Advanced Biomedical Research and Innovation

Research Article

A SCITECHNOL JOURNAL

Molecular Analysis of Biomolecules Pertaining to the Diagnosis of a Human Genetic Disorder: Lesch Nyhan Syndrome

Michael Halim[1*] and Alice Halim[2]

[1]*Department of Biomedical Science, University of Salford, United Kingdom*

[2]*Department of medicine , Shanghai Medical College, Fudan University, Shanghai, China*

Abstract

Molecular analysis of biomolecules has been applied in many areas of research into different genetic disorders. Genomic DNA (Deoxyribonucleic acid) is the molecule of choice for such analyses. This report summarizes the activities carried out in the molecular analysis for the diagnosis of genetic disorders. Emphasis is put on the Lesch-Nyhan syndrome, which is a genetic disorder of purine metabolism. The genomic DNA used in this practical was extracted from the fibroblasts of breast cancer cells individuals (MRC5), normal cells (MCF7), and fibroblasts from Lesch-Nyhan patients (SALMAT/LN). The concentration of the three DNA samples was determined by the use of spectrophotometer and Nanodrop method. The absorbance values recorded were used in the calculation of the concentration and purity of the DNA samples. The samples were then optimized by fragmentation using a Bioruptor sonicating water bath. The DNA fragments were separated on a 1% agarose gel by electrophoresis. However, the experimental findings were not in accordance with the results reported in similar research studies on Lesch-Nyhan disorder. The deviation can be explained by various factors that ought to be controlled to ensure accurate analysis of biomolecules. Nevertheless, the diagnostic approaches based on molecular genetics enable clinicians to select an appropriate test for each patient, generating clinically relevant and optimal results required to define a treatment strategy for patients.

Keywords: Molecular analysis; Genetic disorders; Lesch-Nyhan syndrome

Introduction

Genetic disorders are conditions that stem from changes (mutations) in the genome. The changes can be inherited from parents or acquired from the environment. Inherited genetic disorders affect the children if parents are carriers of the defective gene [1,2]. This paper dwells on Lesch-Nyhan syndrome. Lesch-Nyhan syndrome is a genetic disorder affecting purine metabolism [3,4]. According to [5], purines such as adenine, guanine, cytosine, and thymidine are nucleic acids and are the building blocks of DNA. Lesch-Nyhan syndrome is an X-chromosome linked and recessive disorder affecting males [6]. The females are carriers and may not have symptomatic disease. A few cases have been found to arise from unexplained mutations in cases in which the mother is not a carrier of the defective gene. The Lesch-Nyhan syndrome condition is related to the deficiency or absence of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity since the enzyme is mutated [7,8]. The metabolism of purines occurs in two pathways, namely de novo and salvage synthesis. De novo synthesis is the pathway that builds DNA from scratch while the salvage pathway uses the free circulating purines from degraded DNA to make new DNA. The salvage process "recycles' purines prevent accumulation [9]. The HPRT enzyme is the rate-limiting step of the salvage process. Torres and Puig (2015) observe that defective HPRT1 gene leads to the deficiency of the enzyme and subsequent accumulation of the purines in circulation. The de novo pathway has been found to compensate for the failure of the salvage process by increasing the production of purines [10]. The purines are broken down into uric acid. The increased levels of uric acid are manifested in blood and urine. Gout and kidney disorders are the early symptoms of hyperuricemia. Lesch-Nyhan syndrome also manifests as a neurological disorder affecting muscle control and cognitive function [11-13]. Behavioural abnormalities such as self-harm are common in the patient's first few years of life. Self-harm involves hand biting and headbanging all of which may cause fatal injuries. The diagnosis of Lesch-Nyhan syndrome is by gene testing and other molecular analytic techniques. Genes are studied to identify any mutations or changes in DNA that may cause a genetic disorder. This test is used in genetic counselling to mothers who are carriers of the possibility of begetting a son with Lesch-Nyhan syndrome [14,15]. The ratio of uric acid to creatinine is found to be above 2 and is indicative of uric acid overproduction in the urine sample of the patient. These findings are common in Lesch-Nyhan patients aged less than 10 years. The activity of the HPRT enzyme is found to be less than 2% in cultured normal fibroblasts. Other tests are recommended to rule out other conditions with manifestations similar to Lesch-Nyhan syndrome. Conditions that may be confused for Lesch-Nyhan syndrome include autism, cerebral palsy and dystonia caused by other conditions of the muscular-skeletal system.

