Flurochroms Comparison

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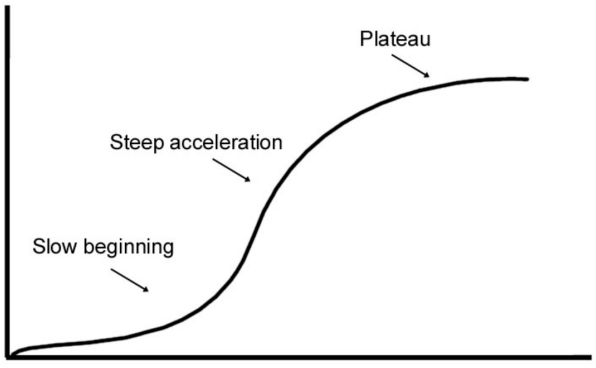
Introduction

There is wide range of fluorochromes on the market. This assay was performed to find the best fluorochrome: with the widest range of detection and specificity. It's highly possible that presence of proteins, salts, RNA, ssDNA and different molecules prompts differences in measurement, even if producent write in mannual that fluorochrome is highly specific only to one molecules (for instance dsDNA). Some of additional components can unspecific bind to fluorochrome and make fluorescence lower or higher.

- For each fluorochrome I will make measurement on spectrofluorometer machine: **Gemini EM Gemini XPS - Dual Scanning Microplate Spectrofluorometer User Guide** in 96 wells microplate. Each well can fit between 70-200 ul.
- Standard curve- to know concentration range which is possible to measure by each fluorochrome – standard curve I will prepare to wide concentrations: from 32000 ng/ml to 0.025 ng/ml (and blank repeated 4 times: fluorochrome + 100St reagent).
- To DNA I will measure standard curve from Lambda DNA. Also important is to remeber that dyes can measure different in circle DNA than linear DNA
- Each standard dilution was prepared in 100ST reagent, which is used in our lab to make direct isolation of all cell components, to be able to make fluorochrome measurement in our samples derived from direct isolation.

Plan of experiment

1. Machine calibration- detection limits of machine using fluorochrome fluoresceine
2. Measurement- standard Curve with dilutions of Lambda DNA in 100ST reagent
3. Using standard curve from experiment calculate LOW (detection limit), which is value of blank fluorescence average + 3x it's standard deviation. All results below this was excluded.



In theory curve should have shape with 3 phases: **slow beginning**, **steep acceleration** and **Plateau**. Measurement points from Plateau and Slow beginning were excluded, because of giving imprecise concentration. (In results are included two curves for each fluorochrome: with all received measurements and second without excluded points.)

4. Comparison of all fluorochromes range. The best fluorochromes will be chose to make specificity essay.
    1. Choose for each fluorochrome point of the middle of steep acceleration and use this concentration for specificity measurement.
    2. Make measurement of triplicate of:
        1. dsDNA
        2. dsDNA + RNA in the same concentrations
        3. dsDNA + ssDNA in the same cocnetrations
        4. dsDNA + protein in the same concentration (compared concentrations was to have the same proportion fo bp per aminoacid)
        5. *for RNA and protein specific assays first point was only alone RNA/protein and the next poits analogically.
    3. Comparison of fluorochromes specificity.

List of fluorochromes:

- DNA fluorochromes:
    - DNAQ DirectQuant
    - EtBr
    - SYBR Safe THERMO
    - Pico Green Thermo
    - EvaGreen Biotium
    - Propidium Iodide
    - Accu Blue Biotium
    - HOECHST33258 10 mM
- RNA fluorochromes
    - Ribo Green Thermo
- Protein fluorochromes
    - Nano Orange Thermo
    - Nile Red Sigma

Methods

Dilutions of fluorochromes was performed according to producent's protocol (PicoGreen, AccuBlue, DNAQ, RiboGreen, Nano Orange). Some of fluorochromes are not dedicated to spectrofluorometry assays and was diluted to concentration using in different assay (for example Eva Green). Fluorochromes dedicated to different assays (Ethydium Bromide, SybrSafe, propidium Lodide, HOECHST33258, Nile Red Sigma) was diluted and prepared using available literature.

In plate was combined 50ul fluorochrome working solution and 50 ul of standard in 100ST.

Emission and Excitation wavelength was chosen from producent's protocol or literature.

Graphs, plots and statistic analysis was performed using programs written in Python in JupyterLab software in Anaconda Navigator (software available for free for all users).

RNA standard isolated from mouse brain using Trireagent. Quality of sample was checked using agarose elctrophoresis, spectrophotometry and spectrofluorometry.

Machine calibration

Serial dilution of fluoresceine in DMSO (highest concentration is 10mM)

Machine: Dual Scanning Microplate Spectrofluorometer Gemini

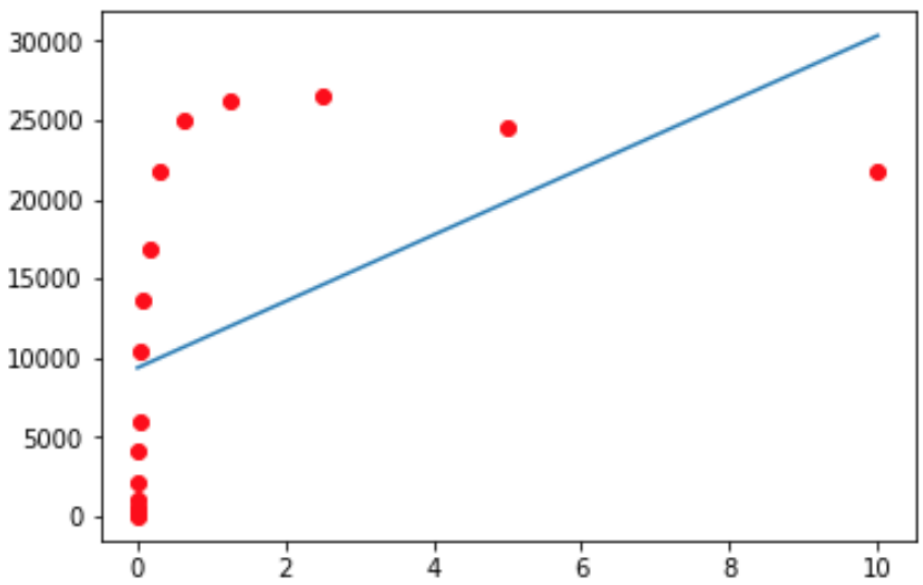
- without Cutoff
- Excitation 475 nm, emission 543 nm
- Auto PMT
- 96 well plate, plastic, clarity
- 200 ul of liquid inside a well

