JOINT INSTITUTE FOR NUCLEAR RESEARCH

Dzhelepov Laboratory of nuclear problems

FINAL REPORT ON STAGE 1 OF THE INTERNATIONAL STUDENT PRACTICE

"PCR and sequencing as tools for detecting intergenic changes induced by ionizing radiation in the vestigial gene of *Drosophila melanogaster*"

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ABSTRACT:

Among the biological effects caused by ionizing radiation, the most important are mutations of individual genes induced in generative cells. Therefore, the problem of studying the genetic effects of rarely and densely ionizing radiation, which affects living organisms. In this project, we used the PCR and sequencing in detecting DNA alterations and mutations induced by radiation in *Drosophila melanogaster*. The results shows that different doses of irradiations induced heritable mutations (deletions, insertion, and substitution) at the genes of *Drosophila melanogaster*.

INTRODUCTION:

Ionizing radiation is radiation that can remove electrons from an atom. Losing an electron charge or ionizes the atom. Sometimes, ionizing radiation takes the form of a wave, like gamma rays or X-rays. But it can also take the form of a particle, like neutrons or alpha and beta particles. When ionizing radiation interacts with a cell, it could prevent the DNA from replicating correctly or damage DNA and cause cell apoptosis (Borrego-Soto *et al*, 2005).

Ionizing radiation can interact directly with DNA molecule's atoms. This prevents cells from reproducing. Alpha particles, beta particles and X-rays can directly affect a DNA molecule in one of three ways: 1) Changing the chemical structure of the bases; 2) Breaking the sugar-phosphate backbone; or 3) Breaking the hydrogen bonds connecting the base pairs (Mavragani *et al*., 2019).

Any chemical or physical change that alters the nucleotide sequence in DNA is called a mutation. Common types of mutations include substitution (a different nucleotide is substituted), insertion (the addition of a new nucleotide), and deletion (the loss of a nucleotide). These changes within DNA are called point mutations because only one nucleotide is substituted, added, or deleted (Mavragani *et al.*, 2019).

Mutations that occur in germ cells (sperm and ova) can be transmitted to future generations and are therefore called genetic or heritable effects. Genetic effects may not appear until many generations later. The genetic effects of radiation were first demonstrated in fruit flies in the 1920s. Drosophila melanogaster is commonly known as the fruit fly or the Vinegar fly. It is one of the best model organism in genetics and radiobiology owing to its rapid life cycle about 10 days at room temperature, relatively simple genetics with only four pairs of chromosomes, and large number of offspring

per generation. In addition, we could understand how mutation works by visualization its phenotypes and sequencing its genotypes (Celniker and Rubin 2003).

Genetic markers are commonly used in Drosophila research, and most phenotypes are easily identifiable either with the naked eye or under a microscope. There are many types of mutants in *D. melanogaster* such as: Vestigial, body color, eye color, body shape. Vestigial gene is charged with normal development of wings. Mutation of this gene conducts to reduction of wings in various degrees (Celniker and Rubin 2003).

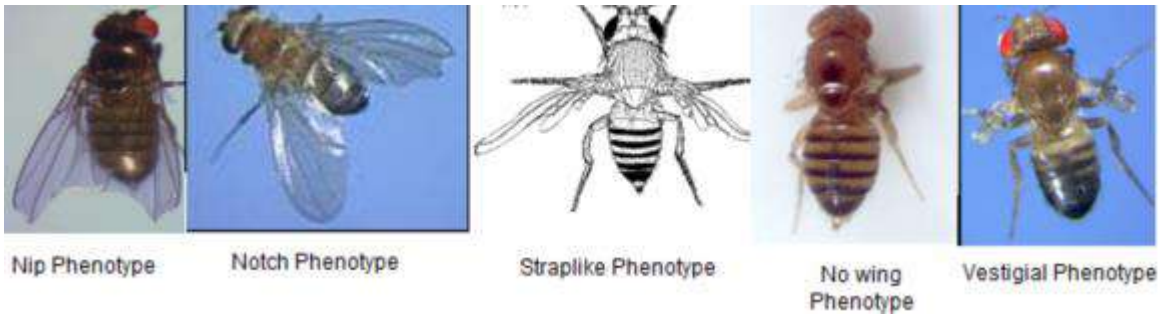

Figure 1: Vestigial gene phenotypes.

