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Purification and Analysis of Folate-Dye Conjugate

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Purification and Analysis of Folate-Dye Conjugate

A Project

Submitted

To

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By

Sravya Mothe

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Dedicated to My Family

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I would like to take this opportunity to thank my committee members Dr. Karen D'Arcy and Prof. Stephen Kent without whose knowledge and assistance this study would not have been successful. I wish to avail myself of this opportunity, express a sense of gratitude to Professors Dr. Addison and Dr. Shailendra Kumar.

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Special thanks to my friends who have offered support throughout my research work and who have always been there whenever I had difficulties.

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My graduate program at Governors State University would not have been achieved without the support and blessings of my family.

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Abstract:

Folate receptor has achieved great importance for use in targeted drug delivery and in vitro and in vivo diagnostic assays[1-2]. Folate receptor (FR) is over expressed on several cancer cell types and activated macrophages, but exists in relatively low abundance on the surface of non-cancerous tissue and non-inflammatory cells. This unique attribute is the hallmark of folate – targeted methodologies allowing the diagnostic agent and/or drug to be ferried to the specific disease sites while preventing "off target" effects in normal tissues[3-4]. Although several folate-based dyes have been reported, simple and detailed synthetic and purification methods for these conjugates have not been reported. Our objective is to develop a new folate-based dye and the corresponding HPLC methods for the purification and analysis of this conjugate. Folate-Dylight™ 800 was successfully synthesized by very simple, inexpensive and safe method. The whole synthesis method can be performed in small laboratories with minimum resources and small quantity of reagents required. The synthesis of Folate-Dylight™ 800 conjugate was successfully purified by HPLC. The purified Folate-Dylight™ 800 conjugate was evaluated by microplate reader, UV-Vis and HPLC.

Introduction:

Folic Acid: (Figure: 1)

Folic acid or vitamin B9 is a water soluble vitamin. Folic acid from food and supplements is biologically inactive in the body. The reduced form i.e. Tetrahydrofolate (THF), which is the active form of Folic acid, plays a key role in the metabolic activity of the body. It is necessary for the normal and healthy growth of cells because it acts as a coenzyme for DNA and RNA synthesis.[5-6] Folate is a vital nutrition for pregnant women as it helps in the reproduction of cells and growth in the foetus. Deficiency of folic acid may result in improper effects on cell division and protein synthesis and therefore causes impairment of growth. In the presence of Folic acid and Cyanacobalmine (Vit B12) conversion of homocysteine to methionine takes place thereby reducing the blood levels of homocysteine and thus decreasing the risk of heart related problems.[7] It maintains a healthy nervous system's by involving in the production of neurotransmitters which regulate mood, sleep and appetite. It also prevents urinary tract infections. Neural tube effects can be avoided if the pregnant women include folic acid into their daily diet[8-9]. Low levels of folic acid may result in cancers of cervix, colon and lungs. Red blood cells are particularly susceptible to folic acid deficiency; hence causing anaemia[10]. Folate also plays a crucial role in the metabolism of many amino acids which include histidine, glycine and methionine[11].

Folate receptor:

Folate receptors are present on the cell surface. They are surface glycoprotein with high affinity for folic acid and folate, which capture the folate from the extracellular liquid and transports to the cell[11-12]. The folate receptors are present on the normal cells, however there is greater receptor presence on malignant cells coupled with the fact that only unique GPI (glycosylphosphatidylinositol anchored folate receptor is the only form capable of uptake of folate conjugated species (reduced folate carrier is incapable of uptake of folate conjugates). This characteristic of malignant cells can be useful for the diagnosis of cancer[13-14]. Folate receptors can be used for many kinds of folate conjugates like targeted drug delivery for cancer treatment and attached conjugate will not release and will not be degraded. Within the endosome, the folate imaging agent conjugate is released from the folate receptor.

Folate Conjugates:

Folate can be coupled with different molecules including chemotherapeutic agents, protein toxins, immunotherapeutic agents, antisense oligonucleotides; liposomes entrapped drug, polymeric drug carrier and imaging agents.[15] Imaging agents can be conjugated to folic acid using variety of linkers and cleavable bonds without affecting the binding affinity of the folate moiety to the folate receptor. (**Figure: 2** shows the schematic representation of folate-dye conjugates) Peptides or carbon chain can be used as spacer depending upon the size of imaging agent.[16-17] Folate based conjugates have exceptionally good penetration power due to its low molecular weight as compared to antibodies. Production cost of antibodies is very high which leads to higher cost for *in vivo* as well as *in vitro* diagnosis.[18]

Folate receptor pathway:

Folate-conjugates bind folate receptor with high affinity[19-20] and are subsequently engulfed in as endosomes and that can reduce disulfide bonds of the linkers. In the absence of any cleavage of the bond, the attached conjugate will not release and will not be degraded. Within the endosome, the folate imaging agent conjugate is released from the folate receptor **(Figure-3).**

Dylight™ 800 Maleimide:

The **DyLight™** 800 belongs to DyLight line of fluorescent dyes which are fabricated by Dyomics in collaboration with Thermo Fisher Scientific. DyLight dyes have a broad range of application including in biotechnology and research applications as biomolecules, cell and tissue labels for fluorescence related studies, cell biology or molecular biology.

Classical fluorophores such as fluorescein and Rhodamine have been used in a wide range of applications. But these dyes have certain limitations for use in microscopy and other applications that require exposure to an intense light source and high temperatures such as a laser, because they photo-bleach very quickly. DyLight family of Fluorophors have similar excitation and emission spectra and are exceptionally photostable, brighter, and less pH - sensitive. The excitation and emission spectra of the DyLight Fluorophores series covers the visible spectrum and also extends into the near infrared region, allowing detection using most of the fluorescence microscopes, as well as special infrared imaging systems.

- Dylight™ 800 products **(Table-1).**

- Dylight^{TM} 800 properties and applications **(Figure-4)**.

Thermo scientific Dylight 800 is near-IR flour that is invisible to the naked eye but increases the staining options when using infrared imaging systems. Dylight 800 has spectral properties[21-24] which are very similar to other near-IR dyes, including Alexa Fluor*790 and IR*Dye 800, and has high solubility for maximal incorporation into the biomolecule of interest.[22-23]

Physical properties of Thermo Scientific DyLight^{TM} 800 dye:[27-29]

Physical characteristics of Thermo Scientific Dylight^{TM} 800 dye, including excitation/emission spectra, emitted color, extinction coefficient, the correction factor used to quantitate labeled protein, the molecular weight of the dye and comparable commercial dyes.[30] Right panel: Excitation (black) and emission (brown) spectra for Thermo Scientific Dylight 800 dye[25-26] **(Figure-4).**

Thermo Scientific Dylight^{TM} 800 derivatives for a broad range of applications:[27-28]

Dylight 800 reactive derivatives for labeling:

- Amine-reactive dye (N-hydroxysuccinimide (NHS) ester-activated).[27-28]
- Sulfhydryl-reactive dye (Maleimide-activated).[27-28]
- Free acid dye – non reactive; used as negative control. [27-28]