Results:

"concentration [ng/ul]": [10,10/2,10/4, 10/8, 10/16,
10/32, 10/64, 10/128, 10/256,
10/512,10/1024,10/2048,10/(2048*2),
10/(2048*4),10/(2048*8),10/(2048*16),10/(2048*32)

"fluorescence [RFU]" : [21794,24583,26513,26189,
24959,21768, 16912,13631,
10450,6009,4113, 2185,
1041, 544.8, 300.9, 150.7, 5.629

Choosen detection range: to receive straight line better fitting:



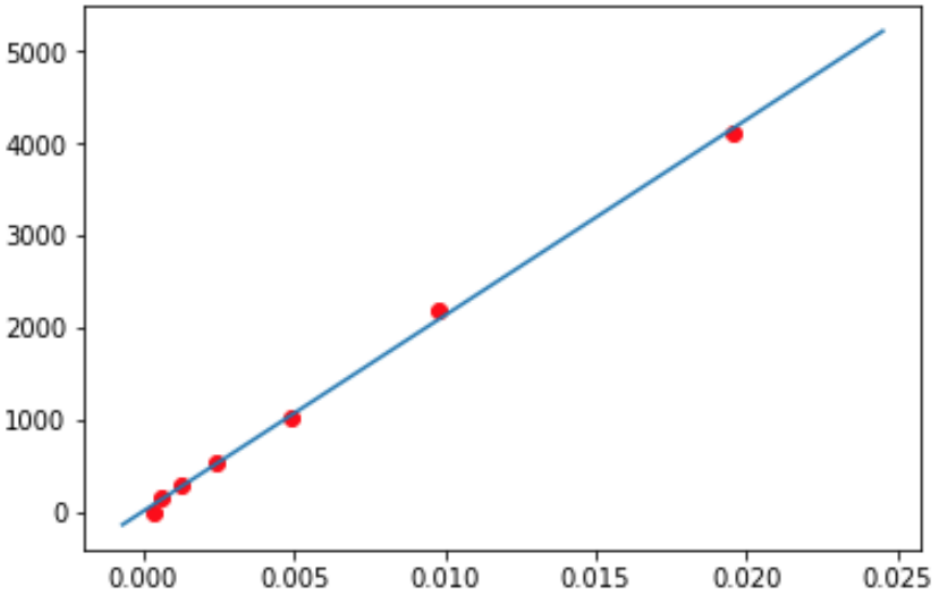
x – concentration

y - fluorescence

Detection limit using 5 repetition of balnks (DMSO):

blank = [4.221, 4.082, 4.201, 3.963, 3.654]

detection limit: 4.6450228732899586



Remarks:

My observation is that this detection limit is different regarding chosen excitation wavelength, because the same wells had completely different emission in different excitations wavelengths. Maybe this experiment of machine calibration is not usefull?

In the next experiments observation is that fluorescence below machine detection limit shows straight line of fluorescence (in Propidium Iodide essay).

None of fluorochromes showed the highest fluorescence close to machine upper detection limit

(machine upper limit is 5000 RFU, for the rest of fluorochromes:

- DNAQ: 123.86,
- AccuBlue: 1158.6
- Ethydium Bromide: 720.31,
- EvaGreen: 502.64

- PicoGreen: 175.43,
- SybrSafe: 80.253)

Dna specific fluorochromes, Results of range assay

DnaQ

"concentration": [32000, 16000, 8000, 4000,
2000, 1000, 500, 250,
25, 2.5, 0.25, 0.025,
0.00],

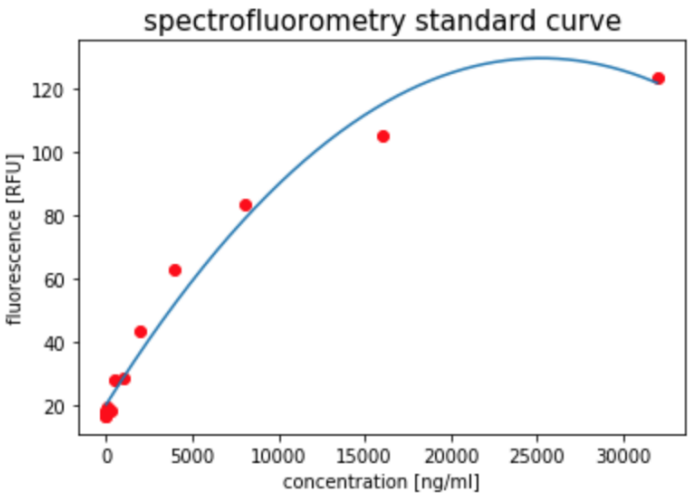
"fluorescence [RFU]" : [123.86, 105.24, 83.604, 62.811,
43.786, 28.986, 28.098, 18.266,
19.419, 17.262, 17.039, 18.528,
16.818],

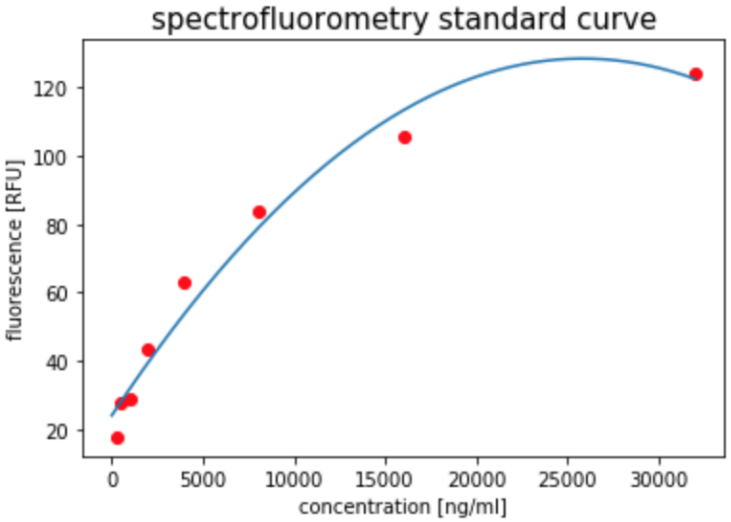
"concentration units" :"[ng/ml]",

"Excitation" : "495 nm",

"Emission" : "535 nm",

"blank" : [14.725, 14.165, 14.220, 14.378, 15.063]






```
blank standard deviation: 0.33840295506984
blank average: 14.510200000000001
detection limit: 15.52540886520952
```

