



SIMPLE AND EFFICIENT CARTRIDGE PURIFICATION OF 6-FAM™ DYE PHOSPHORAMIDITE

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ABSTRACT

We have developed and evaluated a method for the purification of 6-FAM modified oligonucleotide, highly pure oligonucleotide. The full length target oligonucleotide are purified using a method that select for intact 3' and 5' terminal. The new purification method is outlined that further simplify the routine purification of Modified DNA Oligomers. This procedure result in purified DNA linked with 6-FAM™ without the use of PAGE gels or HPLC. The Cartridge purification protocol is applicable for many of the convenient disposable products available for rapid oligonucleotide purification, clean-up by selective adsorption, and elution on solid-phase media. Many of these products are prepackaged, single-use cartridges or columns filled with affinity or size-exclusion media. 6-carboxyfluorescein (6-FAM™) is a single isomer derivative of fluorescein. 6-FAM™ is the most commonly used fluorescent dye for attachment to oligonucleotides and is compatible with most fluorescence detection equipment. Below pH 7, 6-FAM™ becomes protonated, which results in decreased fluorescence. It is typically used in the pH range 7.5-8.5. The quantum yield of fluorescein is measured to 95 % at 0.1 M NaOH at 22°C with excitation at 496 nm. In this method we have examined the oligos of same length, 25-mer labeled with 6-FAM™ are purified- (1) With HPLC, obtained a purity of 94.1% with the peak elution at 37' min 28 'sec and area 93.9 and (2) with the cartridge purification a purity of 100.0% with a peak elution at 50' min 42' sec and area 100. Therefore the conclusion comes the FAM labeled oligo are purified more precisely by the cartridge purification with comparatively short time. The purity check is done by the capillary gel electrophoresis (CE) - Cepro 9600 at Eurofins Genomics India Pvt Ltd.

KEYWORDS: 6-FAM™, CGE-(CE)-Cepro 9600, Cartridge purification, HPLC Purity 94.1%, Cartridge Purity 100% & Labeled Oligos.

INTRODUCTION

6-FAM™ Dye Phosphoramidite is used for the 5' attachment of FAM™ dye during oligonucleotide synthesis. 6-FAM™ Dye Phosphoramidite is an optically pure isomer (not a mixture). The label is treated as if it were simply another base: It is inserted at the appropriate place in the sequence and incorporated automatically. 6-FAM™ is equally useful in applications historically employing isotopic labels. As such, it eliminates the hazard associated with handling, storage, and disposal of radioactive materials. Fluorescein is a synthetic organic compound available as a dark orange/red powder soluble in water and alcohol. It is widely used as a fluorescent tracer for many applications. Fluorescein is a fluorophore commonly used in microscopy, in a type of dye laser as the gain, in forensics and serology to detect latent blood stains, and in dye tracing. Fluorescein has an absorption maximum at 494 nm and emission maximum of 521 nm (in water) (Amanda Collin *et al.*, 1994 & 1997). The major derivatives are fluorescein isothiocyanate (FITC) and, in oligonucleotide synthesis, 6-FAM phosphoramidite. Fluorescein also has an isobestic point (equal absorption for all pH values) at 460 nm. Fluorescein is also known as a color additive

(D&C Yellow no.7). The disodium salt form of fluorescein is known as uranine or D&C Yellow no. 8. The color of its aqueous solution varies from green to orange as a function of the way it is observed by reflection or by transmission, as it can be noticed in bubble levels in which fluorescein is added as a colorant to the alcohol filling the tube to increase the visibility of the air bubble and the precision of the instrument (Atrher *et al.*, 2000 & 2003). More concentrated solutions of fluorescein can even appear red (Gore *et al.*, 2000).

Chemical and physical properties

The fluorescence of this molecule is very high, and excitation occurs at 494 nm and emission at 521 nm. Fluorescein has a pK_a of 6.4, and its ionization balance leads to pH-dependent absorption and emission over the range of 5 to 9 (Banks PR *et al.*, 1995). Also, the fluorescence lifetimes of the protonated and deprotonated forms of fluorescein are approximately 3 and 4 ns, which allows for pH determination from non intensity based measurements. The lifetimes can be recovered using time-correlated single photon counting or phase-modulation fluorimetry (Lakowicz *et al.*, 1991-2003): Fig-1.