Dylight 800-conjugated fluorescent probes:

- Secondary antibody-conjugated- Goat Anti-Mouse or Anti-Rabbit. [27-28]
- Streptavidin-conjugated. [27-28]
- Neutravidin protein-conjugated. [27-28]

Additional fluorescence-based applications: [27-28]

- In-Cell ELISA Near Infrared Detection Kit. [27-28]

Materials and Instrumentation used for Synthesis:

1. DyLight™ 800 Maleimide is the main product 1mg, stored at -200^{0}C Product#46621, Lot#KK12610414
2. DMSO: Sigma Aldrich, 99% purity; Folate Cysteine: Provided by Dr. Henne ; Fluorescein sodium salt(C20H10Na2O5) : Lot# 079K0141V; Multichannel pipetter : RAININ , 20-300µl;
3. UV Plates: BD Falcon microtest 96 well 370µl clear plate, UV Vis transparent film bottom,
4. Non-sterile, no lid, lot No:E1002007; Regular Plates: Generic Bio-One, microplate, 96well flat bottom, Lot No: E091006L; Spin Filters : Sigma Aldrich, Amicon ultra 0.5 centrifugal filter,
5. 3 kDa, Batch No: 3110; PD-10 Columns: Sephadex G-25M columns, contains 0.15% Kathon CG in DI H20, lot no. 393861; Centrifuge: Beckman CS-15R Centrifuge at 8898 RPM for 12 mins.
6. UV Plate Reader and BCA assay Software: Gen 5.1.10 Biotek, Epoch.
7. DIPEA (N,N-Diisopropylethylamine)
8. Synthesized Folate – Peptide (Folate-Asp-Arg-Asp-Asp-Cys-SH supplied by W. Henne), DyLight™ 800 maleimide was supplied by Thermo Scientific, 99% DIPEA (N,N-Dissopropylethylamine) supplied by Aldrich chemicals, DMSO (Dimethylsulfoxide) supplied by fischer swcientific.
9. HPLC instrumentation and UV-visible spectrophotometer.

Method and Analysis:

Synthesis of Folate-DyLight^{TM} 800:

1 mg DyLight^{TM} 800 maleimide was dissolved in 200 ul dimethylsufoxide(DMSO). 2 mg of folate peptide cysteine and 10 ul of DIPEA (N,N-Diisopropylethylamine) were added and mixed overnight using a magnetic stirrer (at room temperature). The product was purified by reverse phase HPLC and analyzed by LC/MS, UV-Vis spectrophotometer. The synthesis of Folate-Dylight 800 maleimide is shown in **(figure -5).**

Preparation of Buffers:

Take 0.45 g of ammonium bicarbonate and add 500 ml of distilled water and then stir to dissolve (10 mM bicarbonate buffer, pH ~ 6.8).

Experimental Parameters for HPLC:

The analysis of synthesized product was done by HPLC (Hewlett Packard, series1050) with YMC, HPLC column with particle size 6µm and dimension 4.6 x 150 mm. Purification was done by Rigel HPLC with particle size 5 µm and column dimension 10 x 250 mm.

Solvents used: ammonium bicarbonate/acetonitrile

Flow rate is 1 ml/min

Detector: DAD with scanning range 200-600

Method used is BIOTINRH.M

Gradient method for Folate - Dylight conjugates purification (HPLC)

Time (min)	% B
0.00	1
1.00	1
20.0	99
40.0	99
41.0	99
50.0	99
52.0	1
60.0	1

Experimental parameters for UV-Visible Spectrophotometer:

Instrument: Epoch Microplate Spectrophotometer

U.V Plate Reader Analysis at $\lambda = 777$nm of stock containing 1:10 dilution of purified dylight 800 in PBS was conducted.

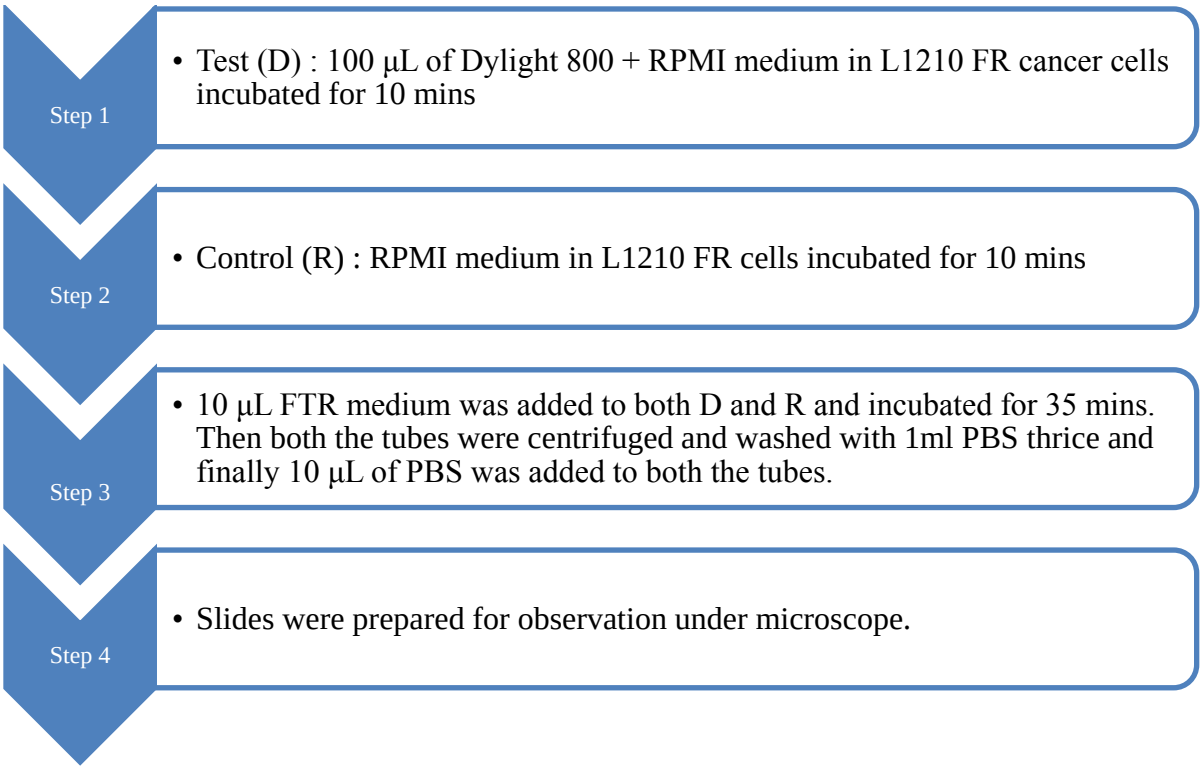
Experimental Parameters for LC/MS:

Initially sample was centrifuged for 7 mins at 12.2 p/min and supernatant sample was extracted. Then 100 μL of folate dylight 800 was injected into LC/MS.