2. PROGECT GOALS AND SCOPE OF WORK:

- Estimate the number and type of mutations caused by different types of radiation in *Drosophila melanogaster* and determine the spectrum of inherited changes in DNA caused by the types of radiation studied.

- Obtain new fundamental data on the relative activity of different mutations in generative cells of eukaryotes after radiation damage, and investigate the mechanisms underlying radiation-induced mutation

3. METHODS:

D. melanogaster males have been irradiated with Gamma radiation of Cobalt^{60} then mate with wild type females to investigate the effect of radiation on DNA mutations in the next generations of the fly. Different morphological changes were occurred in the successive generations due to radiation exposure. These changes can be examined by the naked eyes or under the dissecting microscope. The most prominent

morphological change was the wing degeneration in different manners till become not present. Also, the transform of body and eye color have been appeared.

After the examination of morphological changes, the flies sorted according to the changes occurred and gathered for further investigation of DNA mutations in the responsible genes. The DNA mutation were investigated according to the following protocols and analysis

Figure 2: The image show *D. melanogaster* with black body and cinnabar eye color as morphological changes occurred due to irradiation with Gamma radiation of Cobalt^{60}

Figure 3: The images show the normal wing shape and body color (right) and the vestigial wing with black body (left) after irradiation of *D. melanogaster* with Gamma radiation.

3.1- DNA extraction:

DNA extraction was carried out in three different protocols

The first protocol: genomic DNA isolation by a commercial kit:

1- Put 10-20 flies in 1.5 ml microcentrifuge tube and homogenize with 400 µl lysis buffer.
2- Incubate the sample tube for 30-40 min at 65 °C.
3- Centrifuge the sample at 5000 rpm for 30-40 sec.
4- Transfer the supernatant in a new 1.5 ml tube.
5- Add to the supernatant 20 µl silica solution (Nucleous™) and gently mix (tapping or inverting) the
6- Mix the tube for 10 min on a rocking platform
7- Centrifuge at 5000 rpm for 10 sec
8- Discard the supernatant
9- Resuspend the pellet in 200 µl lysis buffer by vortex
10- Add 1 ml of the washing solution and mix by vortex
11- Centrifuge at 5000 rpm for 10 sec
12- Repeat the steps 10 and 11
13- Discard the supernatant and dry the silica-nucleic acid pellet by opening the tube at 65 °C for 4-5 min in heating block.
14- Add to the pellet in the tube 70 µl "ExtraGene™" solution and resuspend the pellet by tapping and then incubate the closed tube at 65 °C for 4-58 min.
15- Vortex DNA solution and centrifuge at 10000 rpm for 1 min
16- Transfer the supernatant with DNA into new tube and label it.
17- Store the DNA sample at -20 °C

The second protocol: DNA isolation using chemicals (chloroform):

1- Anesthetize 50-100 flies with ether or you can freeze them by butting them in freezer
2- Transfer the anesthetized flies to a tube with 500 µl of crushing buffer
3- Grind the flies, then incubate at 65 °C for 30 min
4- Add 70 µl of 3M sodium acetate or potassium acetate (pH 5.5) then incubate for 30 min on ice (don't mix after incubation)
5- Centrifuge at 14000 rpm for 10 min
6- Transfer thee supernatant to a new tube