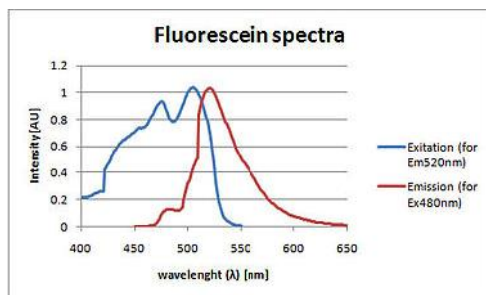
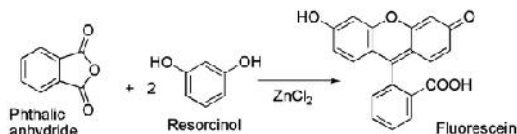


FIGURE: 1 Absorption and emission spectra of fluorescein

Synthesis and Preparation of 5- and 6-Carboxyfluorescein

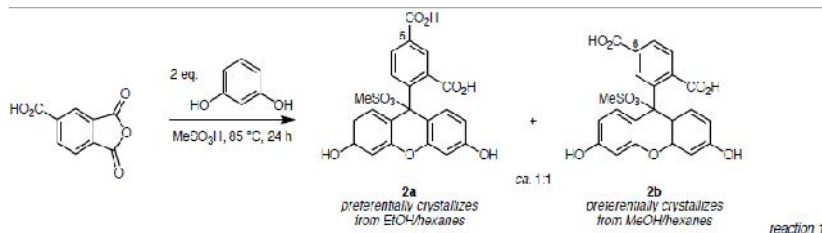
Fluorescein was first synthesized by Adolf von Baeyer in 1871. It can be prepared from phthalic



anhydride and resorcinol in the presence of zinc chloride via the Friedel-Crafts reaction.

Condensation of resorcinol with 4-carboxyphthalic anhydride in methane sulfonic acid gave a mixture of 5- and 6- carboxyfluorescein stereoisomers. These were

separated by recrystallization from methanol- or ethanol-hexane to give 5- and 6- carboxyfluorescein, each in over 98 %purity.



Despite the widespread applications of 5- and 6-carboxyfluorescein as molecular labels, 2-4 it is surprisingly difficult to obtain these compounds as pure regioisomers. They can be purified from one another via preparative HPLC, and the price of commercial samples of these materials implies that this route is used in practice. Large scale procedures for the preparation of these fluorescein derivatives would definitely be preferred. (Yuichiro Ueno *et al.*, 2004)

A second method to prepare fluorescein uses methanesulfonic acid as a Brønsted acid catalyst. This route has a high yield under milder conditions

Application: Biochemical research

In cellular biology, the isothiocyanate derivative of fluorescein is often used to label and track cells in fluorescence microscopy applications (for example, flow cytometry). Additional biologically active molecules (such as antibodies) may also be attached to fluorescein, allowing biologists to target the fluorophore to specific proteins or structures within cells. This application is common in yeast display. Fluorescein can also be conjugated to nucleoside triphosphates and incorporated into a probe enzymatically for in situ hybridisation. The use of fluorescein amidite shown above allows one to synthesize labeled oligonucleotides for the same purpose. Yet another technique termed molecular beacons makes use of synthetic fluorescein-labeled

oligonucleotides. Fluorescein-labelled probes can be imaged using FISH, or targeted by antibodies using immunohistochemistry. The latter is a common alternative to digoxigenin, and the two are used together for labelling two genes in one sample (J.R. Lakowicz *et al.*, 1999).