Column Parameters

Mode	Positive ion mode
Flow rate	5ml/min
Solvent A	Water
Solvent B	Methanol
Column	XDB-C18
Scan range	500 – 2200 m/z
Nebulizer Pressure	40 psi
Threshold	1000
Drying gas Temperature	300C

Competition Studies of DyLight ™ 800 using FTR:



Results and Discussion:

The synthesis of Folate –DyLight™ 800 was a very simple and quick method. In this method, the folate-peptide and DyLight™ 800 was conjugated via maleimide chemistry (thioether linkage). The synthesis method was inexpensive and can be performed in small laboratories. The main advantage of this synthetic approach is that folate-peptide is very stable and can be stored for a long time and can be scaled to 0.5 gram batches.

The purification of Folate-DyLight^{TM} 800 conjugate was accomplished using reversed phase HPLC. Total run time was 1 hour (60 mins). For the analytical run, 10 µl injection of the product was injected in HPLC and analyzed. Peaks were observed at 11.4 min, 15.9 min, 16.4 min, 17, 5 min, 18.6 min. From these peaks, the sample from peaks at 17.5 min and 18.6 min were collected and stored it in the vials and these purified samples we injected 10 µl each in the HPLC for analysis. Also UV-Vis of the collected fractions were assessed using a spectral scan in a UV/ViS micro-plate reader. In the HPLC chromatogram, at 17.7 min we observed the unreacted folate cysteine peak, the compound Folate-DyLight 800 peak at 21.1 min and the unreacted dye peak at 32.3 min **(Figure-6)**. At 18.6 min we have clearly observed few peaks with small intensities. It is largely anticipated that the compound at 21.2 min is folate dylight 800 **(Figure-7).**

By using the microplate reader we observed the characteristic DyLight 800 peak at around 790 nm. We also observed the characteristic folate doublet at 280 and 363 nm. The spectrum is displayed **in Figure-8.**

From LC/MS chromatogram a distinct peak at 3.252 min was observed corresponding to that of Folate -DyLight 800 **(Figure-10).** Offline session was conducted and the DAD spectrum was produced which showed the DyLight – 800 peak at 800 nm and Folate peak at 330 nm **(Figure-9).**

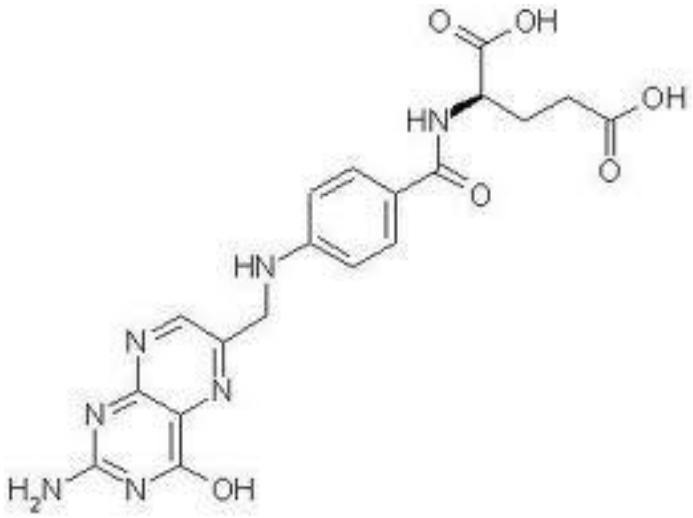
Competition studies with Rhodamine showed that higher concentration of DyLight 800 competes and binds with L1210 FR cancer cells **(Figure-12).** More Dylight 800 prevents FTR from binding with folate receptors on the cancer cells. The other slide (control) showed dark red spots of FTR binding to cancer cells **(Figure-11).**

Conclusion:

Folate-Dylight^{TM} 800 was successfully synthesized by very simple, inexpensive and safe method. The whole synthesis method can be performed in small laboratories with minimum resources and small quantity of reagents required. The synthesis of Folate-Dylight^{TM} 800 conjugate was successfully purified by HPLC. The purified Folate-Dylight^{TM} 800 conjugate was evaluated by LC/MS, UV-Vis and HPLC.

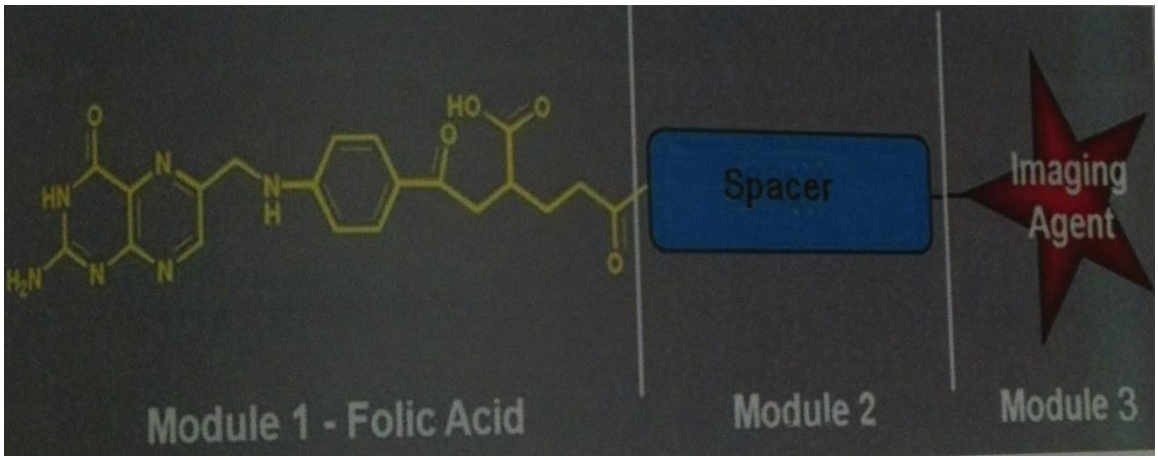
Competition studies of DyLight^{TM} 800 showed that high concentrations of our dye competes with FTR and binds to L1210 FR cancer cells.

Figures:



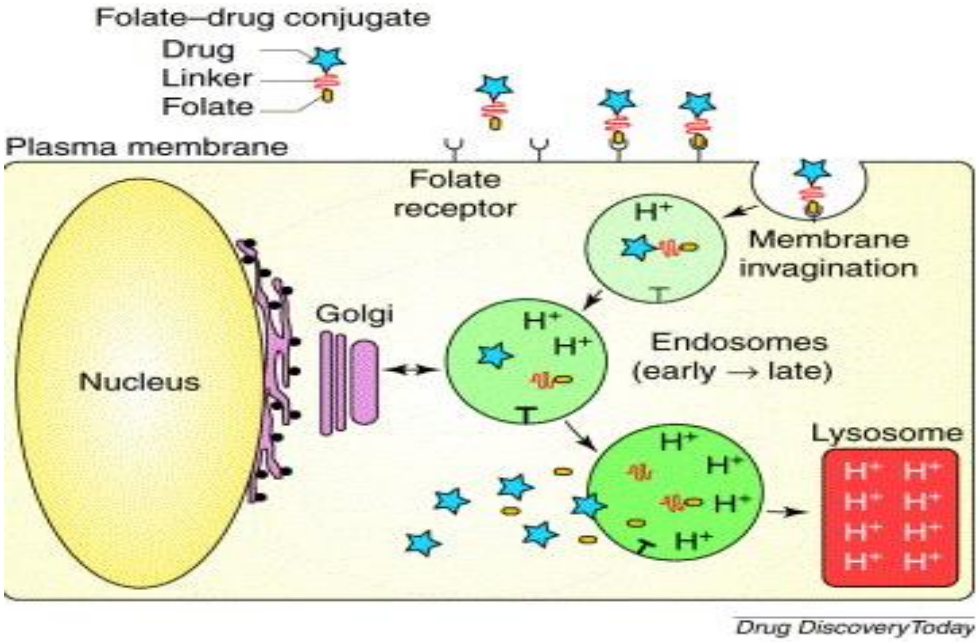
Source: Folic Acid - Bristol University homepage - a place for ... (n.d.). Retrieved from http://www.chm.bris.ac.uk/webprojects2002/schnepp/folic.html

