# The Analogy Between the Effect of Various Dyes for DNA Quantification in QUBIT 4.0

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***Abstract : This study has examined the effect of various fluorescent dyes, to check its feasibility for it's use in a highly sensitive fluorometer. QUBIT 4.0 is a fluorometer that is used to measure the quantity of RNA, DNA or Protein. The QUBIT 4.0 was developed to measure the RNA integrity and quality using its high sensitivity assays. It was also used to measure dilute sample concentrations ranging from 1-20 micro litres; which is why dye selection is crucial. The evolution of dye selection has been discussed in detail, ranging from Ethidium Bromide (commonly used as a fluorescent tag for gel electrophoresis), to the dye chosen here for DNA quantification in QUBIT 4.0, called PicoGreen (ultra sensitive dye which is used for ds - DNA quantification in QUBIT 4.0). An alternative dye is also discussed called C-phycocyanine, which ideally has no literature data cited for being used in QUBIT 4.0; but proof of concept points it out to be feasible as it conforms to most of the properties required for the same. Based on this study, one can understand the main features of QUBIT 4.0, the various dyes used in fluorometers and their key properties and also know what criteria to choose while selecting a dye for a particular assay, about PicoGreen, the main emphasis laid here, and its alternative; C-phycocyanine.***

***Keywords : QUBIT 4.0, Assay, Ethidium Bromide, SYBR dyes, Hoescht 33258, PicoGreen, C-phycocyanine, Fluorescence, Jablonski Diagram, Intercalation, Groove Binding, Major Groove, Minor Groove.***

## I. INTRODUCTION

The University of Utah; Huntsman Cancer Institute's High Throughput Genomics and Bio-informatics Analysis Shared Resource cites data on an experiment performed on ChIP-DNA sample concentration. NanoDrop tends to be very inaccurate due to high concentrations of background measured in the A260 wavelength in dilute concentrations of nucleic acids. The QUBIT 4.0, previously known as Quant- iT is used to measure DNA concentration for very dilute concentrations of sample solution. As mentioned earlier, The QUBIT 4.0 measures RNA Integrity and Quantity. The PicoGreen Assay uses PicoGreen effectively to mainly discriminate between various nucleic acid samples in accurately measurement of the concentration of dsDNA in the sample. This assay can quantitate samples that range from 10 pg/µl to 100 ng/µl. A comparison between various dyes have been drawn and also, the importance of the use of PicoGreen has been discussed. Importance of the use of cyanine dyes and tapping its advantages have been discussed in detail. PicoGreen is a non-sulphonated cyanine dye.

Cyanine dyes have existed from as far as we can think of and it's development has bloomed only in the past few years. They have no fluorescence of their own, but emit a strong fluorescence in the presence of nucleic acids. More the positive charges, more is the affinity of these dyes to bind with non-covalent moieties. Throwing light on some other dyes that have been previously used, an article called [3]"MYTH OF ETHIDIUM BROMIDE", written by Derek Lowe; states that normally, one tends to pay heed to the short term mutagenicity, which makes EtBr safer than another dye that would be discussed, called SYBR Green. The analogy between dyes like EtBr and SYBR dyes and Hoescht dye variants have been discussed. The article suggests that these dyes have structures that tend to be intercalating agents, which would be discussed in the sections below, which tend to disrupt the structure of the given nucleic acid. This brings us to the main focus, PicoGreen as the most appropriate dye to use for high sensitivity as well as dilute concentrations of sample measurement or quantification. PicoGreen is a fluorescent probe that binds dsDNA and forms a luminescent complex of high intensity on binding. On binding with DNA, there seems to be a >1000 fold. Fluorescence increases by >38,000 fold in the presence of silver nano particles; which shows improvement in properties due to metal-enhanced fluorescent effects. This was cited in the Biophysical Journal, Volume 99; Issue 9; Published on November 3, 2010. Therefore, this study helps us understand the effect of the dyes discussed based on its fluorescence, and other properties like extinction coefficient, quantum yield, major or minor groove binding etc.; by doing a comparitive study and also, about the QUBIT 4.0 in detail which can help us connect the dots and understand the working mechanism of the fluorophore and the nucleic acid sample using QUBIT 4.0.