Applications: Biological research Carboxyfluorescein

Commonly called FAM and its aminoreactive succinimidyl esters are favored over FITC in bioconjugations. FAM reagents give carboxamides that are more resistant to hydrolysis. In addition, FAM reagents require less stringent conjugation conditions and give better conjugation yields, and the resulted conjugates have superior stability (Nazarenko, I *et al.* 2002). FITC-labeled nucleotides and peptides tend to deteriorate more quickly than the corresponding FAM conjugates. We found that FAM reagents can be used to substitute FITC reagents in most biological applications. 6-FAM is the other purified isomer of 5(6)-carboxyfluorescein. Complementary to 5-FAM isomer, 6-FAM is mainly used for labeling nucleotides and nucleic acids

Properties

6-carboxyfluorescein is a single isomer derivative of fluorescein. 6-FAM™ is the most commonly used fluorescent dye for attachment to oligonucleotides and is compatible with most fluorescence detection equipment (Valeur *et al.*, 2002). Below pH 7, 6-FAM™ becomes protonated, which results in decreased fluorescence. It is typically used in the pH range 7.5-8.5. The quantum yield

of fluorescein is measured to 95 % at 0.1 M NaOH at 22°C with excitation at 496 nm (Tyagi *et al.*, 1998).

Stability-Photobleaching

Under high-intensity illumination conditions, the irreversible destruction or photobleaching of the excited fluorophore becomes the factor limiting fluorescence detectability. The multiple photochemical reaction pathways responsible for photobleaching (Darzynkiewicz Z *et al* 2001)

Materials and Methods: Cartridge purification – Overview

Polymeric (polystyrene) resin is used to purify primers by reverse phase chromatography. This method is also referred to as OPC (oligonucleotide purification cartridge). Primers that have not had the trityl group removed ("trityl on") during the last synthesis cycle (full length) are purified from the untritylated, "n-x" primers with this option (PE Biosystems. 1997 1996). After purification, the trityl group is removed. Primers >60 bases tend to have some shorter sequences co purified by this method. This purity option is functionally similar and more to HPLC purified oligo (Song *et al.*, 1996).

Cartridge Purification

This is also known as reverse phase purification or OPC. It requires that the trityl group be left on the oligo nucleotide at the addition of the last base. The trityl group is the hydrophobic protecting group on the 5' end of the phosphoramidite that is normally removed by TCA at each cycle to expose 5'OH on the sugar (Seidel *et al.*, 1996). The "tritylated" primer is loaded onto a Polymeric (polystyrene) resin in a dilute ammonia solution and then washed with the same dilute ammonia. At this point, the

capped, non-extended products from each synthesis cycle (which do not have a trityl group) are washed off the polystyrene resin in the column which behaves as a reverse phase purification column. The majority (>95%) of the n-1, n-2, n-3, etc. species will be removed for oligos less than 35 bases. From 40 to 60, partial removal is seen. For >60, most of the impurities remain. After purification, the trityl group is removed. The elution buffer used is acetonitrile - water. The acetonitrile is more volatile than the water. The dried sample should have no acetonitrile remaining in the sample. The cartridges will always remove the shorter products, but on longer oligos, there will be a higher proportion of N-1 and N-2 type failure sequences present (Thomas Horn *et al.*, 1988). Cartridge and HPLC purified oligos will almost be equal in purity for the shorter sequences (15mer). A cartridge purified oligo is typically in the area of 90% full-length oligo. Of course, this will vary with sequence and length of the primer (Rampal *et al.*, 2001).

Significance

However, a less expensive option would be cartridge purification, which also purifies for full-length (~95% full-length) and also gives better yields. In this case, the minimum specs are 2 OD for 50 nmole scale, 10 OD for the 200 nmole scale and 25 OD for the 1 µmole scale which are equivalent to 66 µg, 330 µg, and 825 µg respectively. So for cartridge purification, the 200 nmole scale will guarantee enough amounts. Both HPLC and Cartridge purified oligos are appropriate for Northern blotting applications. Desalted is sometimes used, and will work but may lead to higher backgrounds Table 1 & 2

TABLE: 1

Purification	Description	Benefit
Cartridge	Based on reverse phase chromatography, removes failure sequences from the completed Synthesis.	Provides full-length sequences needed in Some applications.