Detection range: 250-16000 ng/ml

AccuBlue

"concentration": [32000, 16000, 8000, 4000,
2000, 1000, 500, 250,
25, 2.5, 0.25, 0.025,
0.00],

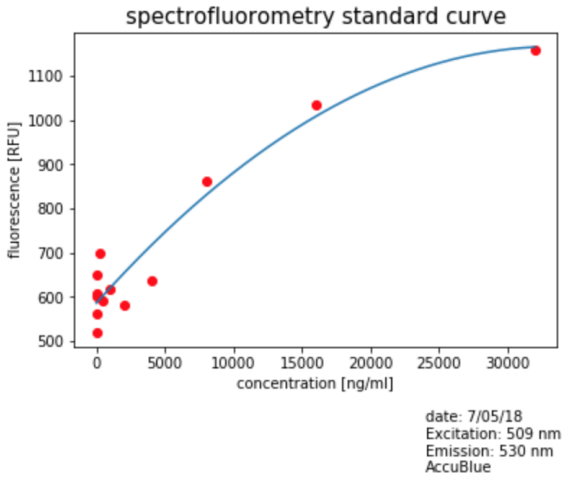
"fluorescence [RFU]" : [1158.6, 1034.3, 861.61, 639.02,
582.45, 618.61, 590.73, 700.22,
521.19, 650.60, 601.44, 562.65,
608.6600000000001],

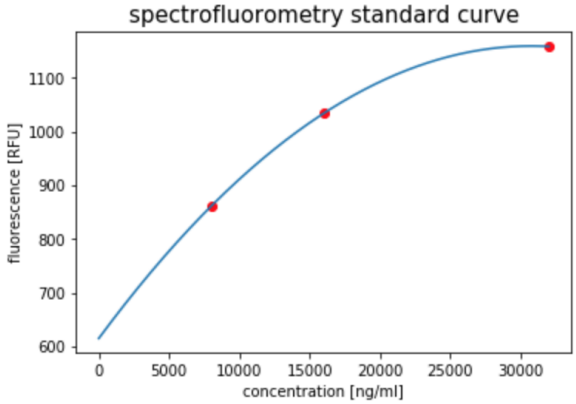
"concentration units" : "[ng/ml]",

"Excitation" : "509 nm",

"Emission" : "530 nm",

"blank" : [564.82, 652.50]






```
blank standard deviation: 43.839999999999975
blank average: 608.6600000000001
detection limit: 740.1800000000001
```

range: 8000- 32000 ng/ml

Remarks: Standard curve is not proper, propably reason is that AccuBlue is only one fluorochrome from the rest that producent in protocol wrote to combine 190 ul working solution and 10 ul sample/standard. (Is more dificult to mix this properly, in opposite to the rest of fluorochromes, combined 50 ul fluorochrome working solution and 50 ul standard).

Ethydium Bromide

Fluorochrome concentration:

"concentration": [32000, 16000, 8000, 4000, 2000, 1000, 500, 250,

250/2, 250/4, 250/8, 250/16, 250/32, 0.00],

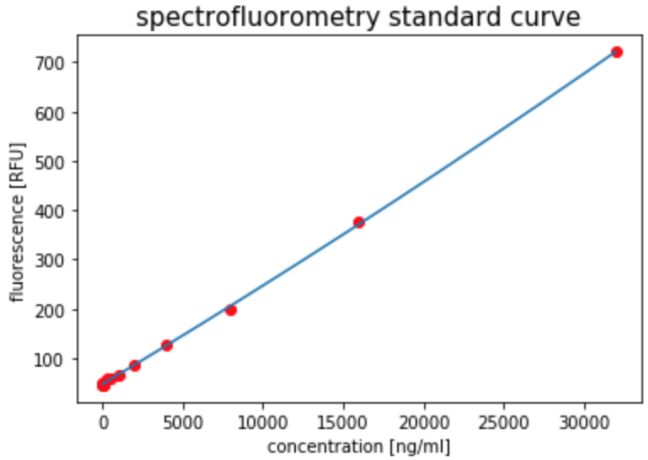
"fluorescence [RFU]" : [720.31, 375.62, 200.24, 126.23, 84.540, 66.349, 56.789, 58.876, 50.245, 47.108, 46.250, 47.216, 45.096, 47.012],

"concentration units" : "[ng/ml]",

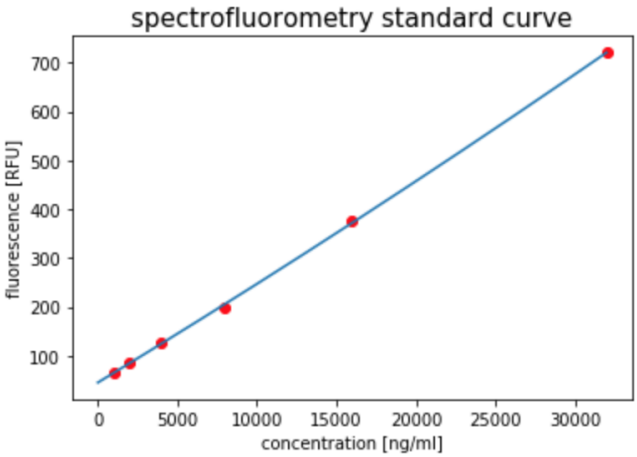
"Excitation" : "302",

"Emission" : "590",

"blank" : [46.146, 47.711, 47.179]




```
blank standard deviation: 0.6497296873828883
blank average: 47.012
detection limit: 48.96118906214866
```




```
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blank average: 47.012
detection limit: 48.96118906214866
```

Detection range: 1000 ng/ml – 32000 ng/ml (upper range can be higher, because standard curve doesn’r reach plateou.