This practical applies analytical skills to study Lesch-Nyhan syndrome at the molecular level. Three DNA samples were nucleic acid extracts from fibroblasts of breast cancer cells (MCF7), normal cells (MRC5) and Lesch-Nyhan cells (SALMAT/LN). These extracts were used to isolate genomic DNA from the three samples, and they were analyzed quantitatively as well as qualitatively [16]. Further analyses involved the determination of the DNA concentration in each sample on a spectrophotometer and Nanodrop. Moreover, the ratio of the absorbance values of each sample (A260/A280) is an indicator of the sample purity.

Since the genomic DNA of an organism is identical, shearing in a sonicator should provide identical products. The cleaved DNA products when electrophoresed on 1% agarose gel, the individual fragments [17]are separated and studied. This lab performed this technique to detect the difference (s), if any, present in the fragment

***Corresponding author:** Michael Halim, University of Salford, Greater Manchester, United Kingdom, Tel: +44 7869646125; Email: michaelhalim1000@gmail.com

Received: 01 October 2020; **Accepted Date:** 15 October, 2020; **Published Date:** 22 October, 2020

lengths of the genomic DNA available in test and control samples. Extraction of the genomic DNA from the parent samples and quality control constitute important steps. Identification of a mutated gene that led to the Lesch-Nyhan condition requires a significant amount of pure genomic DNA for downstream analytical applications, such as Polymerase Chain Reaction (PCR) and gene sequencing. Therefore, quantitative and qualitative analyses form the critical component in the molecular diagnosis of the Lesch Nyhan syndrome.

Materials and Methods

Measurement of DNA concentration using both spectrophotometer and Nanodrop methods.

In the spectrophotometer method, the instrument was set by using a blank to 'zero' the readings before the samples were tested. The elution buffer was used as a blank solution (reference solution upon which sample measurements is based). The samples were prepared by making a 1:50 dilution of the DNA samples in water to a final volume of 100 µL. The samples were separately put into cuvettes and the absorbance readings of each sample at wavelengths 260 nm and 280 nm were recorded. The concentration and purity of the DNA samples were determined by calculation using the data recorded. In the Nanodrop method, the equipment was blanked by 2 µL of elution buffer. The Nanodrop base and arm were cleaned after blanking to get rid of the elution buffer. 2 µL of DNA sample was pipetted onto the pedestal base of the Nanodrop apparatus and the arm lowered into position to read the concentration of the sample. The instrument was cleaned and another sample loaded onto the base. The concentration of all the DNA samples was recorded and compared to the data collected in the spectrophotometer method.

Absobance Ratio	OD Reading		
	MRC5	CF7	SALMAT (LN)
A_{260}	0.142	0.109	0.6
A_{280}	0.183	0.074	0.112
A_{260}/A_{280}	0.78	0.47	5.35

Table 1: Absorbance readings and ratios for DNA samples.

The results of the practical were recorded and tabulated in the tables. The observed results were compared to the results in the literature to ascertain the accuracy of the protocols used in the experiment. Tables 1 and 2 display data obtained from the spectrophotometer method.

At the wavelength of maximum absorbance (A260), 260 nm, samples from Lesch-Nyhan fibroblasts had the highest absorbance of 0.6 whereas the breast cancer cells had the lowest absorbance (0.109). The absorbance at 280 nm is 0.074, 0.112 and 0.183 for breast cancer, LN cells and normal cells respectively. LN cells had the highest ratio of 5.35 followed by breast cancer (1.47) and normal cells (0.78).

Calculation of DNA concentration in the samples

Concentration=standard concentration x OD260 x dilution factor

Standard concentration=50 ng/mL

OD260 is the absorbance at 260 nm, dilution factor is 50

MRC5=50 µg/mL x 0.142 x 50=355 ng/µL

MCF7=50 µg/mL x 0.109 x 50=272.5 ng/µL

LN=50 µg/mL x 0.6 x 50=1500 ng/µL

Absobance Ratio	OD Reading		
	MRC5	CF7	SALMAT (LN)
A_{260}	0.142	0.109	0.600
A_{280}	0.183	0.074	0.112
A_{260}/A_{280}	0.78	0.47	5.35
CONCEN-TRATION (ng/µL)	355	72.5	1500

Table 2: Absorbance ratio and concentrations of DNA samples.

