TECHNICAL REPORT

SERIES 8

The Technology of Reagents in the Automated Hematology Analyzer Sysmex XE-2100™ - Red Fluorescence Reaction-

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Key Words

Automated Hematology Analyzer, XE-2100, Red Fluorescence,
Polymethine Dye, Fluorescence Dye

INTRODUCTION

In the field of clinical laboratory testing, a wide variety of dyes are used for pathological tests, hematologic morphology tests, and urinalysis tests[1]. Well-known dyes include hematoxylin and pyronine-Y for pathologic tests, and azure B and eosin-Y contained in Giemsa's staining solution.

Our products also use such dyes; for example, the fluorescent dye Auramine O[2] is used in the world's first commercially available fully automated reticulocyte analyzer R-1000™ launched in the market in 1988, and a phenanthridine dye and a carbocyanine dye are used in the fully automated urine sediment analyzer UF Series[3].

Being newly launched in the market, the XE-2100™ uses a polymethine dye for 4-part differential WBC counting, reticulocyte measurement, and erythroblast measurement. This dye is excited by a red semiconductor laser (wavelength 633 nm) and emits red fluorescence of 660 nm or higher wavelength[4]. The features of this dye are outlined below.

LIGHT AND COLOR

Before discussing the dye, the differences between light color and solid material color must be understood. The three primaries of light and those of solid material differ and must be distinguished from another. When white light, such as sunlight, is spectrally divided using a prism, the seven rainbow colors appear: red, orange, yellow, green, blue, indigo, and violet in the descending order of wavelength (***Fig. 1***). The shorter the wavelength is, the greater the photo energy.

Using Griffith's color circle, the colors of light are divided as shown in ***Fig. 2***. In this color circle, which provides light-characterizing information, numbers 1 through 9 represent respective spectral colors, whereas number 10 purple represents a mixture of red and violet light. Mixing a pair of colors separated by its intermediate color produces the intermediate color. Mixing the red and yellow colors produces an orange color. Mutually facing colors are complementary; mixing a pair of facing colors produces white light.

The three primaries of light are red (R), green (G), and blue (B). When the cathode ray tube of a television set or a PC display is seen through a magnifying lens, you can readily realize that the display surface consists of a collection of the three colors R, G, and B, which produce the multi-color display in color televisions (***Fig. 3***).

On the other hand, the three primaries of solid material are magenta (M), yellow (Y), and cyan (C); mixing all these colors produces a black (gray) color (***Fig. 4***). It should be noted that light and solid material produce different tones of red or blue color.

Auramine O looks yellow because it absorbs blue light to produce a yellow color as a mixture of the colors (green and red) of unabsorbed light. Aqueous solutions of dyes