EvaGreen

"concentration": [32000, 16000, 8000, 4000, 2000, 1000, 500, 250,
25, 2.5, 0.25, 0.025, 0.00],

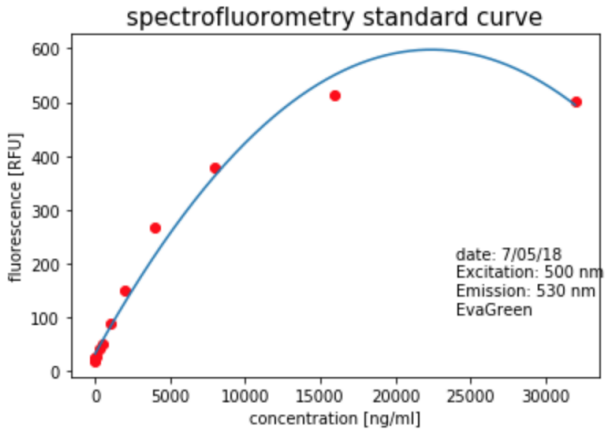
"fluorescence [RFU]" : [502.64, 513.10, 377.65, 268.23, 149.42, 88.487, 50.581, 41.550,
26.423, 23.856, 23.395, 21.013,18.737000000000002],

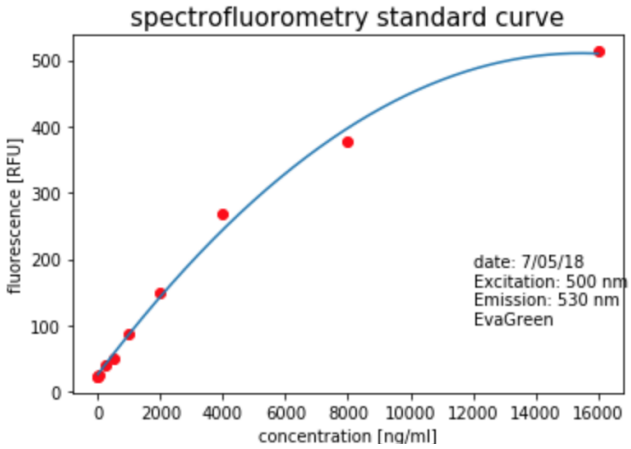
"concentration units" : "[ng/ml]",

"Excitation" : "500 nm",

"Emission" : "530 nm",

"blank" : [19.340, 18.599, 17.683, 19.326]






```
blank standard deviation: 0.6783233004991057
blank average: 18.737000000000002
detection limit: 20.771969901497318
```

Detection range: 0.25 -16000 ng/ml

PicoGreen

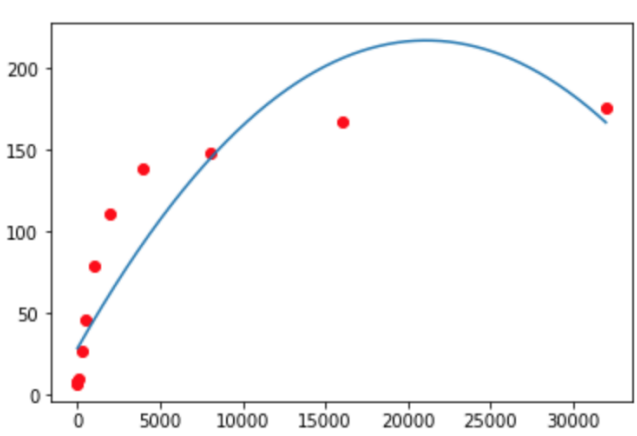
"concentration [ng/ul]": [32000, 16000, 8000, 4000, 2000, 1000, 500,
250, 25, 2.5, 0.25, 0.025, 0.00],

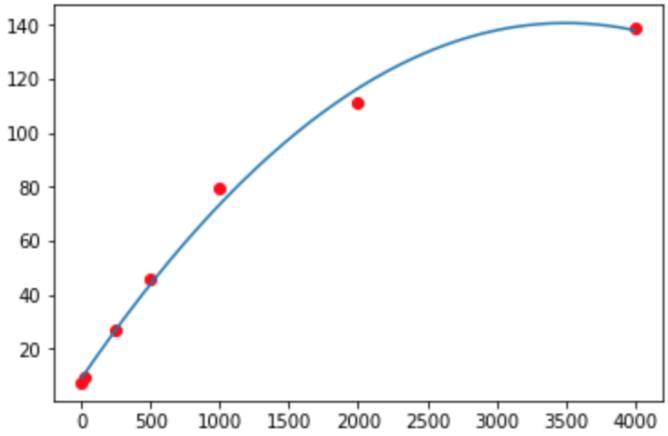
"fluorescence [RFU]" : [175.43, 167.30, 148.42, 139.03, 111.44, 79.572, 46.053, 26.756,
9.490, 7.563, 7.563, 8.116, 6.838]

"Excitation" : "500 nm",

"Emission" : "530 nm",

"blank" : [7.022, 6.838, 6.829, 6.663]






```
blank standard deviation: 0.12704526752303685
blank average: 6.838
detection limit: 7.21913580256911
```

Detection range: 2.5 ng/ml up for 4000 ng/ml

SybrSafe

Fluorochrome concentration:

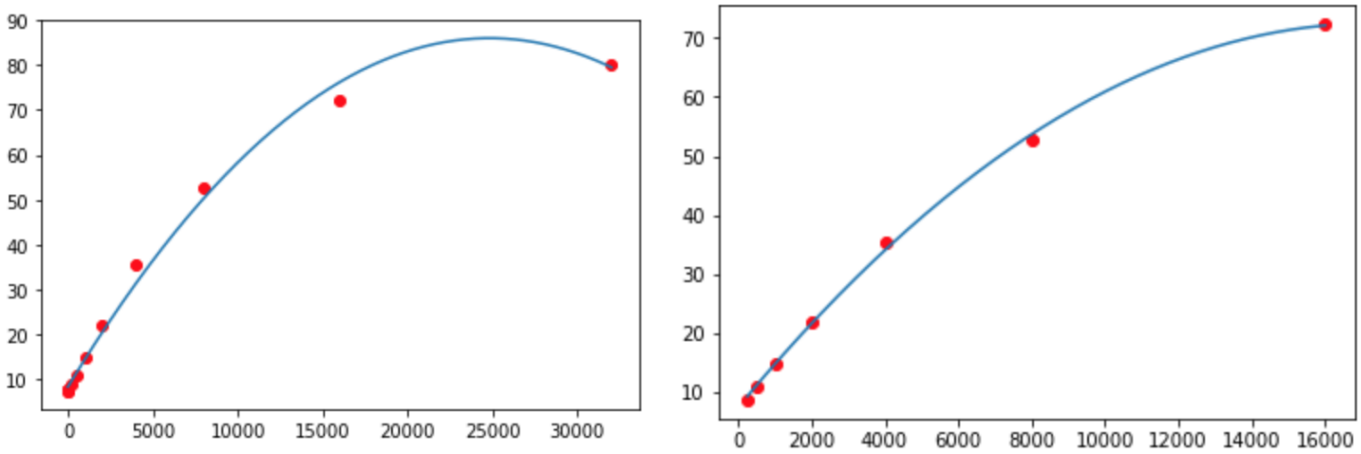
"concentration [ng/ul]": [32000, 16000, 8000, 4000, 2000, 1000, 500, 250,
25, 2.5, 0.25, 0.025, 0.00],