Absorbance/ concentration	MRC5	MCF7	SALMAT (LN)
A_{260}/A_{280}	2.03	2.04	2.01
CONCEN-TRATION (ng/µL)	151.4	282.7	37.6

Table 3: Absorbance ratio and concentration by Nano drop method.

The absorbance ratios (A260/A280) of DNA obtained by Nanodrop method are 2.03, 2.04 and 2.01 for normal cells, breast cancer cells, and Lesch-Nyhan cells, respectively. Since the ratios in each case fall above 2.0, the samples may be contaminated with other types of nucleic acids (such as RNA) or solution is too acidic.

The DNA samples tested in both spectrophotometer and Nano drop method were the same but the results differ. The results for DNA concentration in literature are 214.2 ng/µL, 292.8 ng/µL and 50.8 ng/µL for normal cells, breast cancer cells and Lesch-Nyhan cells.

Discussion of Results

The spectrophotometer and Nanodrop readings are indicative of the purity of genomic DNA more than the amount of DNA available in a sample. Several standardization protocols in the past have already established the accepted observation ratio values for different types of nucleic acids to determine the molecule's purity in a sample. It is now known that pure DNA preparations yield an observational ratio ≥1.8 [4] while pure RNA preparations have absorption ratio value above 2.0 [18-20]. However, the findings of the quantitative analyses performed on the three samples were not concordant with the expected values. The absorbance of all the cell types are within range for the wavelength 260 nm, MRC5; 0.142, MCF7; 0.109, LN; 0.600. At 280 nm, the absorbance of the breast cancer sample (MCF7) is out of range with a reading of 0.074.

Samples from normal and breast cancer cells have ratios below 1.8. Lower ratios imply the presence of impurities and contaminants introduced during extraction. This indicates the genomic DNA preparation was contaminated with other foreign molecules such as RNA. This may be resulted due to manual errors occurred during the DNA extraction process. In using a spectrophotometer to determine absorbance, errors can arise during the cleaning of cuvettes, mixing of the sample solutions and transferring by use of pipettes. Presence of impurities that absorb at the same wavelength also interferes with the reading. The observation is most likely due to inaccurate dilution of the sample. Improper setting of the spectrophotometer can also

interfere with the reading.

These errors can be overcome by proper handling of samples and materials. Samples with lower ratios (below 1.8) may require further optimization to acquire a more accurate reading on the spectrophotometer. The ratio of LN sample was greater than 2. The ratio is indicative of the fact that there could be contamination with RNA and similar proteins from the sample of origin. The presence of RNA contamination can be confirmed on the gel electrophoresis where the RNA migrates ahead of DNA. The results of the Nanodrop method are different from those of the spectrometer method despite the use of the same samples. The results are different because of the three probable reasons. The cleaning of the pedestal was improperly done, the sample lacked homogeneity and the errors that arose during sample transfer through pipettes. These are common errors in such experiments and are to be avoided to ensure accurate results.

However, choosing a more reliable method for DNA quantification is essential for sample preparation to ensure performance as well as reproducibility of downstream assays. Since the path length of the light traversing through the sample is much smaller than a traditional UV-spectrophotometer (wherein path length=1.0 cm) and can adjust is as per sample absorption, this lab acknowledges the results obtained from the Nanodrop is more precise for high nucleic acid-concentrated samples. In traditional spectrophotometers, it is not advisable to measure the low concentration of nucleic acid preparations in a cuvette, as readings may be inaccurately incurred by the cuvette which, if unclean/damaged, may absorb the traversing UV light. This problem is not present with the Nanodrop setup.

Human tissue extracts are known to be rich in nucleic acids. So, a small amount of their preparations can be easily detected by Nanodrop with more accuracy. Therefore, Nanodrop becomes a method of choice as it works on minimal volume, consuming fewer source resources.

The DNA samples tested in both spectrophotometer and Nanodrop method were the same but the results differ. The spectrophotometry method gave 355, 272.5 and 1500 µg/µL for normal cells, breast cancer cells and diseased cells respectively. The Nanodrop method produced the following results: 151.4 normal cells, 282.7 breast cancer cells and 37.6 µg/µL for the Lesch-Nyhan sample. The results for DNA concentration in literature are 214.2 ng/µL, 292.8 ng/µL and 50.8 ng/µL for normal cells, breast cancer cells and Lesch-Nyhan cells respectively. The concentrations recorded by the two methods are expected to be similar but that is not the case for this practical. The samples were carefully kept separate to avoid mix up. The likely reason for the difference is the pipetting errors in the transfer of the samples to the instruments. The pipetting errors include contamination from one sample to another and pipetting varied volumes of the sample. To avoid these errors, strict adherence to the methods and the corresponding protocols is essential. As discussed earlier, precise quantified samples are essential for the optimization of downstream assays on it.