TABLE: 2 Scale of synthesis

Scale	Bases
10 nmol	N/A
50 nmol	7-60
200 nmol	7-60
1 µmol	7-60
10 µmol	7-60

Procedure for Cartridge purification of 6-FAM oligos:

1. Materials Needed

- DMT-on oligonucleotide with 6'-fluorescein labeled. Ammonia may be present.
- Glen-Pak™ or Fluoro-Pak™ Cartridges.
- Luer adaptor or other vacuum based adapter (available with columns).
- 3 mL PE/PP syringe.
- Vials for catching wastes.
- Acetonitrile (MeCN)-HPLC grade.
- 2 M aqueous TEAA (triethylammonium acetate).
- NaCl- Concentration -100mg/ml.
- 2% aqueous TFA (trifluoroacetic acid).
- Milli Q Water.
- 50%-MeCN with 0.5%NH₄OH.

2. Sample preparation:

Without removing the ammonia used in the deblocking step, dilute the crude deprotected oligonucleotide is diluted with a 5X (Volume of oligo) with 100mg/ml of NaCl. The final sample volume should be near about -6 mL

3. Conditioning the column:

Pass the following through the column to waste at a flow rate of 2-4 seconds per drop.

Add 500 µL of MeCN to the column and on the vacuum. Step repeated twice.

1.0 mL of 2.0 M aqueous TEAA-On the vacuum.

4. Loading the DMT-on 6-FAM -labeled oligonucleotide

Using the sample as prepared above, pass it through the pre-conditioned column at a flow rate of 5 seconds per drop, conditions that allow one-pass loading. Most of the failure sequences pass through the column during loading

5. Eluting remaining failure sequences

Pass the following through the column to waste at a flow rate of 2 seconds per drop:

1. Wash it with 1 ml of 100mg/ml NaCl

2. 0.5 mL of water

The NaCl and water solution selectively washes the remaining failure sequences from the resin while the fluorescein-labeled oligonucleotide is retained. The water

step washes the NaCl out of the resin prior to the acid-catalyzed detritylation in the next step.

6. Elution of the final detritylated oligonucleotide

Pass the following through the column at **2 seconds per drop**, collecting the eluate in an

Appropriate sample tube:

1 mL of 2% TFA on the column, on the vacuum immediately do not wait.

Wash it with 1ml Milli-Q water twice.

Elute it with 50% acetonitrile with 0.5% NH₄OH.

The oligo should elute in the first 15-20 drops.

Observation

Determine the optical density units at 494 nm to quantify the final oligonucleotide. Use as-is or lyophilize for short-term storage. If the oligonucleotide needs to be stored, add 100 mL of 10x TE buffer (100 mM Tris, 10 mM EDTA, pH 8) and store at 4 °C for a ready-use solution, or freeze at -20 °C for longer periods

RESULT & DISCUSSION

The overall study signified that the FAM-Labeled Oligos with the Cartridge purified are more pure as the purity check gives the 100% Purity with a single sharp peak with a Migration time of 50:42 min:sec, Height 102.9, Area 144.2, Area Percentage 100% and Corrected Area percentage 100% while in the case of the HPLC purified the purity level is 94.1% with a multiple Peak and Migration Time 37:28 min:sec, Height 42.7, Area 26, Area percentage 93.9 and Corrected Area percentage 94.1%. Chromatogram (1 & 2). However for the Quality check of the FAM labeled Oligos the PAGE QC is also been done, the results are parallel as the Oligos purified with the Cartridge (Well-1, 2&3) are showing no extra or free dyes but in the case of the Oligos purified with the HPLC (Well-4,5,6&7) are showing the free dyes at the lower ends. Fig-2.