(Figure: 1) Structure of folic acid



(Figure:2) General Scheme for Folate-Dye conjugates

Source: ScienceDirect.com - Advanced Drug Delivery Reviews - Optical ... (n.d.). Retrieved from http://www.sciencedirect.com/science/article/pii/S0169409X05000566



Source: Christopher P. Leamon. *Drug Receptor pathway. Sciencedirect.com. 17 Nov 2012.* **<http://www.sciencedirect.com/science/article/pii/S135964460001944>**

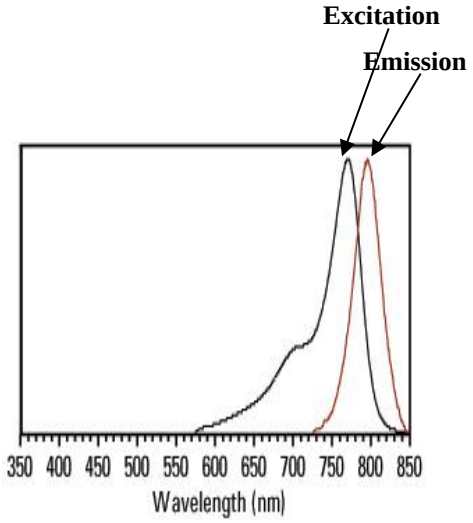
(Figure:3) Transportation of Folate-conjugates via Folate receptors.

Product	Color	mass (g/mol)	Absorb (nm)	Emit (nm)	ε ($M^{-1}cm^{-1}$)
DyLight 350	violet	874	353	432	15,000
DyLight 405	violet	793	400	420	30,000
DyLight 488	green	1011	493	518	70,000
DyLight 550	yellow	982	562	576	150,000
DyLight 594	orange	1078	593	618	80,000
DyLight 633	red	1066	638	658	170,000
DyLight 650	red	1008	654	673	250,000
DyLight 680	far-red	950	692	712	140,000
DyLight 755	near-IR	1092	754	776	220,000
DyLight 800	near-IR	1050	777	794	270,000

Source: DyLight Fluor Labeling Reagents and Kits - Protein ... (n.d.). Retrieved from http://www.piercenet.com/browse.cfm?fldID=294EFD98-4461-4352-A046-50B522F52952

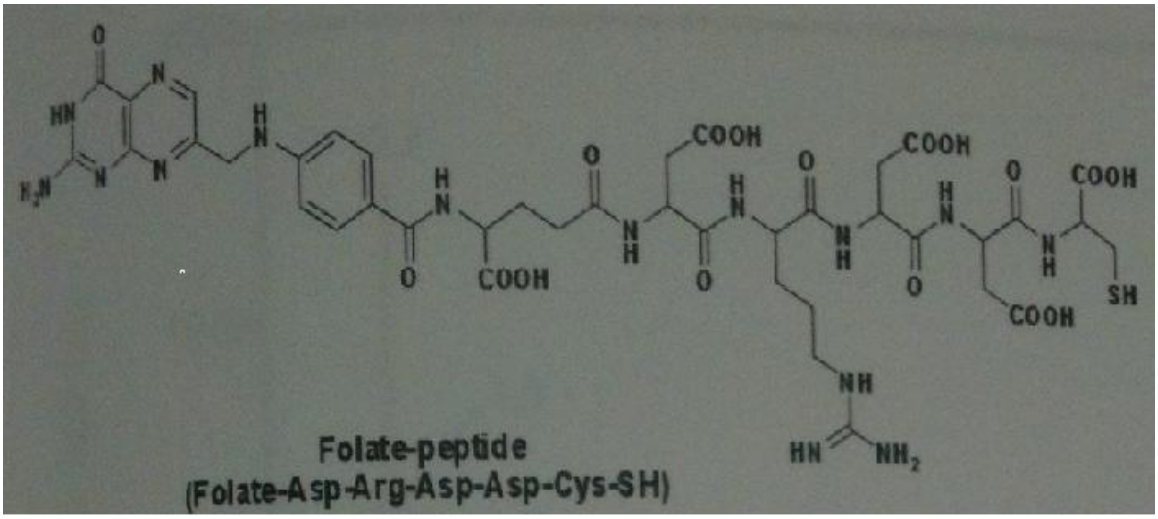
Table-1 Dylight Products

Thermo Scientific Dylight 800 Properties	
Excitation(nm)	777
Emission(nm)	794
Color	Near-IR
€†	270,000
CF‡	0.045
Mol.Weight(g/mol)	899.15
Spectrally similar Dyes	Alexa Fluor*790, IR Dye*800
†Molar extinction coefficient (M^{-1} cm^{-1})	
‡ Correction factor(A_{260}/A_{777})	



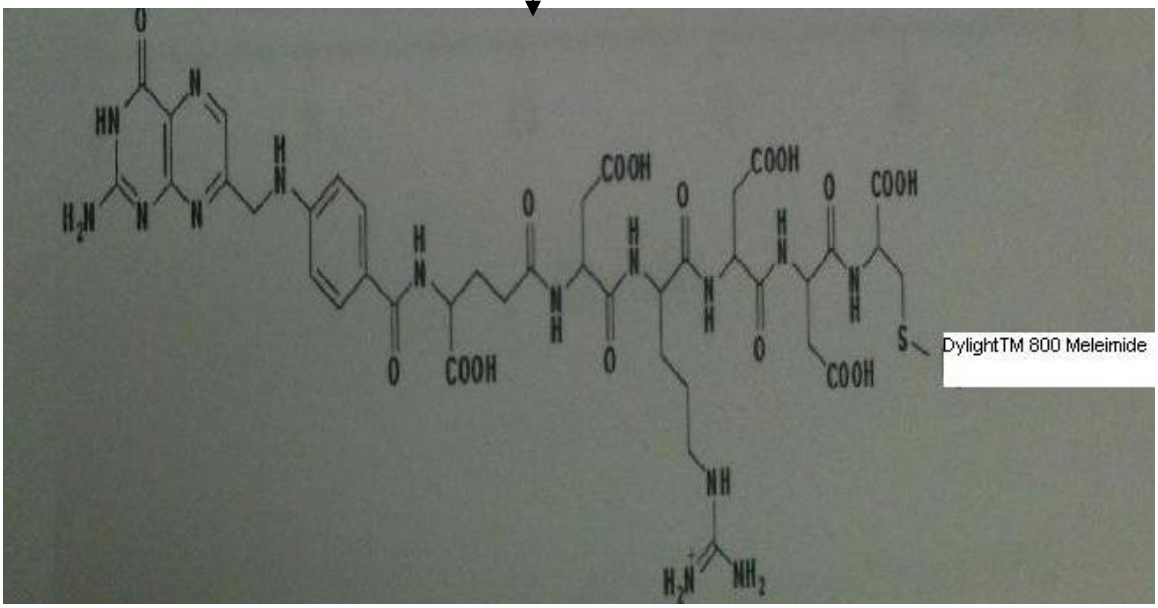
(Figure-4) Thermo Scientific Dylight™ 800 Properties

