0.5×TBE buffer, was run for 25 min. The gel was observed for DNA conformation change under UV light by a UV trans-illuminator (an Ultra Violet Product), as shown in Figure 3.17.

Figure.3.16. Photograph of electrophoresis system.

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Figure.3.17. Photograph of a UV trans-illuminator

3.7. Electroporation for transferring plasmid DNA to competent cell

The gene transfer technique is the electroporation. Electroporation is a dynamic phenomenon that depends on the local transmembrane voltage at each point on the cell membrane. This experiment used Bio-Rad Gene Pulser II Electroporation System, Bio rad, ltd. It is generally accepted that for a given pulse duration and shape, a specific transmembrane voltage threshold exists for the manifestation of the electroporation phenomenon (from 0.5 V to 2 V). This leads to the definition of an electric field magnitude threshold for electroporation (E_{th}). That is, only the cells within areas where $E \geqq E_{th}$ are electroporated.

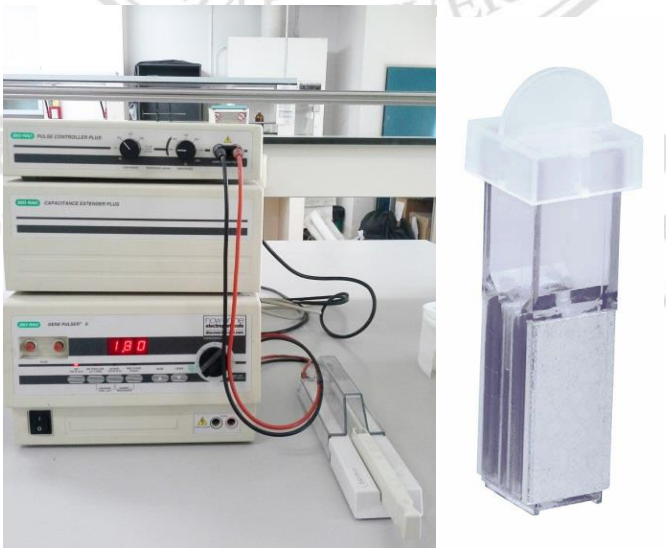

Figure.3.18. Electroporation systems in Left: Device and Right is Cuvette which use for fill sample.

The bio-rad gene pulser II electroporation system has 3 parts. Firstly, Pulse controller plus is on the top of the system. On the middle of the system is capacitance extender plus and the last one is Gene pulser.

The steps of experiment are following. The treated plasmid DNA was separated 2 parts, first one used for gel electrophoresis as previously mentioned and another one for electroporation. The competent cells used were in about 10 µl per conditions. The treated plasmid DNA was mixed with the competent cells in a ratio 1:2 in cuvette. Inside cuvette, there was a small gap of electrode to keep cuvette in an ice cooler box. Next, insert the cuvette into the shocking chamber as shown in Figure 3.19. Set up electroporation device at 1.8 volt and 5 seconds for transformation by pushing a red bottom twice on the Gene pulser. After 5 seconds, the plasmid DNA was sucked and added to 3 ml of LB media.

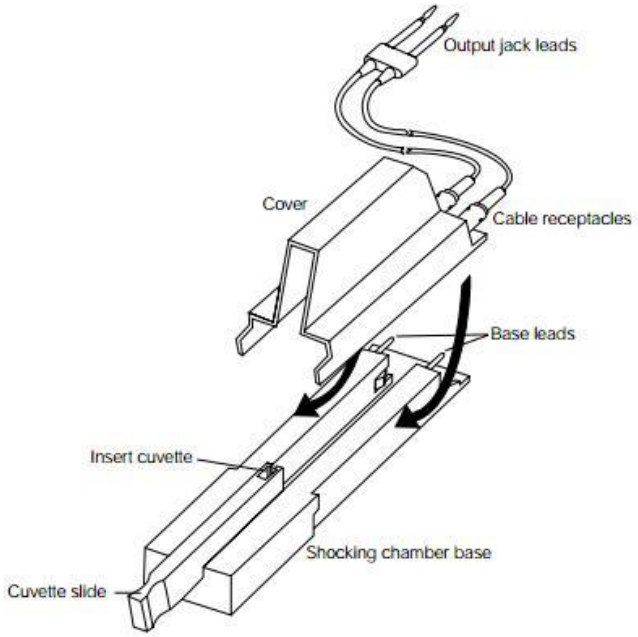

Figure.3.19. Schematic of Gene Pulser II unit shocking chamber

3.8 Observation and Selection of DNA mutation

The 3- ml *E.coli* in LB media was incubated in 37 °C for about 2 hours and added with ampicillin 3 µl per tube then incubated for 1 hour. The LB media gel was prepared in a petri dish, which was added with ampicillin 3 µl to 40 µl LB media gel. After incubating done, the LB media was diluted by using the distilled water as shown in figure 3.20.

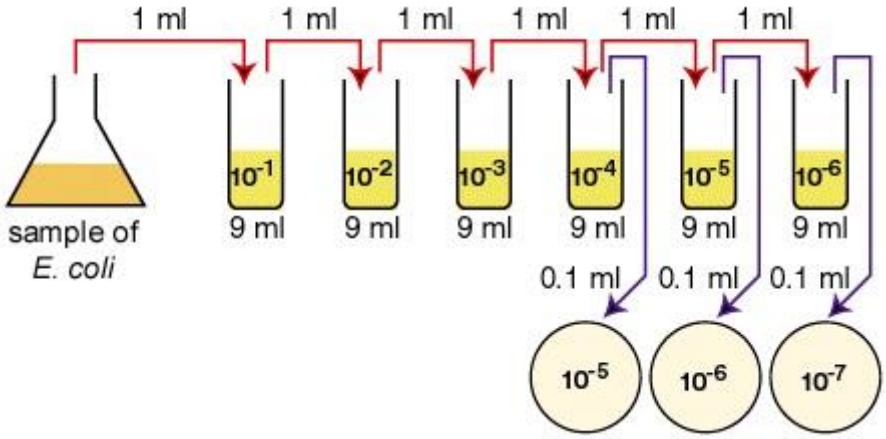

Figure.3.20. Step of plate count dilution procedure. Inside tube has the distilled water about 9 ml then added 1 ml of *E.coli*. Do the same step for deceasing concentration

After dilution, the *E.coli* was dropped on LB media gel at about 10 µl and spread all area on petri dish. All of petri dishes were left in the incubator at 37° c overnight. Mutation selection was indicated by the white bacterial colonies by using a UV trans-illuminator.

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