7- Add 500 µl of chloroform and mix gently by inverting the tube 30-40 times then centrifuge for 10 min at 14000 rpm
8- Transfer the aqueous (top) layer to a new tube
9- Repeat the steps 7 and 8
10- Add 300 µl of isopropanol and mix gently by inverting the tube 30-40 times then spin at 14000 rpm for 10 min
11- Remove the supernatant carefully
12- Add 300 µl of 70% ethanol and spin for 10 min at 14000 rpm
13- Remove the supernatant then add 300 µl of 70% ethanol and centrifuge for 5 min at 14000 rpm
14- Remove the ethanol and let the tube to dry at room temperature
15- Dissolve DNA in Tris-EDTA buffer

The third protocol: genomic DNA isolation by a modified procedure:

1- Put the flies in a tube contains a suitable known amount of A:SDS solution and grind them by a pestle for 5-7 min
2- Add another amount of A:SDS solution till the volume 500 µl reached
3- Add 5 µl proteinase K solution then mix and incubate for 30 min at 55 °C
4- Incubate for 30 min at 70 °C
5- Add 100 µl of 8M potassium acetate solution with shaking
6- Put the sample in the ice path for 30 min
7- Centrifuge for 10 min at 14500 rpm (tabletop centrifuge, preferably with cooling at 4 °C)
8- Transfer the supernatant to a clean tube and repeat the step (7)
9- Add 2 µl of RNase solution then mix and incubate for 5 min at room temperature
10- Add 50 µl of 8M potassium acetate solution and then add 1 ml of 96% ethanol with shaking and centrifugation for 20 min at 14500 rpm
11- Remove the supernatant
12- Add 300-500 µl of 80% ethanol and centrifuge for 10 at 14500 rpm
13- Remove the supernatant
14- Dry the precipitate at 50-60 °C for 15 min and dissolve the DNA in free nuclease water or TE buffer

3.2- DNA concentration and quality determination:

The concentration and quality of the extracted genomic DNA were measured using two methods:

A. Using the nanodrop spectrophotometer
B. Using agarose gel electrophoresis.

3.3- Polymerase chain reaction (PCR):

PCR was carried out to amplify the DNA fragments of the target genes to get a suitable concentration for DNA sequencing. The PCR primers for each fragment were designed using an online tool called "primer BLAST" present in the following link: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

PCR steps was carried out in three tubes as positive control, sample and negative control according to the following steps:

1- Dilute the master mix by adding 10 µl of diluent solution
2- Then, add 7 µl of free nuclease water,
3- Add 1 µl of forward primer
4- Add 1 µl of reverse primer
5- All the forementioned components were added to all tubes, then in the positive control tube, 1 µl of DNA which approved that it contains the target sequence was added.
6- Add 1 µl of the isolated genomic DNA to the sample tube
7- Add 1 µl of H_2O to the negative control tube
8- Then the tubes putted in the thermal cycler instrument

3.4- Gel documentation:

Agarose gel electrophoresis was performed according to the following steps:

1- Agarose gel was prepared as 0.7 g of agarose was dissolved in 70 ml TE buffer solution by the aid of the microwave. Then, the solution left to cool and poured in its tray till solidified.
2- The gel placed in the electrophoresis chamber filled with TE buffer
3- 3 µl of each DNA sample loaded in the gel slots carefully
4- The DNA ladder loaded
5- The power source turned on for 30 min
6- The resulted gel bands were visualized under UV lamp

After the detection of PCR products quality and concentration, they were purified using a commercial kit to be sequenced.

3.5- Sequencing analysis:

PCR products were sequenced using Sanger sequencing instrument in Moscow.

3.6- Data analysis:

The mutations occurred due to the irradiation of the flies were investigated from the Sanger sequence files. Each sample sequence was aligned with the control sequence. The alignment and detection of mutation were estimated using different programs (Genescanner, BioEdite, Chromas, UGENE and the online tool EMBOSS water).

4. RESULTS

After comparing the three methods we found that the third protocol is the best for extraction and getting a high concentration of DNA.