Source: DyLight Fluor Labeling Reagents and Kits - Protein ... (n.d.). Retrieved from http://www.piercenet.com/browse.cfm?fldID=294EFD98-4461-4352-A046-50B522F52952



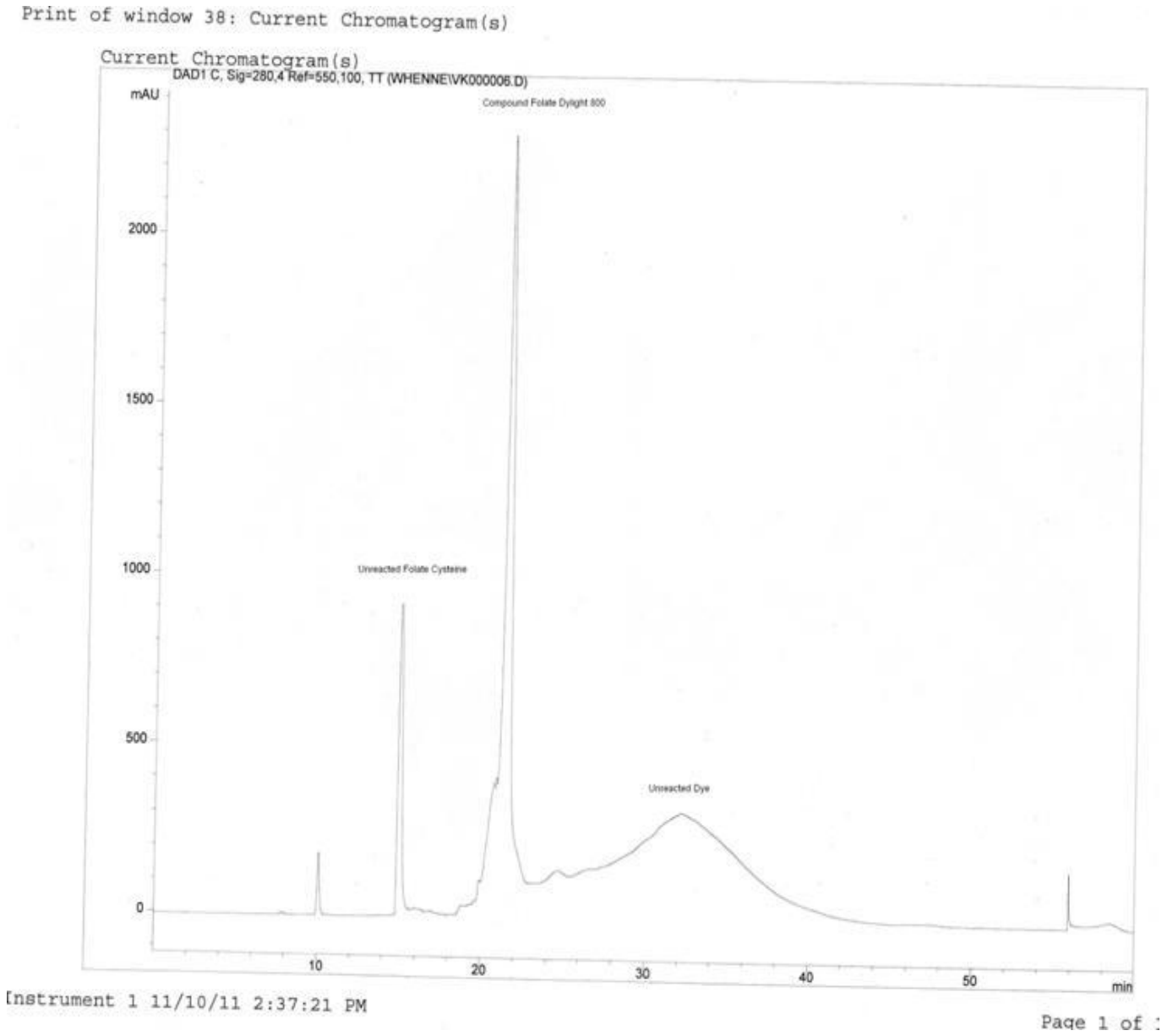
+

Dylight™ 800 Maleimide