## II. UNDERSTANDING QUBIT 4.0

### *A. Principle*

The design allows one to precisely measure RNA, DNA and protein quantity. The Qubit 4 also easily measures RNA integrity and quality.

### *B. Key features*

1. Fast and accurate measurement of RNA, DNA and Protein quantities.
2. Produces output in less than 5 seconds per sample.
3. All the assays that are run are stored in the device's database which helps in procuring the data at any point of time using a USB cable or whatever mean seems suitable and one can re-run assays off the device's memory.

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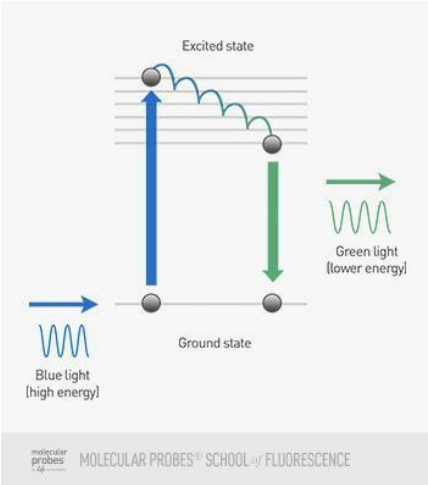
4. The device can be operated in multiple languages to suit the needs of customers from all over the world.
5. A reagent calculator is present which helps us accurately calculate the amount of working solution and buffer that we require to run a set number of samples.

## III. FLUORESCENCE MECHANISM AND ANALOGY OF DYES

Before we begin to understand about the dyes taken into consideration, we need to understand how fluorescence basically works and then we proceed to the analogy drawn between the dyes to finally arrive to the desired dye and its alternative.

### *A. Fluorescence Mechanism*

The Jablonski diagram is used to explain the above mechanism. It illustrates the electronic states and the transitions between the molecules. They are arranged vertically by energy and grouped horizontally by spin multiplicity. The right amount of energy is required from the source for the donor dye molecule. This energy is absorbed and it is used to transition to a higher energy state (Es). The energy released at this level is in the form of a photon and this transfer of energy occurs after the donor molecule moves back to the lower energy level. The photon emitted photon interacts wirh the tandem dye molecule, which excites this molecule to a higher energy level and it emits fluorescence of the complementary colour.

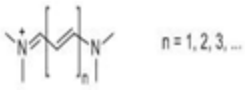


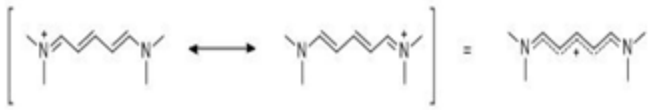

**[2]Fig 1: Jablonski Diagram to explain the fluorescence phenomenon. Absorption, excitation, first emission, second absorption and emission is shown.**

Some important points to remember are :
1. Loss of energy can occur through rotational, vibrational motions as well as heat transfer.
2. The first emission spectrum that is released has a wavelength more towards the red side as compared to the previous wavelength.
3. These photons emitted are absorbed and excited again to ultimately emit photons of another different characteristic wavelength that we see.

### *B. Introduction to Cyanine Dyes*

The development in the last several years of cyanine dyes as fluorescent labels is vast. Cyanine dyes are molecules that contains nitrogen atoms, connected by a polymethine bridge. The presence of delocalised charges on the atoms is the reason why these dyes are so widely considered. The non- covalently binding labels have no fluorescence of their own, but a fluorescence is emmitted when bound to a target like nucleic acids. Another fact to note is that more the number of positive charges, more is their binding affinity to nucleic acids. These charges can be of the order of tri, penta and heptamethine). These labels have large molecular absorptivity, high quantum yield, large stoke's shift, good stability to photobleaching as well as moderate pH sensitivity for the label to work as desired when bound to a substrate, due to their structure as sited by Lumiprobe[2]:



**[2]Fig 2: Resonance in cyanine dye structures. The polymethine bridges are chosen to depict the resonance structure which further shows the molecular donor and acceptor sites.**

Cyanines have high extinction coefficients and long cyanines have higher absorbance and emission wavelengths.