The DNA of a total of thirty-one fragment were amplified (Table 1); six fragments for the gene *S6*, five fragments for the gene *S4*, four fragments for the genes *KL* and mutant line *vg54*, two fragments for the mutant lines; *vg25*, *vg110*, *vg152 and vg57* and one for the fragments of the mutant lines; *vg29*, *vg48*, *vg157* and *vg135*. The reverse primer for the fragment 25 in a previous trails didn't give a good result in sequencing analysis so new sets of primers were designed with our group and submitted to the company for synthesis and a new PCR trail for this fragment will be processed.

Gene	Fragment number																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
	ex1.1 (845b)	in1-1 (630b)	in1-2 (600b)	ex2.1 (794b)	ex2.2 (717b)	in2-1S (844b)	in2-2S (656b)	in2-3S (624b)	in2-4S (750)	in2-5S (771b)	in2-6S (852b)	in2-7S (726b)	ex3.1 (710b)	in3 (812b)	ex4.1 (738b)	in4s1 (787b)	in4s2 (810b)	in4s3 (786b)	in4s4 (808b)	in4s5 (736b)	in4s6 (858b)	in4s7 (753b)	ex5 (670b)	ex6-7 (782b)	ex8 (620b)
KL																									
S 4																									
S 6																									

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
	ex1.1 (845b)	in1-1 (630b)	in1-2 (600b)	ex2.1 (794b)	ex2.2 (717b)	in2-1S (844b)	in2-2S (656b)	in2-3S (624b)	in2-4S (750)	in2-5S (771b)	in2-6S (852b)	in2-7S (726b)	ex3.1 (710b)	in3 (812b)	ex4.1 (738b)	in4s1 (787b)	in4s2 (810b)	in4s3 (786b)	in4s4 (808b)	in4s5 (736b)	in4s6 (858b)	in4s7 (753b)	ex5 (670b)	ex6-7 (782b)	ex8 (620b)
vg25																									
vg29																									
vg48																									
vg54																									
vg69																									
vg110																									
vg152																									
vg157																									
vg57																									
vg135																									

Table 1: Illustrate all the mutant cells of the different lines of four genes in *Drosophila melanogaster*, red color fragment which their DNA was extracted and amplified with our team and express a positive result