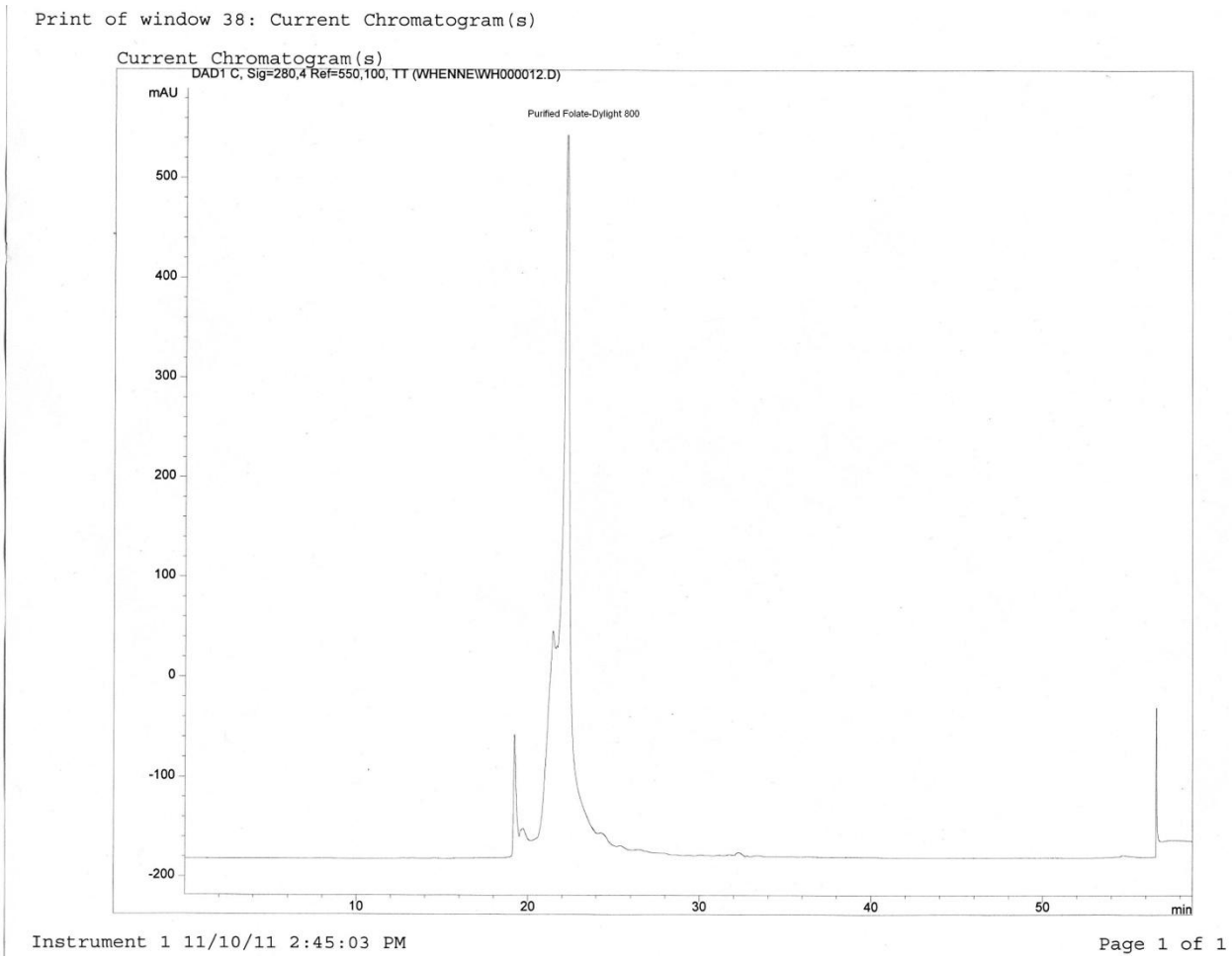


Folate Dylight™ 800 Maleimide conjugate

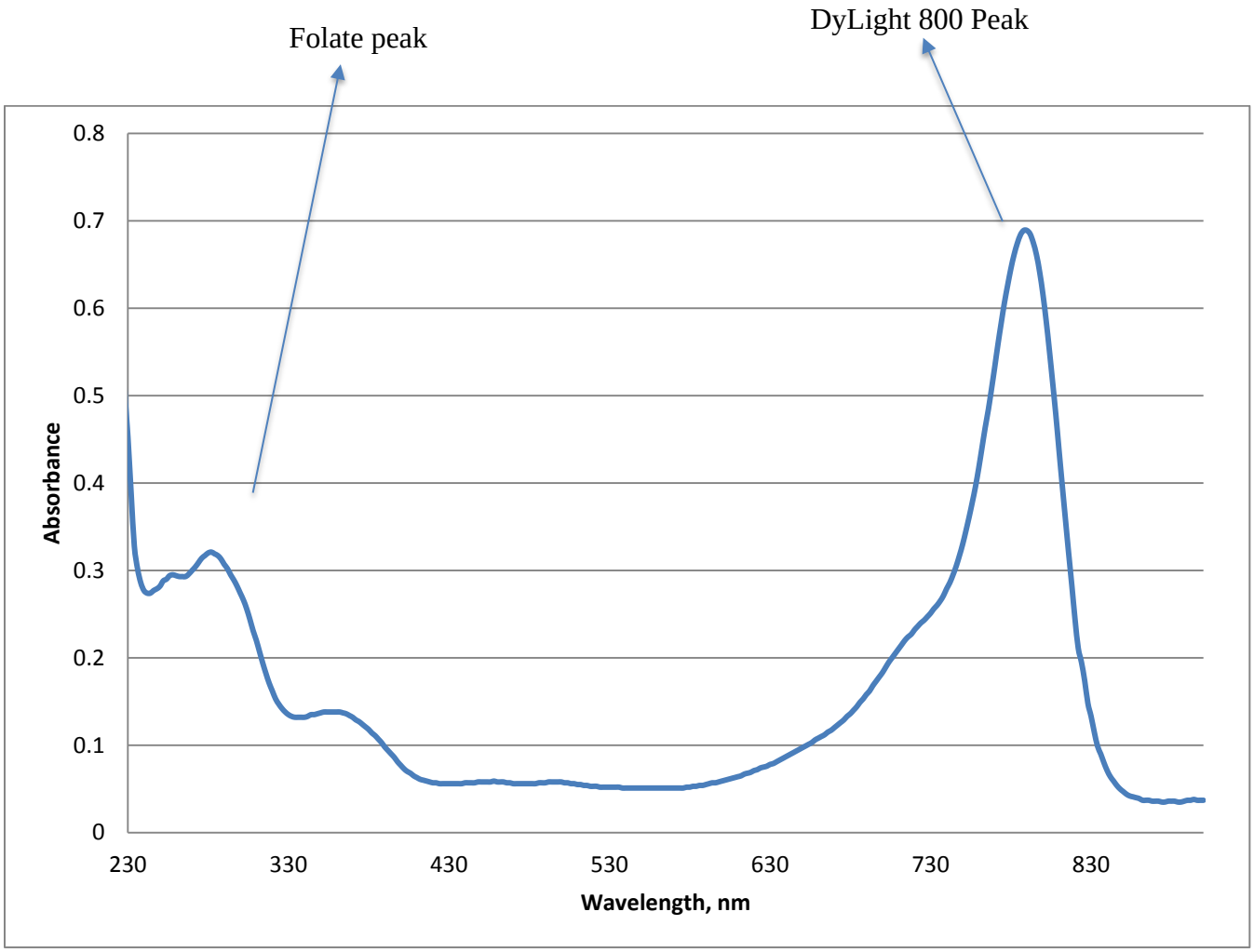
(Figure:5) Schematic Representation of Folate Dylight™ 800 Maleimide conjugate synthesis