"fluorescence [RFU]" : [80.253, 72.295, 52.659, 35.334, 21.789, 14.813, 10.962, 8.683,
7.251, 7.251, 7.618, 7.647, 7.66725

"Excitation" : "280 nm",

"Emission" : "530 nm",

"blank" : [7.646, 7.900, 7.307, 7.816]



Range of detection is from 250 ng/ml to 32000ng/ml

Propidium Iodide

Fluorochrome preparation:

"concentration": [32000, 16000, 8000, 4000, 2000, 1000, 500, 250, 25, 2.5, 0.25, 0.025, 0.00],

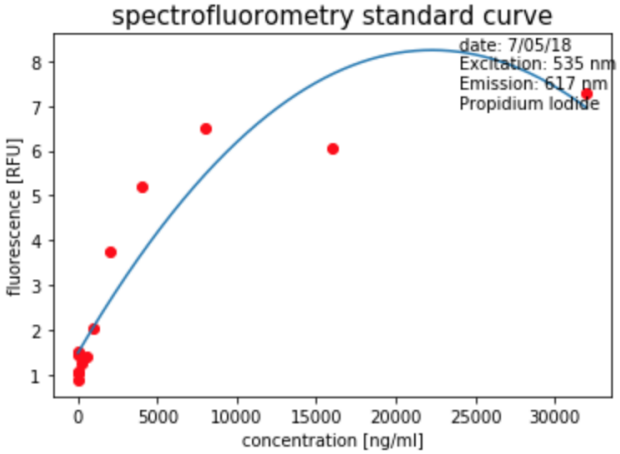
"fluorescence [RFU]" : [7.280, 6.052, 6.502, 5.196, 3.743, 2.050, 1.392, 1.247, 1.437, 1.498, 1.033, 0.882, 1.0605],

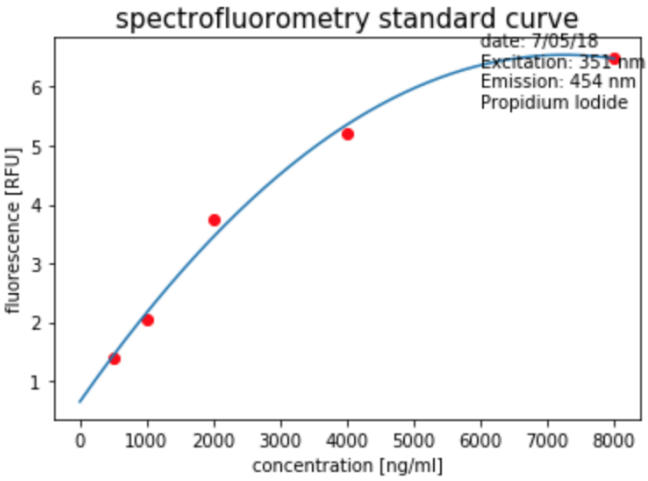
"concentration units" : "[ng/ml]",

"Excitation" : "535 nm",

"Emission" : "617 nm",

"blank" : [1.143, 1.174, 0.962, 0.963]






```
blank standard deviation: 0.09861161189231216
blank average: 1.0605
detection limit: 1.3563348356769365
```

Detection range is from 500 to 8000 ng/ml.

Hoechst 33258

Fluorochrome preparation:

"concentration": [32000, 16000, 8000, 4000, 2000, 1000, 500, 250, 25, 2.5, 0.25, 0.025, 0.00],

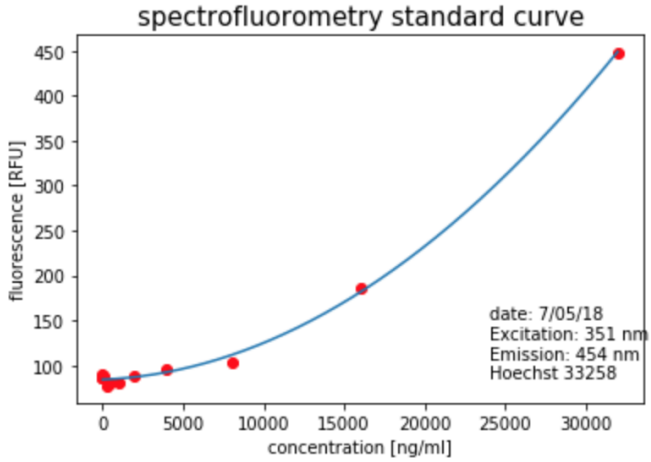
"fluorescence [RFU]" : [448.15, 186.65, 103.92, 95.257, 88.229, 81.404, 80.163, 77.141, 87.672, 90.449, 85.529, 86.477, 86.5135],

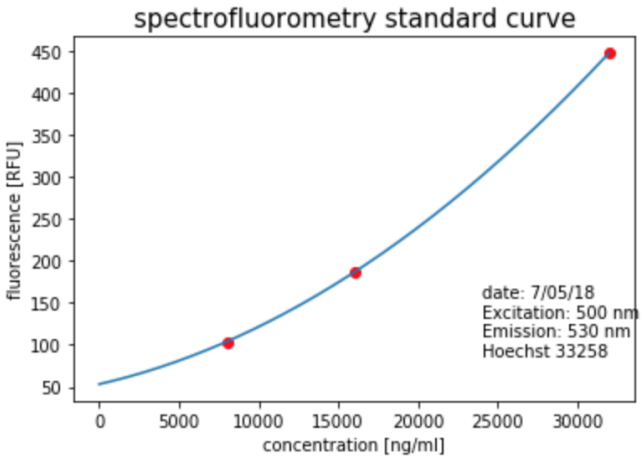
"concentration units" : "[ng/ml]",

"Excitation" : "351 nm",

"Emission" : "454 nm",

"blank" : [86.857, 89.219, 89.156, 80.822]