Discussion

To analyze the quality of the three DNA samples for downstream preparation using Bioruptor sonicating water bath and electrophoresis. The DNA fragmentation was done using a sonicating instrument. The samples were diluted to a concentration of 15 ng/µL using PCR (polymerase chain reaction) grade water to a final volume of 100 µL. The diluted DNA samples were mixed thoroughly and kept on ice for 10 minutes before being placed in the Bioruptor sonicating water bath. The settings of the instrument were put at 2 cycles with on/off time in seconds being 15/90 and the total sonication time set at 3.5 minutes. The tubes with DNA samples were stored at a temperature of -20°C overnight after fragmentation.

Analysis of DNA on 1% Agarose gel was done by preparing the gel and running the samples on the gel in an electrophoresis tank. Gel preparation: the buffer was diluted appropriately and mixed with a Parafilm solution. 0.24 g of agarose was mixed with 30 mL of 1 X TAE buffer in a conical flask plugged with cotton wool. The Agarose was dissolved by placing the flask in a microwave while swirling at an interval of 30 seconds. The agarose was left to completely dissolve and then cooled. The cool solution was thoroughly mixed with 30 µL of gel red before pouring into a cast. Wells of desired sizes were created on the cast and left to stand for 30 minutes.

DNA analysis 20 µL of each DNA sample was separately mixed with 4 µL of loading buffer and pulse spun. The gel was laced in an electrophoresis tank with wells orientated at the cathode end. 6 µL of a hyper ladder was loaded in lane 1 while the DNA samples were loaded in the remaining lanes and the order of loading recorded. The tank was closed by a lid and the power switched on to run the tank at 100 V. The process was stopped when the blue dye was 25 mm from the end of the gel. The gel was taken out of the tank and the DNA bands visualized in a UV trans-illuminator. Images were taken and saved for reference.

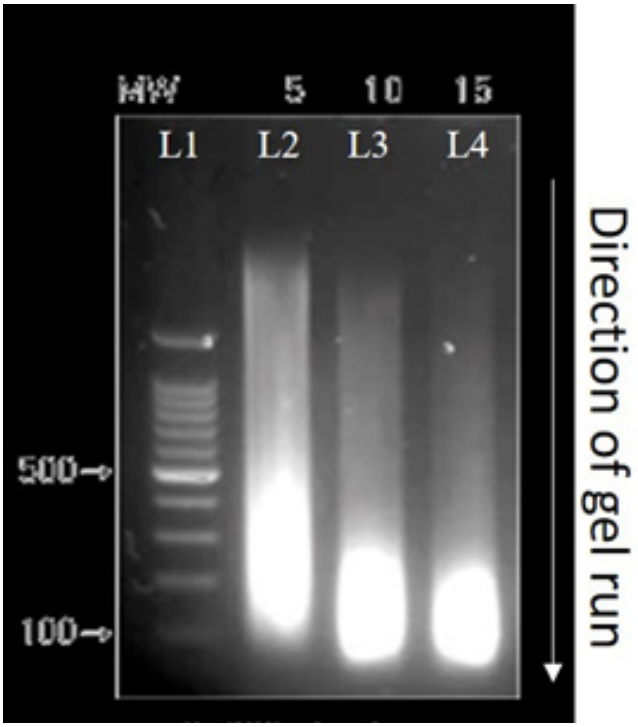

Figure 1: DNA fragments on 1% agarose gel (expected). Lane 1 (L1) show different DNA bands present in the standard molecular weight markers (ladder). L2, L3 and L4 shows DNA in samples.