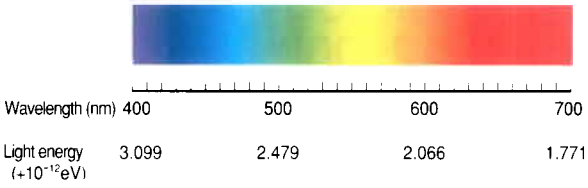

Fig. 1 *Light wavelength and color*

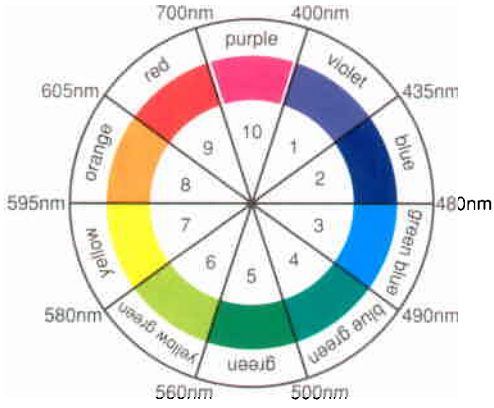

Fig. 2 *Griffith's color circle*

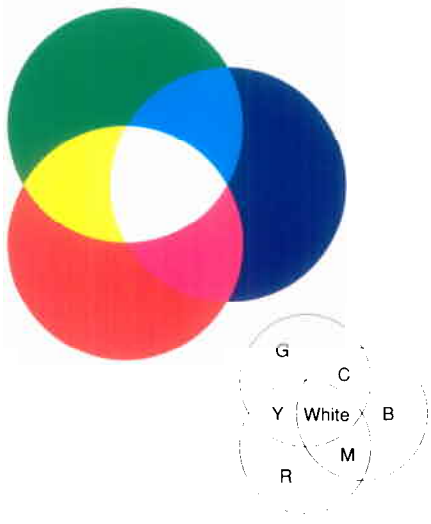

Fig. 3 *The three primaries of light (additive color process)*

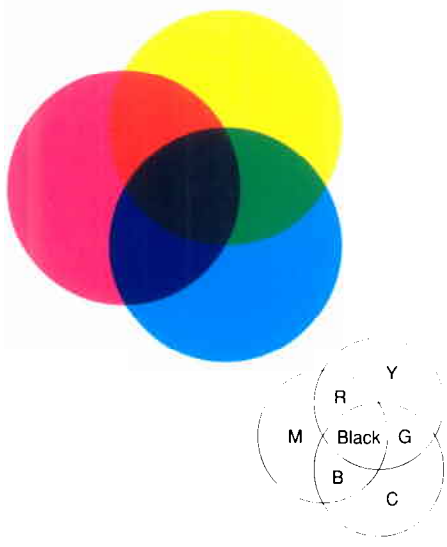

Fig. 4 *The three primaries of color material (subtractive color process)*

absorbing argon laser light (488 nm, blue light) show a yellow color, whereas those of dyes absorbing helium neon laser or red semiconductor laser light (633 nm, red light) show a blue color. When the argon laser is used to screen for a dye suited for a particular light source, yellow to orange dyes are selected. When a red semiconductor laser is used, as in the XE-2100, blue dyes are preferred.

CLASSIFICATION AND STRUCTURES OF DYES

There are numerous synonyms for the various dyes, including long-used traditional names, and designations given by developers or manufacturers; this aspect has caused much confusion for dye users. Currently, efforts are made to provide well-organized designations and numbers for dyes via the Color Index[5]. On the basis of the characteristics of dyes, Color Index Generic Names are given, *e.g.*, Basic Orange 14 (Acridine Orange) and Acid Red 87 (Eosin-Y). On the other hand, Color Index Constitution Numbers are given according to structural formula: from 10,000 for the nitroso group to 77,999 for inorganic pigments. For example, Auramine O is designated as C.I. 41,000 and Basic Yellow 2. However, there are many unregistered functional dyes for color photography, color copying, color liquid crystal displays, etc. The dye used for the XE-2100 is a basic dye structurally classified under Methine and Polymethine Dyes.

FLUORESCENCE AND FLUORESCENT DYES

A fluorescent dye absorbs light of the same wavelength as that of the light source used; its electron state is excited by the light energy absorbed. The light emitted upon return to the ground state is fluorescent, having a longer wavelength than that of the light absorbed[6, 7]. When a fluorescent dye is used, it is advantageous to have a light source whose wavelength is as short as possible, because higher photo energy is obtained, and

because the variety availability for fluorescence increases. However, some problems arise from the light source; the durabiling life of mercury and halogen lamps is at most several hundred hours (up to several thousand hours for argon and helium neon lasers), and the light source size is large.

On the other hand, the red semiconductor laser is the best for configuring a compact long-serving apparatus because its light source is as small as 9 mm in diameter and 5 mm in depth, and because its durabiling life is more than several tens of thousand hours. Our immunology analyzer PAMIA-10™ (sold only in Japan), launched in the market in 1987, incorporates a red semiconductor laser of 780 nm wavelength[8]. Also, our automated multi-parameter hematology analyzer SF-3000™, launched in 1995, incorporates a red semiconductor laser of 650 nm wavelength[9]. These analyzers uses scattered light as a means of measurement, whereas the XE-2100 uses red fluorescence, forward scattered light, and side scattered light generated by irradiating polymethine-stained cells with a red light beam from a red semiconductor laser of 633 nm wavelength.

FEATURES OF POLYMETHINE DYES

As functional dyes[10] supporting up-to-date industrial technology, polymethine dyes are widely used as near infrared-absorbing dyes, dye lasers[11], LB membranes (monomolecular membranes, used to produce CD disks), and color photo film sensitizers, but are in poor demand for use in the essential application of fabric dyes. Polymethine dyes are structurally characterized by the binding of a heterocyclic nucleus (cyclic compound containing nitrogen, sulfur, and oxygen) and a methine chain ($-CH_2=$) (***Fig. 5***). They are also characterized in that the wavelength of the light absorbed can theoretically be calculated from the kind of heterocyclic nucleus, binding side chain, length of the methine group, etc. For this reason, when a color photo film of high sensitivity to a particular wavelength is prepared, a polymethine dye synthesized on the basis of such calculation is added as a sensitizer. There are a wide variety of heterocyclic nuclei used in polymethine dyes; representative examples are given in ***Fig. 6*** in the ascending order of absorption wavelength. As the number m for the methine chain $-(-CH_2=CH_2-)_m-$ to which the heterocyclic nucleus is joined increases by one, the absorption wavelength of the dye shifts toward the longer-wavelength side by about 100 nm. For absorption wavelength changes among different designations and structural formulas of polymethine dyes, please refer to the summarized information given in the appendix, following this article.