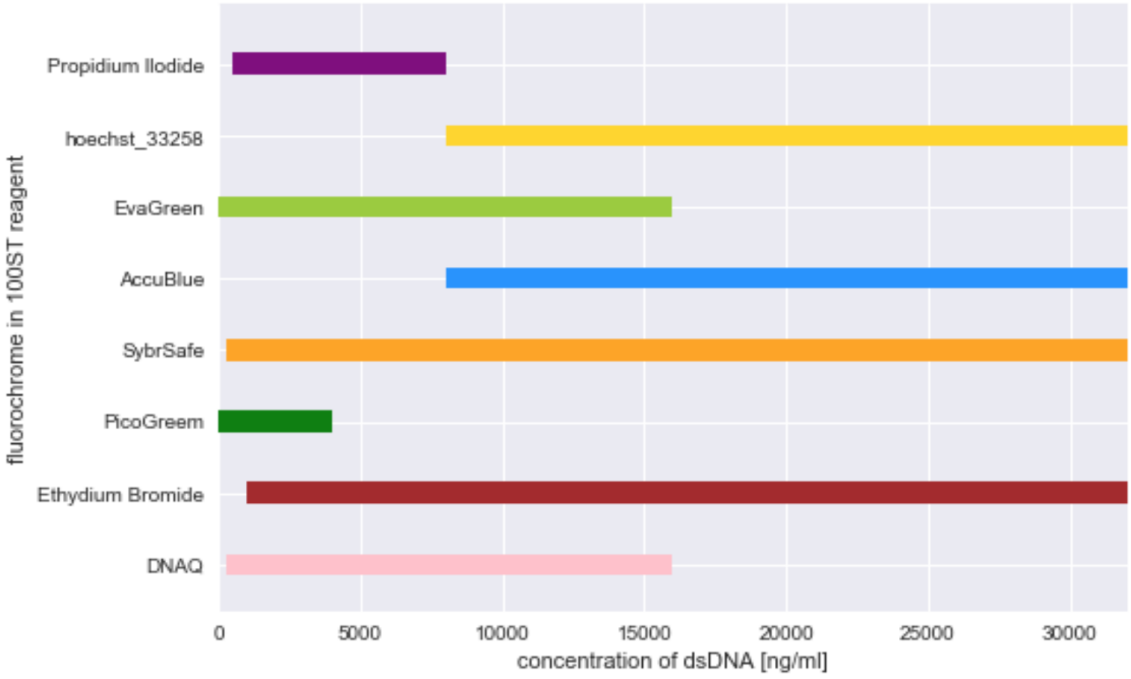


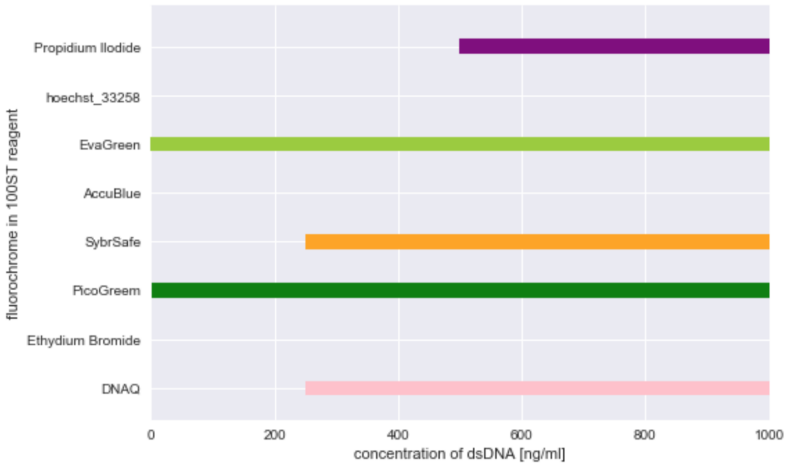



```
blank standard deviation: 3.4210269291544595
blank average: 86.5135
detection limit: 96.77658078746337
```

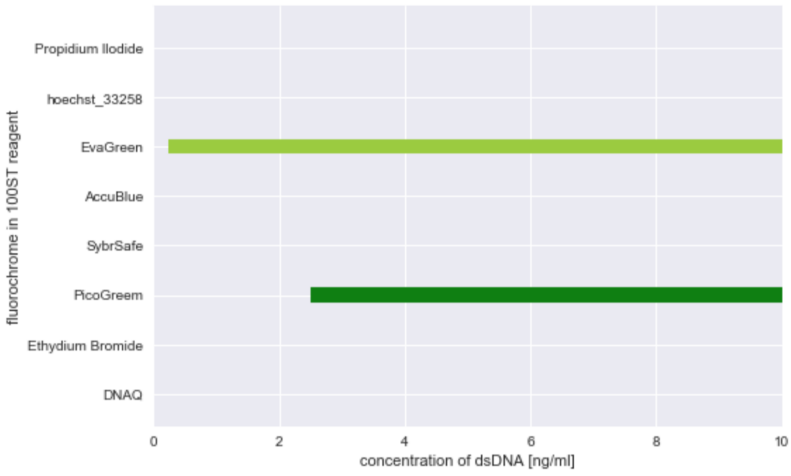
Detection range from 8000 – 32000 ng/ml, can be upper because of curve shape.

DNA specific fluorochromes range comparison





Left: graph with zoom up to 1000 ng/ml.



Left: graph with zoom up to 10ng/ml.

Graph above compare detection range for all used fluorochromes. To the spacificity essay was choosen:

- PicoGreen and EvaGreen – as the most sensitive
- DNAQ, SybrSafe, and Ethydium Bromide – with detection range proper to measure concentrations usually presented in cells and tissues.

Specificity of DNA fluorochromes

First experiment was performed to see general fluorescence tendency of grow of different molecules concentration with the presence of dsDNA. Chosen fluorochrome was picoGreen, to compare my results with prodecents informaions about specificity of this. Fluorescence line with concentration changes should be stable.