### *C. Properties of Cyanine Dyes*

1. High molar absorptivity.
2. Low intrinsic fluorescence.
3. Large fluorescence enhancements of over 1000- fold.
4. Quantum yield of about 0.9.
5. Moderate to high affinity to nucleic acids, causing no damage to other bio polymers.

## IV. INTERCALATION AND GROOVE BINDING

Dyes that bind DNA fall into two mechanistic categories:
In chemistry, intercalation means inserting a molecule or an ion into a layered structure, like Graphite.
Groove Binding involves temporary non-covalent interaction through intermolecular physical forces of attraction by a protein or low molecular weight ligand with double-stranded DNA in either the major groove or minor groove formed by the double helical structure, in either a sequence dependent or independent fashion.

### *A. An Insight into Groove Binding*

1. The DNA had two grooves which are responsible for its binding properties. It is a simple consequence of the geometry of the base pairs.
2. The angle between the glycosidic bonds is 120 degrees and 240 degrees.
3. As more and more base pairs stack up one on top of another, the smaller on one end forms the minor groove and the larger angle forms the major groove, between sugars.

***B.*** *Analogy between A-T and T-A base pairs and G-C and C-G base pairs*

Binding can happen through both A-T as well as T-A base pairs. The difference can be made out in it's structure as shown below. A T-A base pair will have the palindromic sequence of the A-T base pair, both in the major and the minor groove. Edges of an A-T base pair :

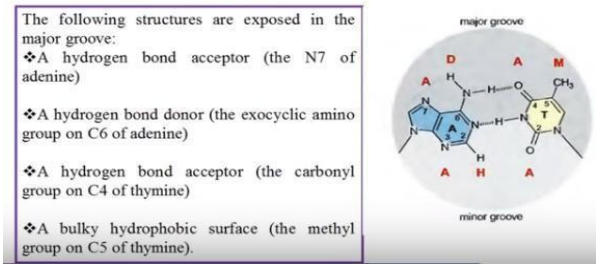



**Fig 3: A-T base pair**

Edges of a G-C base pair :

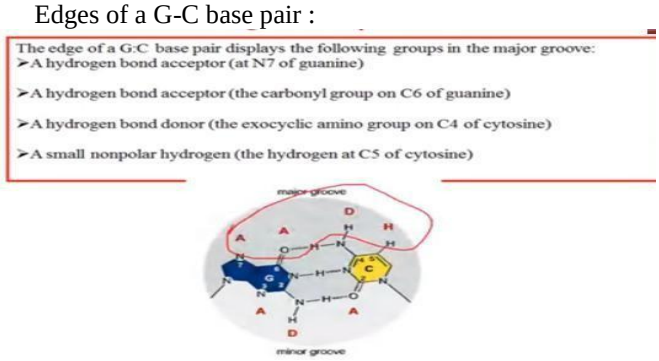



**Fig 4: G-C base pair**

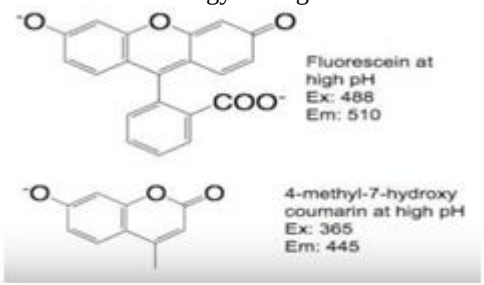
The main question is, why is major groove binding preferred over minor groove binding, and it can be most certainly answered this way.

1. The major groove as seen before has more number of "recognizable sites" which contains an abundance of chemical information.
2. This allows the dye to unambiguously recognize the sequence without having to actually open and disrupt the structure.
3. The minor groove does not provide a lot of chemical information and hence cannot distinguish between base pairs.
4. Also, the small size of the minor group also makes it unfavourable for binding purposes.
5. For "specific binding proteins", i.e; for groups that choose to bind with A-T base pair and not T-A base pair for instance, it is very important that the binding happens through the major groove as one can clearly distinguish where the binding is occurred.
6. Major groove binding is preferred because it is wide enough to fit an alpha-helix or a 2 strand beta sheet and also, can distinguish all 4 base pairs.
7. However, many non sequence specific binding agents bind to the minor groove and often unwind or bend the molecule.