POLYMETHINE DYE APPLICATION TO CYTOLOGY

Polymethine dyes are seldom used for staining purposes in hematologic morphology tests or pathological tests. Rather, they are widely used in membrane potential determination for research purposes[13]. Waggoner[12, 14-16] and Kamino[17-19] presented a number of reports of the use of polymethine dyes as showing slow response to membrane potential changes. Also available are some reports using DiOC6(3) for investigation of mitochondria and endoplasmic reticulum[20-24]. Used for Plasmodium assay and reticulocyte counting by flow cytometry, Thiazole Orange is also a polymethine (methine) dye[25, 26], having a structural formula characterized by asymmetric heterocyclic nuclei (***Fig. 7***). Almost all light sources used are argon lasers or fluorescent microscopes for blue light.

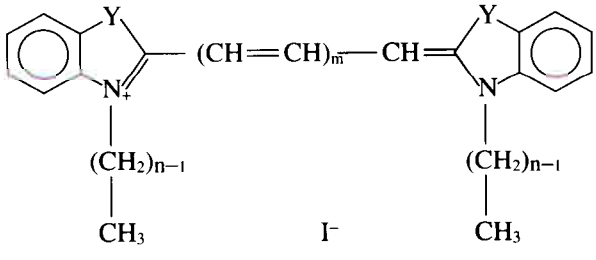

Fig. 5 General structural formula of polymethine dyes

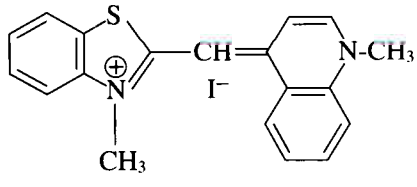

Fig. 7 Structural formula of thiazole orange

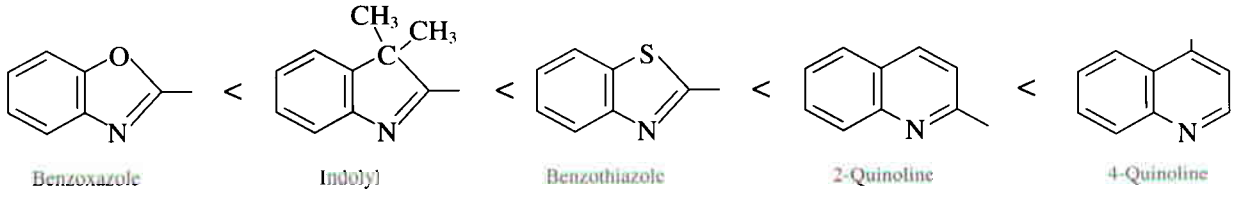

Fig. 6 Polymethine dye heterocyclic ring and absorption wavelength shift (as the heterocyclic ring shifts to right, the absorption wavelength is longer)

Combining a red semiconductor laser and a polymethine dye, the XE-2100 is capable of achieving 4-part differential WBC counting, erythroblast measurement, and reticulocyte measurement (platelet measurement). As stated above, polymethine dyes permit alteration of their maximum absorption wavelength by changing the structural formula of the dye. The longer the polymethine chain is, the more bathchromic shift (blue color side, red absorption) the color obtained has. Because increasing the polymethine chain length results in increased molecular weights, decreased solubility, and decreased chemical stability, measures for improving the water solubility and storage stability must be taken.

REAGENTS FOR WBC 4-DIFFERENTIAL COUNTING (STROMATOLYSER-4DL, STROMATOLYSER-4DS)

The surfactant contained in STROMATOLYSER-4DL lyses and dissolves erythrocytes and platelets and makes holes in the leukocyte membrane, after which the polymethine dye contained in STROMATOLYSER-4DS enters white blood cells and stains nucleic acids and cytoplasmic organelle, such as endoplasmic reticula. Because immature granulocytes appear in zones of higher fluorescence intensities than neutrophils, and because atypical lymphocytes also appear in zones of higher fluorescence intensities than lymphocytes and monocytes, polymethine dyes are assumed to stain not only nucleic acids but also cytoplasmic organelles (***Fig. 8***).