Producent's information:

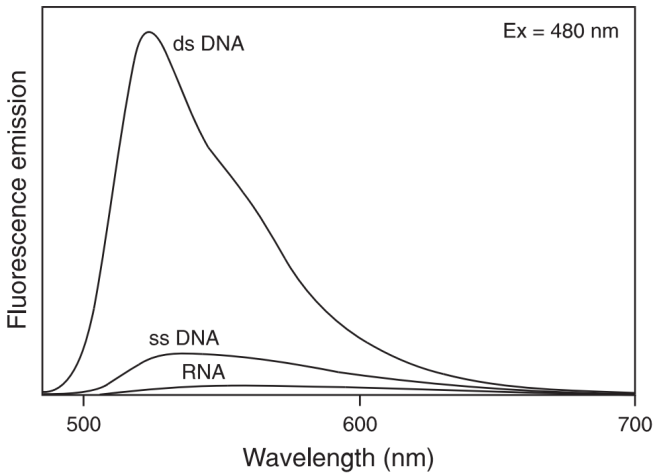
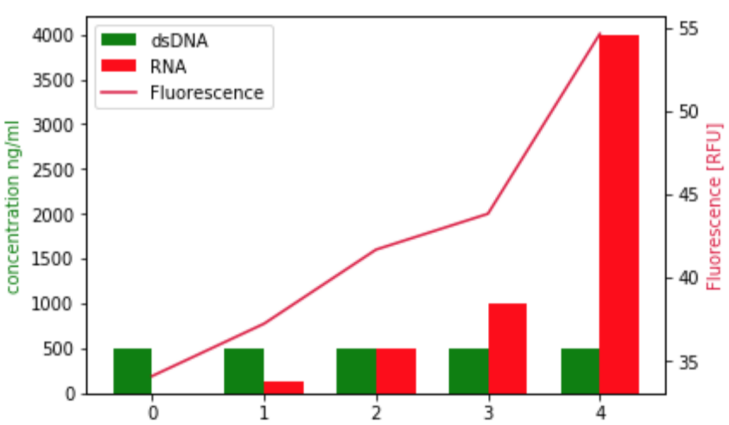
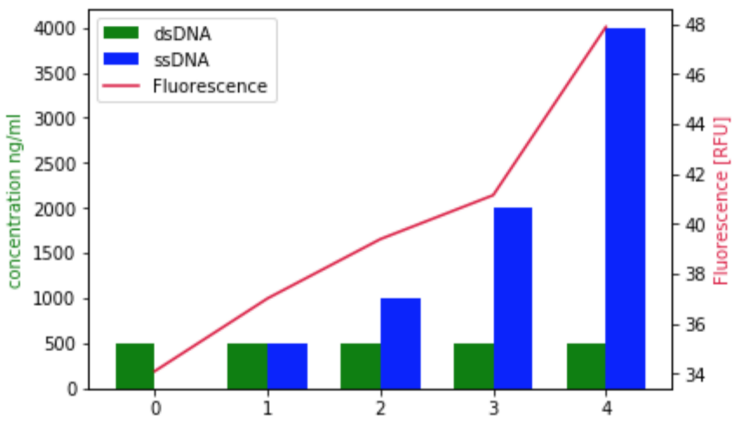


Figure 2. Fluorescence enhancement of Quant-iT™ PicoGreen® reagent upon binding dsDNA, ssDNA, and RNA. Samples containing 500 ng/mL calf thymus DNA, M13 ssDNA, or *E. coli* ribosomal RNA were added to cuvettes containing Quant-iT™ PicoGreen® reagent in TE. Samples were excited at 480 nm and the fluorescence emission spectra were collected using a spectrofluorometer. Emission spectra for samples containing dye and nucleic acids, as well as for dye alone (baseline), are shown.

My results:

('dsDNA concentration ng/ml', [500, 500, 500, 500, 500]), ('RNA concentration ng/ml ', [0, 250/2, 500, 1000, 4000]), ('Fluorescence', [34.095, 37.239, 41.686, 43.838, 54.643])	('dsDNA', [500, 500, 500, 500, 500]), ('ssDNA', [0, 500, 1000, 2000, 4000]), ("Fluorescence", [34.095, 37.010, 39.384, 41.144, 47.886])





In the presence of the same amount of RNA fluorescence grows 22%. In the presence of the same amount of RNA fluorescence grows 8%.

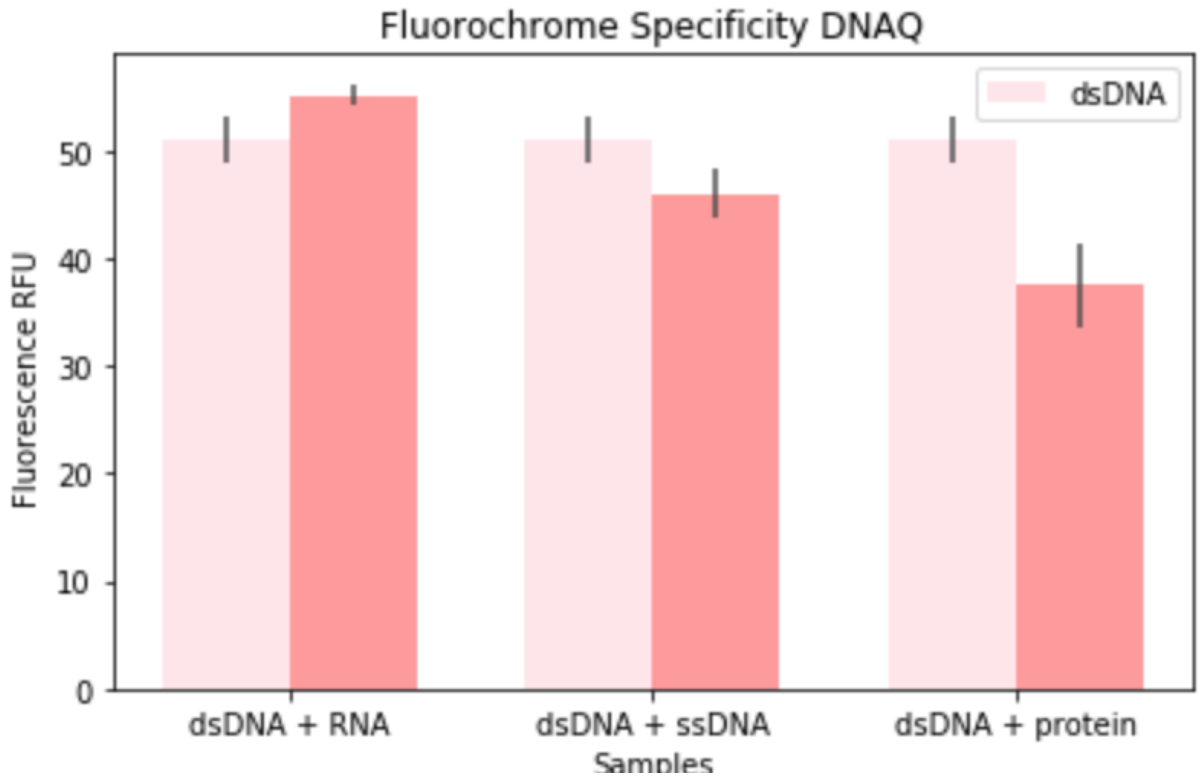
Next assays was performed according to planned protocol. First columns in graph are dsDNA in concetration x, second is dsDNA with concentration x + additional component in concentration x.