(Figure:6) Chromatogram of crude Folate-Dylight^{TM} 800

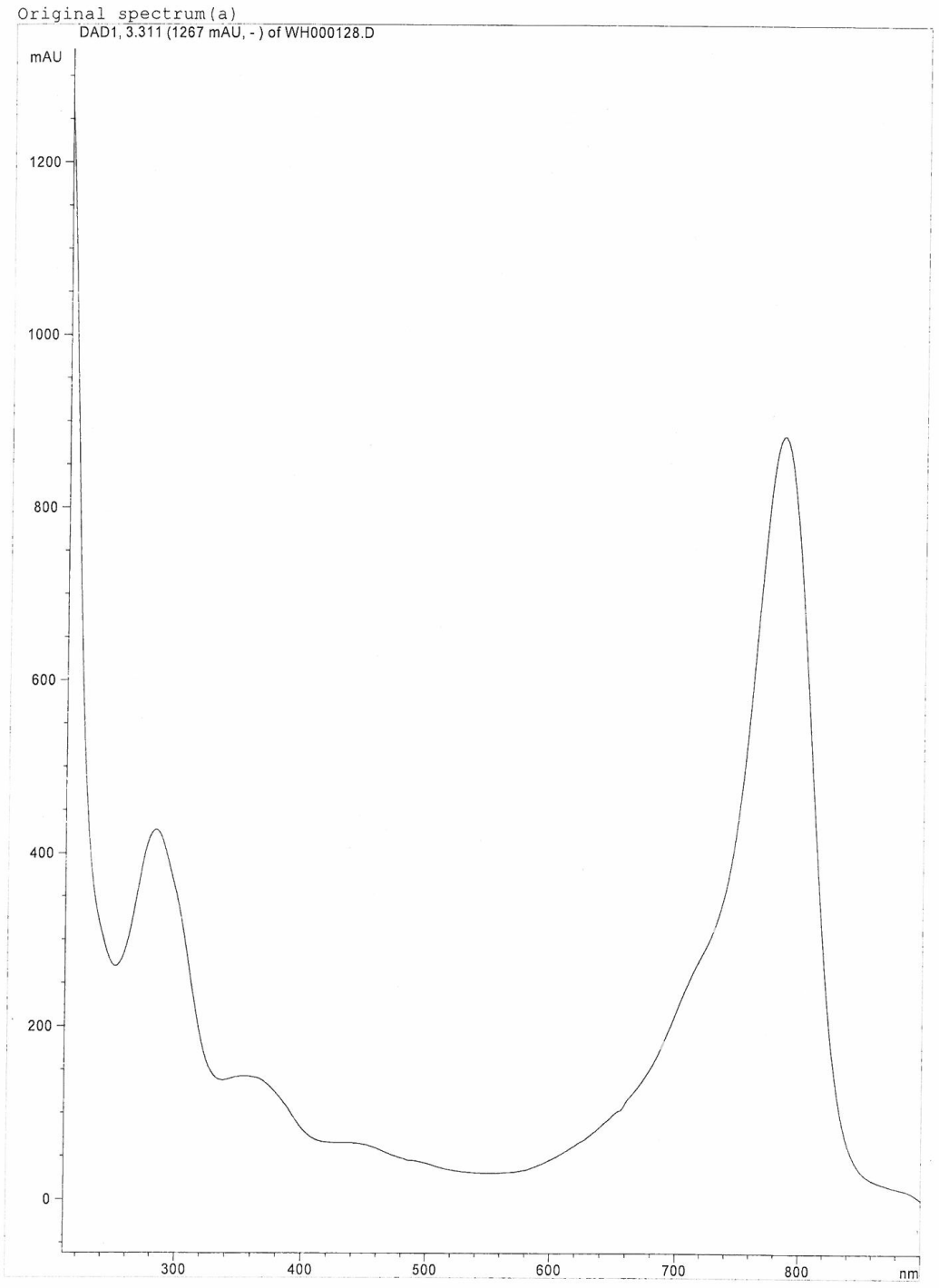


(Figure: 7) Chromatogram of purified Folate-Dylight^{TM} 800 conjugates at 21.1 min.



(Figure: 8) UV-Vis of Folate-Dylight™ 800 conjugates

Print of window 42: Original spectrum(a)