REAGENTS FOR ERYTHROBLAST MEASUREMENT (STROMATOLYSER-NR, LYTIC AGENT, STAINING LIQUID)

The surfactant contained in the STROMATOLYSER-NR lytic agent lyses erythrocytes, exposes erythroblast nuclei, and make holes in the leukocyte membrane. The polymethine dye contained in the staining liquid then enters the cells and stains nucleic acids (nuclear membrane). Because basophilic erythroblasts appear in zones of higher fluorescence intensities than orthochromatic erythroblasts, this dye is also assumed to stain not only nucleic acids (nuclear membrane) but also cytoplasmic organelle (***Fig. 9***). STROMATOLYSER-NR is characterized by its capability of clearly distinguishing erythroblasts from leukocytes (particularly lymphocytes). We previously developed an assay system for erythroblasts in which erythroblast lysis with acidic hypotonic solution is followed by erythroblast nuclei staining with PI (propidium iodide) or EB (ethidium bromide); however, it failed due to the inability to discriminate erythroblasts from damaged (dead) lymphocytes[27, 28]. STROMATOLYSER-NR enables us to obtain quantitative data on the erythroblast count and ratio without the influence of dead lymphocytes.

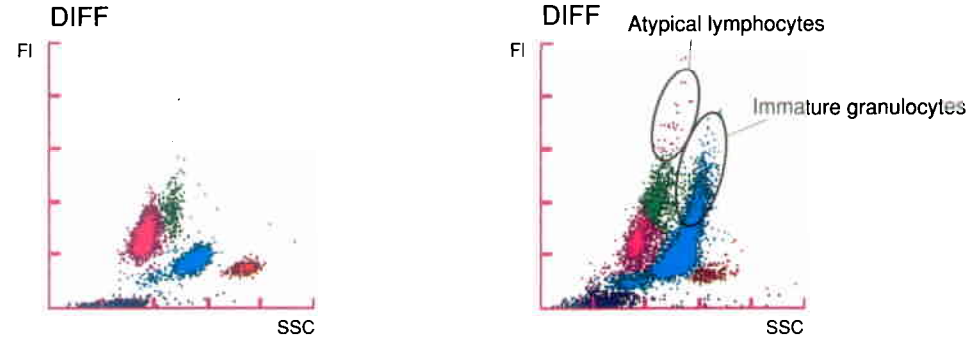

Fig. 8 Diff scattergram from a normal healthy subject (left) and diff scattergram with immature granulocytes amd atypical lymphocytes

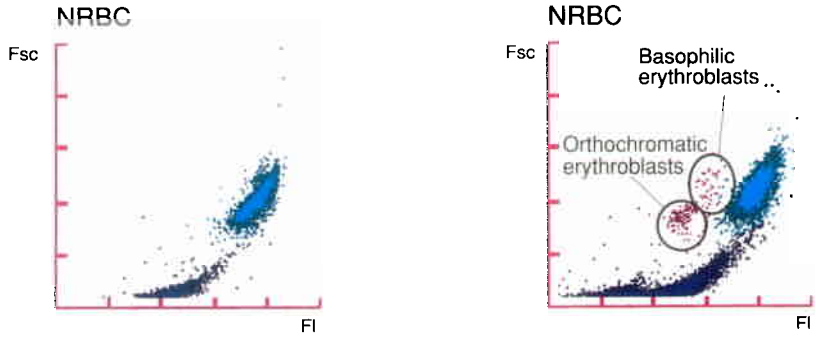

Fig. 9 NRBC scattergram from a normal healthy subject (left) and NRBC scattergram with orthochromatic erythroblasts and basophilic erythroblasts (right)

REAGENTS FOR RETICULOCYTE MEASUREMENT (RET-SEARCH II, DILUENT AND STAINING LIQUID)