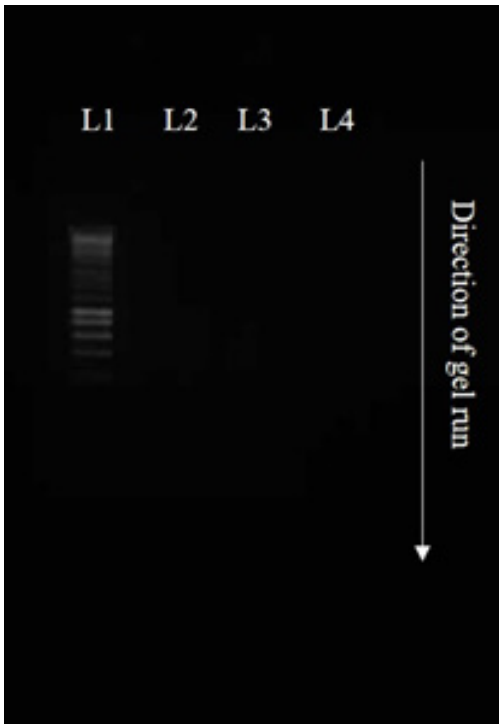

Figure 2: DNA fragments on 1% agarose gel (observed result). L1 has DNA bands of known molecular bands present in the reference ladder. L2- L4 shows no band for the samples.

Since mutation may cause change (s) in normal base-pair (bp) sequence of the genomic DNA, the sonicated products of each sample were electrophoresed to analyze the differences, if any, in the corresponding DNA segments in the respective samples [21]. The gel shows no DNA bands in the lanes corresponding to the sample wells except the ladder ones. No visualization may be resulted due to the loading of lower (than recommended) quantity of the sonicated products in the sample wells. It may also be probable the sample loading dye was used in very low concentration, and therefore, bands remained elusive to the detection [22-24].

Conclusion

The DNA samples were sheared and separated on 1% agarose gel for separation by electrophoresis. The Bioruptor sonicating water bath works on the principle that exposure of molecules to sound energy agitates the molecules. The agitated particles in a solution are sheared by sonication when the physical vibrations break the particles apart. The sonicating apparatus is set to achieve particles of the desired size. Sonication is suitable for the generation of DNA fragments that are longer than other methods of shearing. Gel electrophoresis has been used and proved to be suitable in the separation of DNA molecules in samples In gel electrophoresis, the DNA particles are separated based on the individual sizes and charge. The sheared DNA samples are loaded into wells on the gel at one end and the electric current applied on the other end. The electric current pulls the fragments across the gel towards the positive electrode (DNA is negatively charged). Small fragments are separated faster than the big fragments. The gel is stained by a DNA binding dye before loading. Gel red was used in this practical as the binding dye. The dye appears as a band when fragments of the same size are moved to the same spot. The characteristics of the gel can be varied to achieve the desired parameters in the isolation of DNA. The rationale of using the gel electrophoresis is based on the premise that some samples have very low concentrations of DNA and require a method other than spectrophotometry to produce accurate and reproducible results. Another application of gel electrophoresis is to isolate and catch DNA molecules in its matrix. As a result, contaminants that are present in the loaded samples and absorb traversing light at 260 nm during DNA quantification are removed during the electrophoresis. The agarose gel is preferred at a concentration of 1-2% because whole DNA is separated and seen as high-density robust bands. The preference of the gel electrophoresis does not mean that the process is devoid of errors. However, like every technique, gel electrophoresis is susceptible to errors and limitations that might lead to incorrect visualization of stained bands. The concentration of the gel must be kept between 1-2% to ensure the complete migration of the DNA. The migration is also affected by the steadiness of the electric current applied on the gel. Uniform migration requires a steady current devoid of fluctuations throughout the practical. The buffer should be of a suitable pH and of appropriate ionic composition to ensure complete separation of the fragments. The results on the gel were not visible and could be associated with how the practical was conducted. The errors need to be minimized while performing similar experiments in the future. However, the DNA ladder resolved into molecular reference bands that indicate the gel concentration used was correct, and the buffer was fresh. The electrophoresis ran well at 100 V and the separation most likely occurred but the visibility was limited. This lab was significant as it provides insights into fundamental molecular bio-techniques. It is evident as DNA quantification as well as quality analyses, such as sonication and gel run, is routine experiments carried out in studies wherein DNA is extracted from raw samples and prepared for the next-generation sequencing. Nevertheless, attributed to their significance in molecular studies, these techniques find wide application in the professional practice of biomedical scientists.