## V. CHOOSING CRITERION BASED ON SPECTRAL AND PHYSICAL PROPERTIES

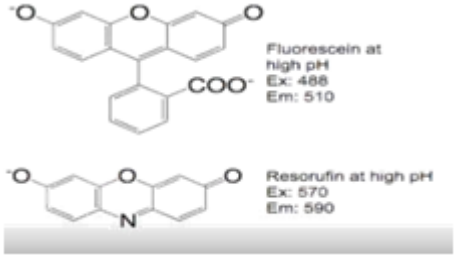
[7]Factors while choosing a fluorophore dye are listed below.
Extinction Coefficient : High extinction coefficients often exceeding 100,000 $M^{-1}cm^{-1}$

Quantum Yield : Ratio of the decided event to the number of photons absorbed by the system.
Stoke's shift : Difference between the excitation and emission maxima. A large shift is favourable to prevent the leakage of the excitation maxima into the emission detector.
Stability to photo bleaching : Which means that the fluorophore should not disintegrate during the process.
pH sensitivity : pH should not be maintained as too high or low a pH as it can lead to photobleaching.
As cited by [7]Tim Mitchison from Harvard Medical school, some important points to remember are that an extended conjugated structure is always favourable for a fluorescence mechanism due to the presence of delocalised pi orbitals that allow particularly useful excitation and emission properties. Larger conjugated structures give rise to a larger wavelength. Symmetry is also a key factor.
Symmetry can result in a small stoke's shift which can be a problem in case of identifying any sort of fluorescence but it is advantageous as the molecule does not rotate and hence there are less losses of energy through bond rotation.




**[7]Fig 5: Fluorescein vs 4-methyl-7-hydroxy coumarin to explain symmetry. Fluorescein having a complete structure has lesser stoke's shift due to its orientation.**

Presence of a heteroatom in the chain also determines the energy difference and give out higher wavelengths as a result. The presence of nitrogen in the given molecule shifts the wavelength more towards red which is why nitrogen based dyes as preferred as they give out longer wavelengths with larger stoke's shift.
Rigidity of the molecule is also important. Incase of the example given here in the image, phenolphthalein is devoid of oxygen in the centre which results in bond rotation along COO- which is the result of it being highly coloured (deep pink) and also, absence of its fluorescent properties. This occurs because, as mentioned earlier; bond rotation results in the loss of energy and in this case, it is very high which results in the absence of fluorescence.




**[7]Fig 7: Fluorescein vs Phenophthalein to explain molecular rigidity. Bond rotation can result in the loss of energy and hence absence of fluorescence.**

### *A. Analogy between different dyes that have been used*

*1. Ethidium Bromide Solution vs SYBR dyes:*

Introduction to SYBR Green I (SG): It is an asymmetrical cyanine dye stain. The SYBR family of dyes is a subsidiary of Life Technologies Corporation. The resulting DNA-dye-complex absorbs blue light at 497 nm and emits green light at 520 nm. The stain selectively binds to double-stranded DNA. SYBR-like dyes are known to work slightly better than EtBr. The EtBr:DNA complex has a fluorescence quantum yield of ~0.15 as compared to SYBR Green I:DNA has a fluorescence quantum yield of ~0.8.

SYBR Green I, taking dsDNA into consideration, shows higher sensitivity than EtBr using a standard transilluminator. SYBR Green dyes are compatible with many scanners and illuminators.

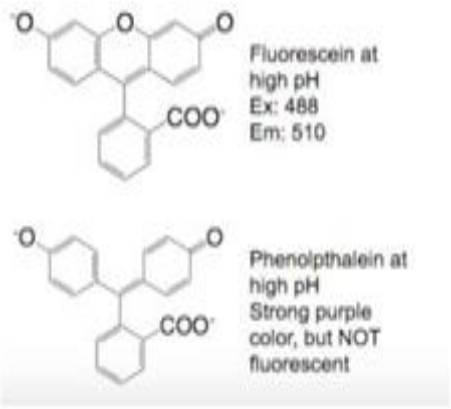
Eventhough EtBr shows a 30 fold enhancement or binding with a fluorescent dye, SYBr is still a better option as it shows a 1000 fold enhancement on binding with the same.