The surfactant contained in the RET-SEARCH II diluent swells erythrocytes and leukocytes and slightly damages the cell membrane to facilitate dye entry in the cell, after which the polymethine dye contained in the staining liquid stains RNA and DNA. Mature erythrocytes and reticulocytes are fractionated by their RNA content difference, whereas reticulocytes and leukocytes are fractionated by DNA and RNA content differences. In addition, because platelets are also stained intensely, they are counted as PLT-O as discriminated from small erythrocytes and fragmented erythrocytes (***Fig. 10***).
Our reticulocyte analyzers R-1000, R-2000™, R-3000, and R-3500™, all based on argon laser, use the fluorescent dye Auramine O. Having a structure in which a phenyl group (dimethylaniline) is bound to the central carbon atom (***Fig. 11***), Auramine O is characterized by its behavior in aqueous solution. It normally does not emits fluorescence due to consumption by intramolecular rotation diffusion of the light energy absorbed, but produces strong fluorescence when its molecular movement is interfered with as it binds to membranes or nucleic acids. Polymethine dyes are also characterized in that when their molecular movement is interfered with as they bind to membranes or nucleic acids, they absorb more light and emit more intense fluorescence, because their structure comprises heterocyclic nuclei bound via methine chains. Although there is some misconception that the use of a fluorescent dye results in poor signal/noise (S/N) ratios due to strong background fluorescence (fluorescence from aqueous solution alone), cell measurement using Auramine O or polymethine dyes results in good S/N ratios.

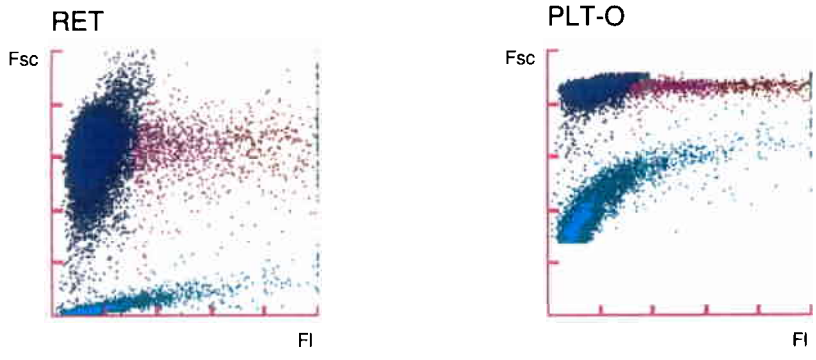

Fig. 10 RET scattergram and PLT-O scattergram

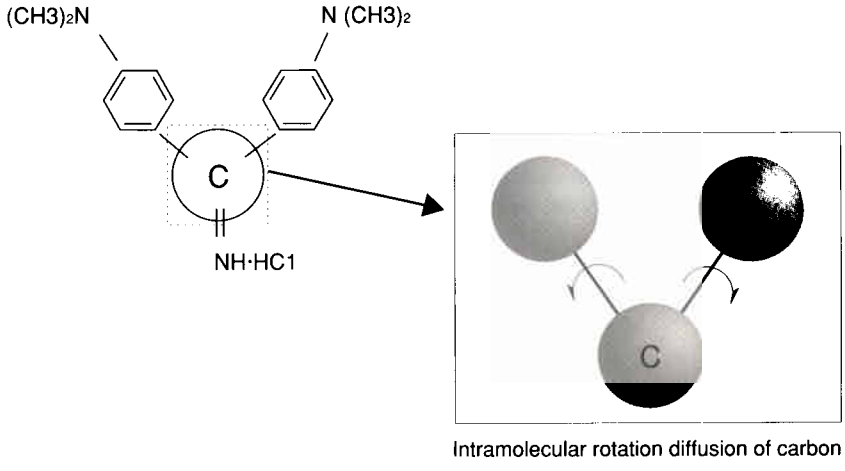

Fig. 11 Structural formula of Auramine O

CONCLUSION

Red fluorescence analysis using the XE-2100, based on a combination of a semiconductor laser and a polymethine dye, has been outlined. Traditionally, the argon laser (wavelength 488 nm, blue light) has formed the mainstream of laser-based fluorescence analysis, and the mainstream of red lasers has been the helium-neon laser (633 nm). Keeping pace with the recent trend toward shorter oscillation wavelengths for semiconductor lasers, the XE-2100 has been brought into practical application in cell measurement based on red fluorescence detection using a semiconductor laser of 633 nm oscillation wavelength.

As a sophisticated flow cytometry analyzer based on a combination of optics, electrical engineering, electronics, software technology, fluid technology, and reagent technology, the XE-2100 also incorporates unique reagents, mainly surfactants, initially developed for the SE-9000™, and the IMI channel (RF/DC detection method). As the development of semiconductor lasers advances steadily, it can be expected that the three primaries of light (R, G and B) will be obtained using semiconductor lasers. The author hopes that this commentary will help you to understanding the reagent technology of the XE-2100 and its contribution to facilitating cytologic studies using light and dyes.