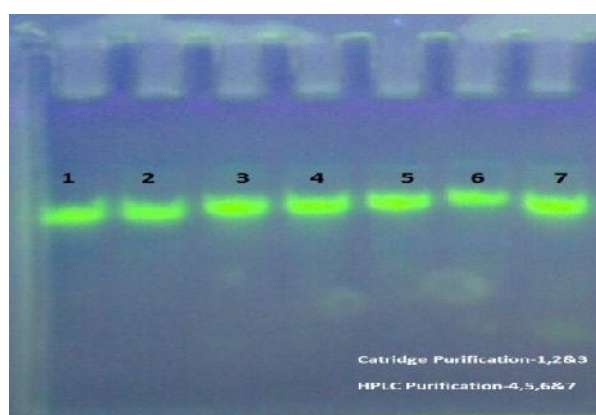


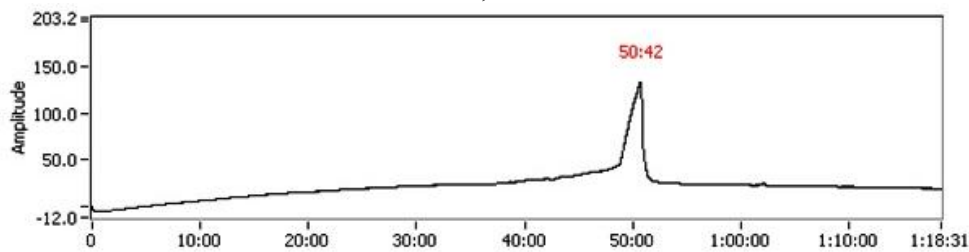
Fig-2

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File Created Time	2:54 PM
Frame Rate (f/s)	3
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Pre-run Period (min)	0
Injection Voltage (kV)	12.00
Injection Time (s)	110
Separation Voltage (kV)	13.00
Total Run Time (min)	79
Sensitivity of PDA (High)	5
Sensitivity of PDA (Low)	0
Filter Type	Moving Average
# of Point for Averaging	9
Background Correction	No
VACE (psi)	0.00

CGE Parameters for FAM-QC

Capillary 5. Total of 1 peak found (Cartridge Purified)

Date Created: 03/08/2013, Time Created: 2:54 PM

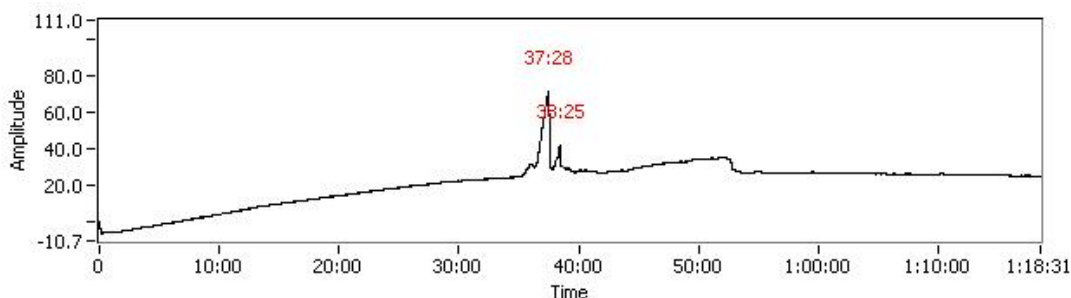


	Migration Time (h:m:s)	Height	Area	Area %	Corrected Area %
Peak 1	50:42	102.9	144.2	100.0	100.0

Chromatogram-1

Capillary 10. Total of 2 peaks found (HPLC Purified)

Date Created: 03/08/2013, Time Created: 2:58 PM



	Migration Time (h:m:s)	Height	Area	Area %	Corrected Area %
Peak 1	37:28	42.7	26.0	93.9	94.1
Peak 2	38:25	10.4	1.7	6.1	5.9

Chromatogram-2

Method for FAM labeling

Once the primer, 25mer is synthesized it is ready for the linking to the 6-FAM dye. FAM 250ug was dissolved in 1ml of Acetonitrile in an Argon gas chamber, now the dissolved Fam should be protected from light (light sensitive) and connected to the SM-3 Machine and the linking program is started. Almost it takes 15 minutes for the complete linking of the FAM to the 25 mer oligonucleotide. The efficiency of labeling is checked by the chromatogram visualized in the machine.

CONCLUSIONS

Cartridges purification for processing of labeled DNA was demonstrated. It has the potential of reducing DNA purification to an easy-step protocol on an existing laboratory. In order to complete the whole sample preparation process in the same cartridge. Further it is the time-controlled process approximately 12.0 minutes. This creates added value for the supplier of standard lab equipment and reduces market entry barriers for organizations developing micro fluidic solutions. Users of the Cartridges purification only require minimal pipetting effort while profiting from pre-stored reagents and standardized assay performances.

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