Both of these dyes, being intercalating agents; have no significance even with the properties mentioned above as these dyes would ultimately end up disrupting the structure of the nucleic acid.

*2. PicoGreen vs Hoescht 33258:*

Hoechst 33258 has been well known as a counterstain for nucleic acids. The dyes Hoechst 33258 and Hoechst 33342 are the ones are most commonly used. The dyes bind to the minor groove of double-stranded DNA with a preference for adenine and thymine. Although the dyes can bind to all nucleic acids, AT-rich double-stranded DNA strands seem to enhance fluorescence.

PicoGreen dsDNA reagent enables researchers to quantitate as little as 25 pg/mL of dsDNA. This sensitivity exceeds that of Hoechst 33258 by 400-fold.

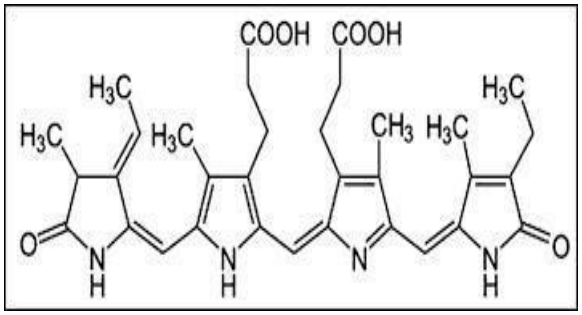



**[7]Fig 6: Fluorescein vs Resorufin to explain the effect of a heteroatom. Addition of heteroatoms alters the spectral characteristics, raising the absorption and emission maximum.**

The Hoechst 33258– based assay is somewhat selective for dsDNA and it can easily distinguish between other nucleic acid moieties present in a given sample.

Even PicoGreen can perform the same function, of differentiating between the various moieties used in a sample, but it can do so for extremely dilute concentrations, which makes this dye stand out as compared to the other dyes discussed above.

## VI. C-PC, A POTENTIAL ALTERNATIVE (DYE)



**[4]Fig 8: C-phycocyanine structure**

Here, the use natural fluorophores as a substitute to organic ones, which is stated here to be; for example- EtBr as these chemical dyes can be a cause of biohazards and also can be extremely expensive.

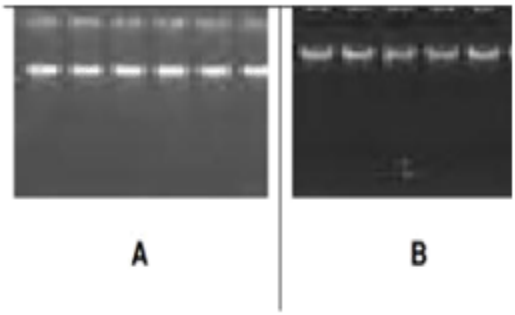
C-PC, is a dye that can be naturally obtained from an algae called Spirulina by various methods and was analyzed with human lymphocytes, fowl blood cells and genomic DNA of plant (guava) as well as human DNA.

D-PC is an "antenna pigment" found in many cyanobacteria and eukaryotic algae to increase the rate of photosynthesis as chlorophyll cannot tap all light wavelengths. (Glazer, 1981). C-PC contains multiple chromophore groups, which are responsible for the fluorescent properties of these proteins. CPC belongs to the family of phycobiliproteins. (Hardy, 1986; Glazer, 1994; Kulkarni et al., 1996).

The speciality of this dye is that it does not disrupt the performance of the dye in it's presence. [4]This was cited in the International Research Journal of Biotechnology; titled "An efficient method for extraction of C-phycocyanin from Spirulina sp. and its binding affinity to blood cells, nuclei and genomic DNA"; written by P. Singh, M. Kuddus and G. Thomas, published on the the 20th of December 2010.