References

1. Aranda PS, LaJoie DM, Jorcyk CL (2012) Bleach gel: A simple agarose gel for analyzing RNA quality. Electrophoresis 33(2), pp.366-369.
2. BalasubramaniamS, Duley JA,Christodoulou J (2014) Inborn errors of purine metabolism: Clinical update and therapies. Journal of inherited metabolic disease 37(5), pp.669-686.
3. Bell S, Kolobova I, Crapper L, Ernst C (2016) Lesch-Nyhan syndrome: Models, theories, and therapies. Molecular syndromology 7(6), pp.302-311.
4. Boesenberg-Smith KA, Pessarakli MM, Wolk DM (2012) Assessment of DNA yield and purity: An overlooked detail of PCR troubleshooting. Clinical Microbiology Newsletter 34(1), pp.1-6.
5. FasulloM, Endres L, (2015) Nucleotide salvage deficiencies, DNA damage and neurodegeneration. International journal of molecular sciences 16(5), pp.9431-9449.
6. Fu R, Sutcliffe D, Zhao H, Huang X, Schretlen DJ, et al. (2015) Clinical severity in Lesch-Nyhan disease: The role of residual enzyme and compensatory pathways. Molecular genetics and metabolism 114(1), pp.55-61.
7. Göttle M, Burhenne H, Sutcliffe D, Jinnah HA (2013) Purine

metabolism during neuronal differentiation: The relevance of purine synthesis and recycling. J neurochemistry 127(6), pp.805-818.

8. Green MR, Sambrook J (2019) Analysis of DNA by agarose gel electrophoresis. Cold Spring Harbor Protocols 2019(1).
9. Jinnah HA, Van Den Berghe G (2013) Metabolic disorders of purine metabolism affecting the nervous system.In Handbook of clinical neurology (Vol.113, pp. 1827-1836). Elsevier.
10. LakhaW, PanteleevaI, Squazzo S, Saxena R, Kroonen J, et al. (2016) DNA fragmentation and quality control analysis using diagenode shearing systems and fragment analyzer. Nature Method 13(10), p.890.
11. LeePY, CostumbradoJ, Hsu CY, KimYH (2012) Agarose gel electrophoresis for the separation of DNA fragments. JoVE(Journal of Visualized Experiments), (62).
12. Liu X, Harada S (2013) DNA isolation from mammalian samples. Current protocols in molecular biology 102(1), pp.2-14.
13. Magdeldin S, ed, (2012) Gel electrophoresis: Principles and basics. BoD-Books on Demand.
14. McCarthy GT, Green EM, Ogunbona O, et al. (2011) A population study of Lesch-Nyhan disease in the UK. Developmental Medicine & Child Neurology 53(1), pp.34-39.
15. Nguyen KV, Nyhan WL (2015) Lesch-Nyhan Syndrome in a Family with a Deletion Followed by an Insertion within the HPRT1 Gene. Nucleosides, Nucleotides and Nucleic Acid 34(6), pp.442-447.
16. Paéibo S (2012) Amplifying ancient DNA. PCR protocols: a guide to methods and applications, 159.
17. Pang B, McFaline JL, Burgis NE, Dong M, Taghizadeh K, et al. (2012) Defects in purine nucleotide metabolism lead to substantial incorporation of xanthine and hypoxanthine into DNA and RNA. Proceedings of the National Academy of Sciences 109(7), pp.2319-2324.
18. Quigley L, O'Sullivan O, Beresford TP, Paul Ross R, FitzgeraldGF (2012) A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese.Journal of applied microbiology 113(1), pp.96-105.
19. Sampat R, LarovereLE, Torres RJ, Ceballos-Picot, I,FischbachM, et al. (2011) Mechanisms for phenotypic variation in Lesch-Nyhan disease and its variants. Human genetics 129(1), pp.71-78.
20. SedlackovaT, Repiska G, CelecP, Szemes T, Minarik G (2013) Fragmentation of DNA affects the accuracy of the DNA quantitation by the commonly used methods. Biological procedures online 15(1), p.5.
21. SilvaLP, Lorenzi PL, Purwaha P, Yong V, Hawke, et al, (2013) Measurement of DNA concentration as a normalization strategy for metabolomic data from adherent cell lines. Analytical chemistry 85(20), pp.9536-9542.
22. Simbolo M, Gottardi M, CorboV, Fassan M, Mafficini A, et al, (2013) DNA qualification workflow for next generation sequencing of histopathological samples.
23. Torres RJ, Puig JG (2015) Hypoxanthine deregulates genes involved in early neuronal development. Implications in Lesch-Nyhan disease pathogenesis.Journal of inherited metabolic disease 38(6), pp.1109-1118.
24. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney L J (2012) Evaluation of methods for the extraction and purification of DNA from the human microbiome.