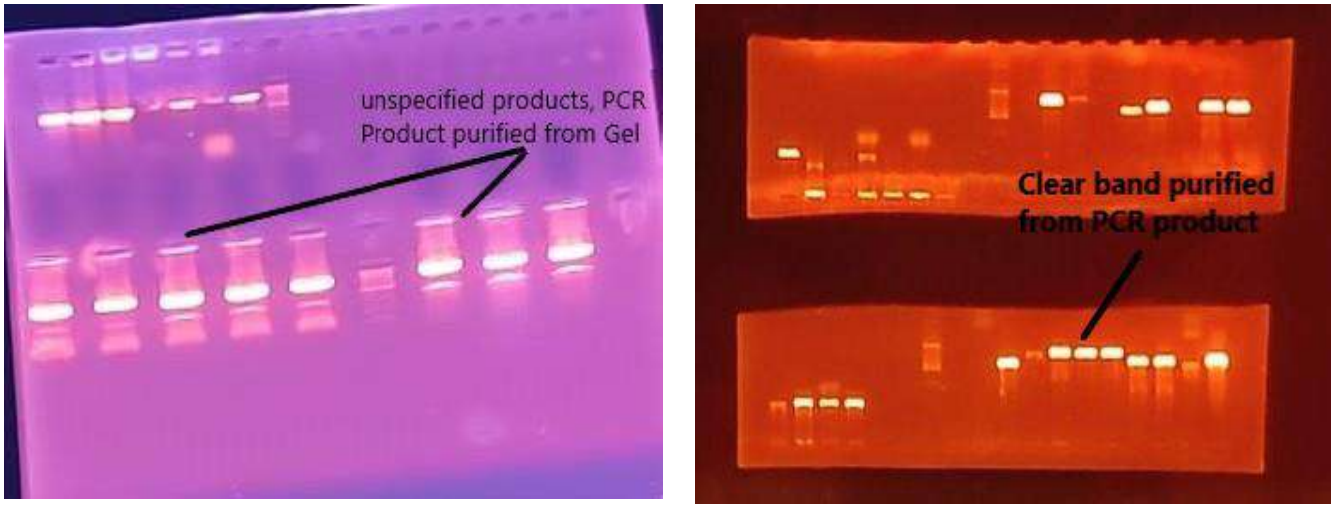

Figure 4: Illustrate the gel electrophoresis results of different fragment s PCR products results

No.	Problems in PCR results	How we could resolve?
1	**Get a negative PCR product result with positive control.**	Repeat the samples as the mistake almost with handling, if it isn't ok, check PCR product DNA concentration and quality.
2	**Get a negative PCR product result with even negative control.**	-Check the primers quality and preparation. - Change some PCR conditions.
3	**Get an additional product in gel.**	- Check the annealing temperature of the primers and may increase by one or two degrees. - Purify the DNA from the band appeared in the gel after separation.
4	**Get a faint band.**	- Check the DNA concentration - Increase the number of cycles of denaturation, annealing and extension.
5	**Get a positive "negative control"**	-Check the handling and sample contamination -loading the sample carefully on the gel combs as some samples smears may affect the results.
6	**Get noisy sequence results.**	-Check the PCR conditions. -Prepare a new set of primers, especially if one of them work and other don't.

Table 2: The problems appeared in PCR results and how it could be resolved.

Figure 5: The alignment process the fragment number four of vestigial gene 54 (*vg54*) of *Drosophila melanogaster*, the forward, reverse and control sequences with BioEdit program.