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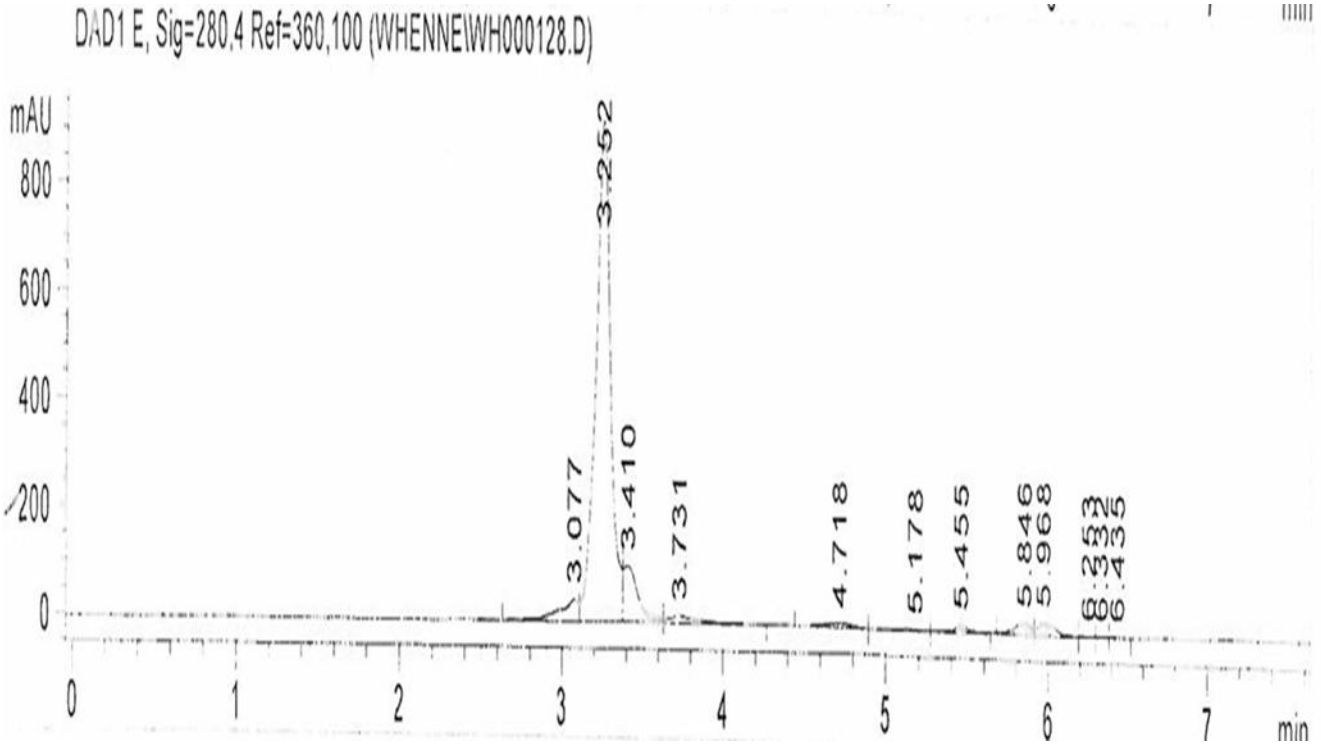
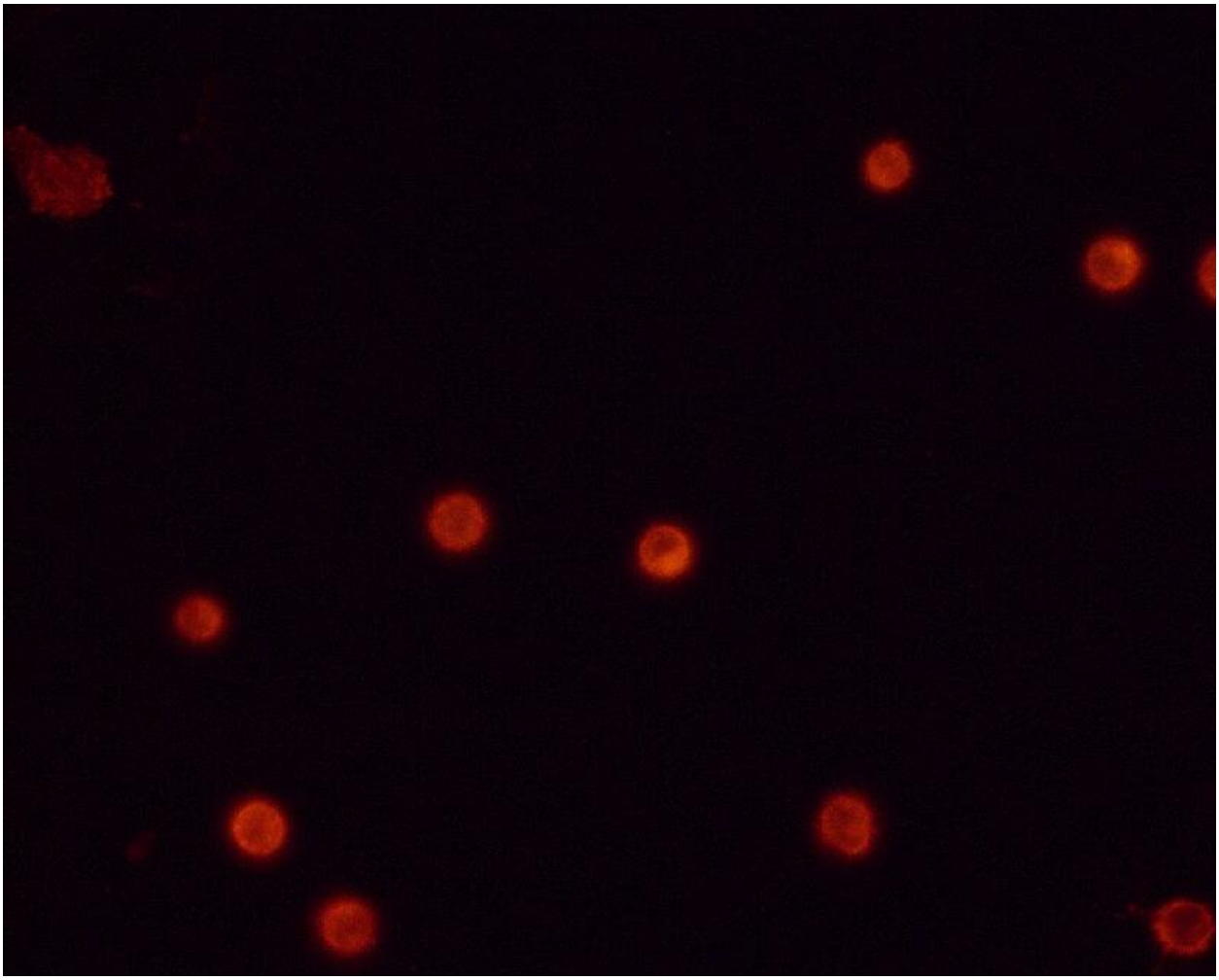
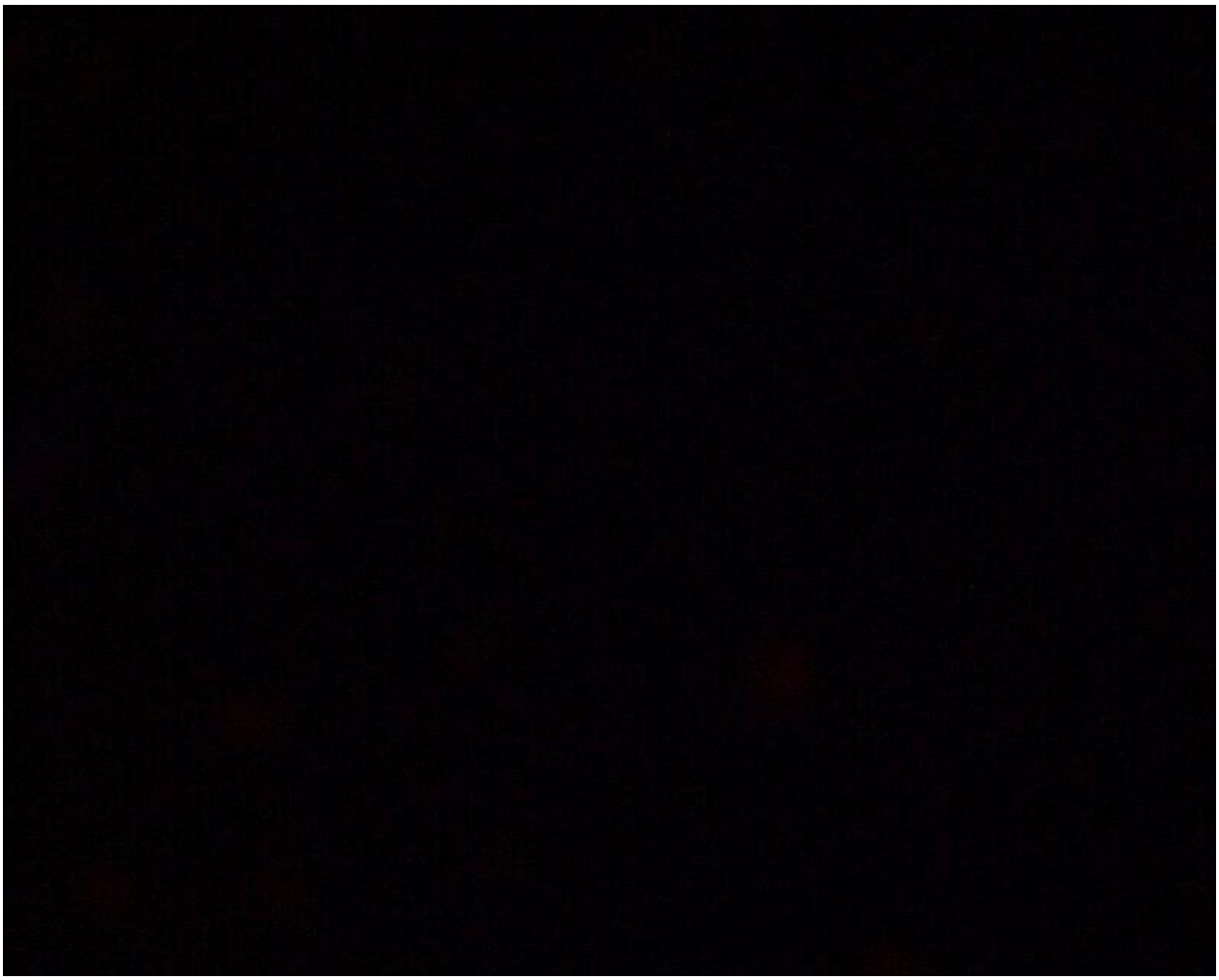
(Figure: 9) DAD spectrum of Folate Dylight™ 800



(Figure: 10) LC/MS chromatogram of Folate DyLight 800 Conjugate



(Figure: 11) Slide R containing L1210 FR cancer cells in FTR



(Figure: 12) Slide D showing a light red spot due to binding of DyLight™ 800 with L1210 FR Cancer cells.

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