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APPENDIX

Designation of Polymethine Dyes

For example, the polymethine dye having the structural formula given in ***Fig. 13*** is designated as 3-ethyl-2-[3-(3-ethyl-3-benzolenylidene)-1-propenyl]benzoxazolium iodide or 3,3'-diethyl-2,2'-oxacarbocyanine iodide. To avoid naming complexity, Waggoner, et al. have proposed the following abbreviation system:
With respect to the general formula of polymethine dyes given in ***Fig. 5***, Di indicates the particular heterocyclic nucleus of the symmetric dye, the heterocyclic ring is abbreviated as O, S, or I when the atom (molecule) corresponding to Y is oxygen O (benzoxazole), sulfur S (benzothiazol), or C(CH3)2 (indolyl), respectively, and the abbreviation Q is used when heterocyclic nuclei is 2-quinoline. The carbon atom number for the side chain adjoining the N atom of a heterocyclic nucleus is represented by Cn. The carbon number for the methine group (-CH=) tying the two heterocyclic nuclei is expressed by a figure in parenthesis. The counterpart anion is not shown. This abbreviation system can be summarized in ***Fig. 14***. The aforementioned polymethine dye is given the abbreviation DiOC2(3). ***Table 1*** shows different polymethine dye heterocyclic nuclei and polymethine chains and corresponding maximum absorption wavelengths.

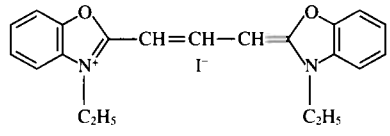
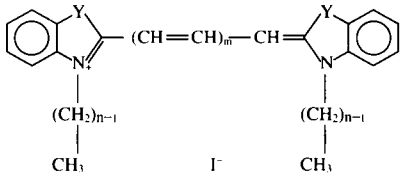


Fig. 13 *Structural formula of DiOC$_2$(3)*

General structural formula



Abbreviated formula :DiYCn (2m+1)

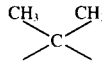
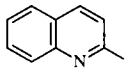
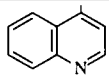
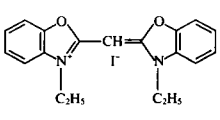
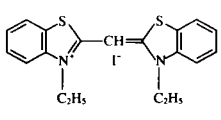
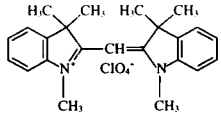
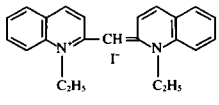
Y=	O	S	I	Q	L
Heterocyclic ring designation	Benzoxazole	Benzothiazole	Indolyl	2-Quinoline	4-Quinoline
Structure					

Fig. 14 *Abbreviations for polymethine dyes*

Table 1 *Structural formulas and maximum absorption wavelengths of various polymethine dyes*
Using Waggoner's abbreviation system, the relationships between polymethine dye structural formula and maximum absorption wavelength are shown (source: sales catalog of Nihon Kankoshikiso Co.,Ltd.)

	DiOCn (2m+1)	DiSCn (2m+1)	DiICn (2m+1)	DiQCn (2m+1)
				
m=0				
Abbreviation	DiOC2 (1)	DiSC2 (1)	DiIC1 (1)	DiQC2 (1)
Maximum absorption wave length	375 nm (methanol)	423 nm (methanol)	433 nm (methanol)	523 nm (methanol)
Product name	NK-863	NK-88	NK-3212	NK-757
m=1				
Abbreviation	DiOC2 (3)	DiSC2 (3)	DiIC1 (3)	DiQC2 (3)
Maximum absorption wave length	483 nm	557 nm	545 nm	608 nm
Product name	NK-85	NK-76	NK-79	NK-3
m=2				
Abbreviation	DiOC2 (5)	DiSC2 (5)	DiIC1 (5)	DiQC2 (5)
Maximum absorption wave length	580 nm	652 nm	638 nm	707 nm
Product name	NK-580	NK-136	NK-529	NK-1456
m=3				
Abbreviation	DiOC2 (7)	DiSC2 (7)	DiIC1 (7)	DiQC2 (7)
Maximum absorption wave length	682 nm	759 nm	741 nm	817 nm
Product name	NK-1511	NK-126	NK-125	NK-123