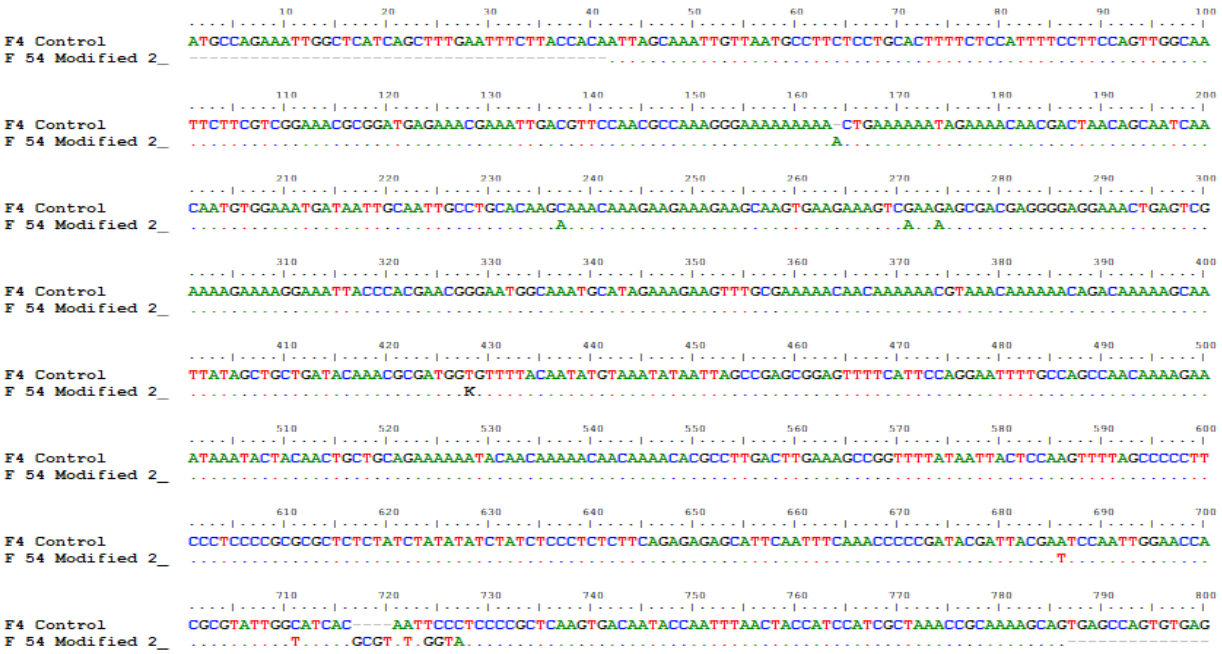

Figure 6: The alignment process of the fragment number four of vestigial gene 54 (*vg54*) of *Drosophila melanogaster*, the forward sequence after modification and control sequence with BioEdit program.