### *A. Binding efficiency of C-PC with blood cells and Genomic DNA*

Fluoresence microscopy was the technique used to test whether C-PC can penetrate through the the nucleus. Anucleated cells show no fluorescence like RBC's and platelets. However, it was observed that human lymphocytes and fowl blood cells showed a reactivity greater than $10^{-4}$ The reason these cells showed fluorescence was because the dye was able to penetrate through the cell nucleus. Since this fact was established, the binding efficiency of diluted C-PC with genomic DNA was tested. The dilution was taken to be 10-6 and it was feasible at 25oC. The staining is shown in the figure given below: economics comes after testing the feasibility as to whether it is viable to extract the natural dye for the dye or formulate a similar synthetic dye with the same characteristics but at less cost; is also a challenge. The amount of time the fluorescence stays once initiated, the exact binding mechanism of the dye and the specificity of binding to just DNA is still unknown. Research in this field of study can truly change the approach to the way C-PC is used as a dye.




**[4]Fig 9: Human (A) and plant (B) DNA stained with C-PC (10-6) at 25oC. This is the result of the binding of the dye C-PC to DNA on gel electrophoresis.**

***B.*** *Feasibility*

As per the experiment, Human genomic DNA was isolated from blood and plant DNA from guava leaf was isolated by the method of Porebski et al. (1997). The isolated DNA samples were quantified by a spectrophotometric method. Binding efficiency of very small concentrations of C-PC have been found when DNA was stained with serially diluting C-PC at different temperature (20, 25 and 37ºC) and separated by agarose gel electrophoresis that was observed under a gel documentation system. The electrophoresis result also highlights the binding affinity of genomic DNA to C- PC. The dye shows great results on electrophoresis without dissociation at microlitre dilutions. C-PC has no specificity for any other proteins so it can be safely used as a DNA specific stain and in genomic DNA analysis.

## VII. RESULT AND DISCUSSION

PicoGreen vs C-PC (Spectral characteristics):\

| Property | PicoGreen | C-PC |
|---|---|---|
| Absorption maximum (nm) | 480 | 621 |
| Emission maximum (nm) | 520 | 642 |
| Molar extinction coefficient (ε) | 70,000 $M^{-1} cm^{-1}$ | $1.54x10^6 M^{-1} cm^{-1}$ |
| Quantum Yield | 0.5 | 1.8 |

The above table shows that while PicoGreen has a higher stoke's shift than that of C-PC, both the stoke's shift are still relatively small. However, the quantum yield of C-PC is much higher than PicoGreen which is much more preferred as it is greater than unity. The small stoke's shift states that both the molecules are rigid and symmetric structures with no bond rotation and hence, minimal loss of energy and maximum fluorescence output. Although experiments show that C-PC is ideal to be used as an alternate, the scope of the future would be to tap all of its important characteristics to see if its truly ideal or not. No information about what bio assay (combination of working solution and buffer) can be used in combination with the same to test out the feasibility of this dye with QUBIT 4.0 as well as prove it to be an appropriate dye as a replacement for PicoGreen. Novelty of the same cannot be ensured without testing it on a substrate. A kit needs to be formulated with C-PC as the dye, instead of PicoGreen or with the help of engineering techniques, a new dye can be synthesized based on the desired properties we need to obtain, most preferably on the same lines as some of the natural dyes. The challenge lies in formulating the kit which is indeed the future scope of research. The

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## AUTHORS PROFILE

**Sowmya Seshadri,** has been an important contributor in improving the lives of the under privileged by actively aiding those in need, providing them with quality education, primarily focusing on their verbal and communication skills in the English language for the past two years. The above profile consists of teaching as well as being a mentor to the potential candidates who wish to achieve the same. U&I, a non-profit organization has paved the way for Sowmya in fulfilling her dream of being an active member of the society starting as a volunteer and now acquiring a leadership role in the, 'Student Impact Vertical', 2019-20. Her education from CMR National Public School, Bengaluru nudged her towards identifying her strength and interest in chemistry which in turn was a key factor in opting her undergraduate field of study in Chemical Engineering from SRM Institute of Science and Technology, Chennai. The subjects offered in her college sparked her willingness to focus not just on Chemistry but also explore various domains with respect to the field of bio-chemistry. An internship in TheraCUES Innovations Pvt. Ltd.,Bengaluru was the guiding force behind the success of this paper. TheraCUES was equipped with advanced technology actively used only by a few organizations in India unlike some other nations. The novelty of this device in her country was the stimulus in pursuing her research to improve the working characteristics of the device referred to in the paper called,'QUBIT 4.0'.