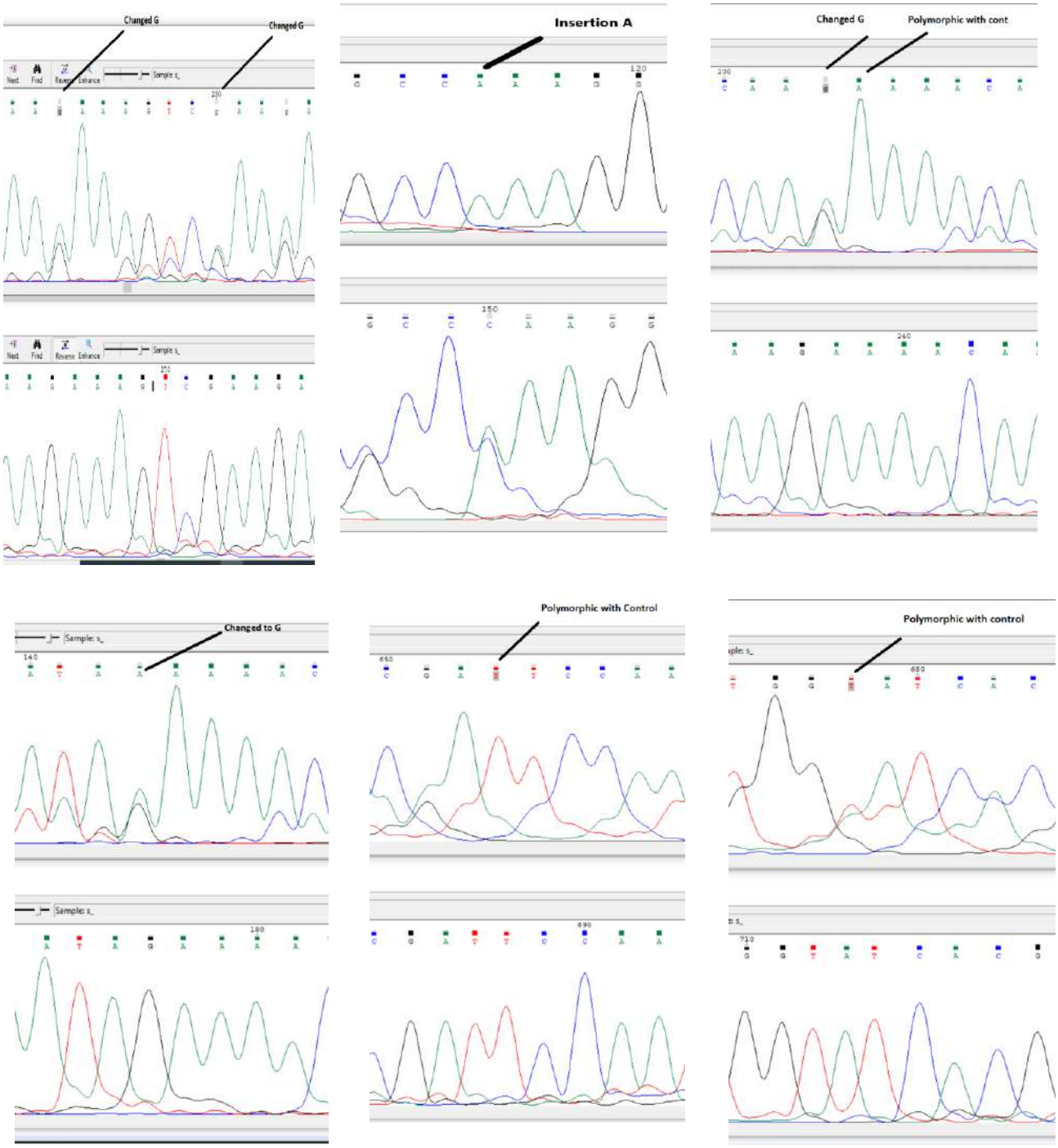

Figure 7: Illustrate the analysis of the forward and reverse primers sequencing results of the fragment number four of vestigial gene 54 (*vg54*) of *Drosophila melanogaster* using Chromas program.

5-CONCLUSION

The Radiation and Molecular Genetics Group (JINR-Egypt) conducts studies on molecular-genetic analysis of gamma and neutron induced mutations with an assessment of the contribution of mutation systems for a number of *Drosophila*

melanogaster gene after irradiation of generative cells and study the DNA mutations of genes in generative cells in *Drosophila melanogaster* after using different radiation doses. For these studies team focus on using different molecular technique like, Polymerase Chain Reaction, sequencing and other methods of molecular biology are used. Finally, radiation have clear impact on intragenic distribution of different DNA alterations relative to the exon-intron structure of the gene. Also results identify the quality and frequency patterns of DNA alterations and deletions of the greater part or a whole of different mutant lines of vestigial genes of *Drosophila melanogaster*.

6. REFRENCES:

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Mavragani, I. V., Nikitaki, Z., Kalospyros, S. A., & Georgakilas, A. G. (2019). Ionizing Radiation and Complex DNA Damage: From Prediction to Detection Challenges and Biological Significance. *Cancers*, *11*(11), 1789.

https://saylordotorg.github.io/text_the-basics-of-general-organic-and-biological-chemistry/s22-05-mutations-and-genetic-diseases.html

7. ACKNOWLEDGMENT:

First of all we wish to offer our heartfelt thanks to our supervisor Dr. Kristina P. Afanasyeva for allowing us to be a part of such an interesting project. Her serious work and continuous discussions were very informative and useful for our experiences. Also, we would like to express our great thanks to the Academy of Scientific Research and Technology (ASRT) and the Joint Institute for Nuclear Research (JINR) for giving such an opportunity for young researchers to broaden their knowledge of nuclear research and its biological applications. Finally, all thanks to everyone in Egypt and Russia facilitated our training and residence in Russia.

Solutions preparation:

Crushing buffer 1: 100mM Tris-HCl, pH 7.5 100mM EDTA	Crushing buffer 2: 0.1M Tris-HCl (pH 9.0) 0.1M EDTA 1% SDS	Crushing buffer 3: 0.1M NaCl 0.2M sucrose 50mM EDTA 0.1% SDS 0.1M Tris-HCl (pH 9.2)

Solution <A>		Vt = 10 ml	Vt = 50 ml
EDTA	0.5M	2 ml	10 ml
Tris-HCl	1M	1 ml	5 ml
Distilled H_2O		7 ml	35 ml

<SDS solution>	10 %
SDS	100 g
Distilled water	1000 ml

<A:SDS>	proportion	Vt= 500 µl (1 sample)	Vt= 500 µl (5 sample)	Vt= 500 µl (11 sample)
A	9	450 µl	2250 µl	4950 µl
SDS	1	50 µl	250 µl	550 µl

Potassium acetate solution	8M
K Acetate	40 g
Distilled water	50 ml

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