DNAQ

dsDNA

'average': 51.08866666666666,
'cv': 0.04187761133288799,
'fluorescences': [48.161, 53.214, 51.891],
'name': 'dsDNA',
'standard_deviation': 2.1394713261821368}

	average	cv	fluorescences	name	standard_deviation	unspecificity	specificity	fluorochrome
0	55.205333	0.018581	[55.14, 56.493, 53.983]	dsDNA + RNA	1.025744	8.057886	91.942114	DNAQ
1	45.981333	0.049667	[48.784, 43.19, 45.97]	dsDNA + ssDNA	2.283755	9.996999	90.003001	DNAQ
2	37.505667	0.104262	[42.664, 36.653, 33.2]	dsDNA + protein	3.910422	26.587110	73.412890	DNAQ

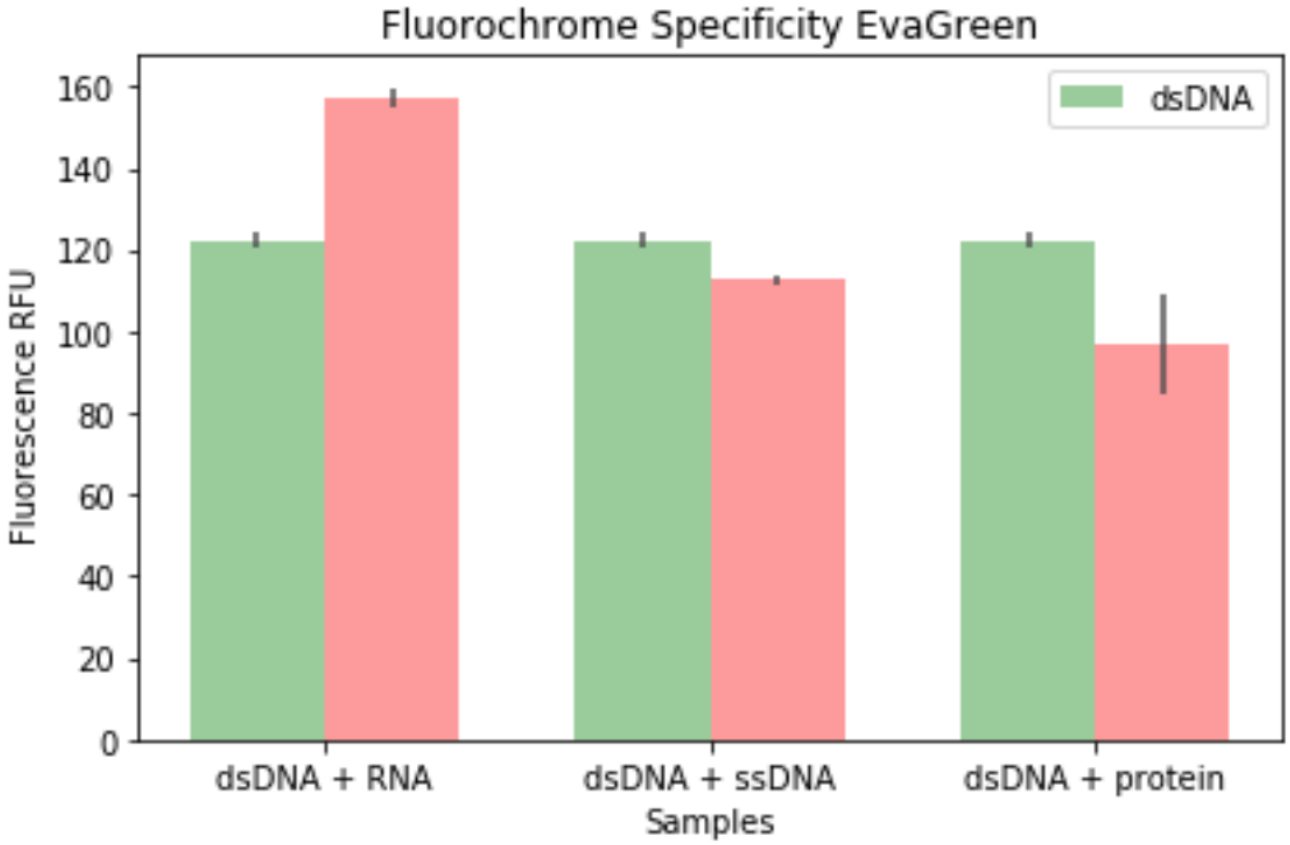


EvaGreen

dsDNA

{'average': 122.27666666666666,
'cv': 0.02873806050680054,
'fluorescences': [121.96, 126.73, 118.14],
'name': 'dsDNA',
'standard_deviation': 3.513994245236547}

	average	cv	fluorescences	name	standard_deviation	unspecificity	specificity	fluorochrome
0	157.123333	0.031559	[163.57, 156.29, 151.51]	dsDNA + RNA	4.958611	28.498214	71.501786	EvaGreen
1	112.560000	0.024698	[115.34, 109.78]	dsDNA + ssDNA	2.780000	7.946460	92.053540	EvaGreen
2	96.867000	0.247895	[125, 59, 105.84, 97.628]	dsDNA + protein	24.012857	20.780471	79.219529	EvaGreen


Fluorochrome Specificity EvaGreen
dsDNA
Fluorescence RFU
160
140
120
100
80
60
40
20
0
dsDNA + RNA
dsDNA + ssDNA
dsDNA + protein
Samples

PicoGreen

dsDNA

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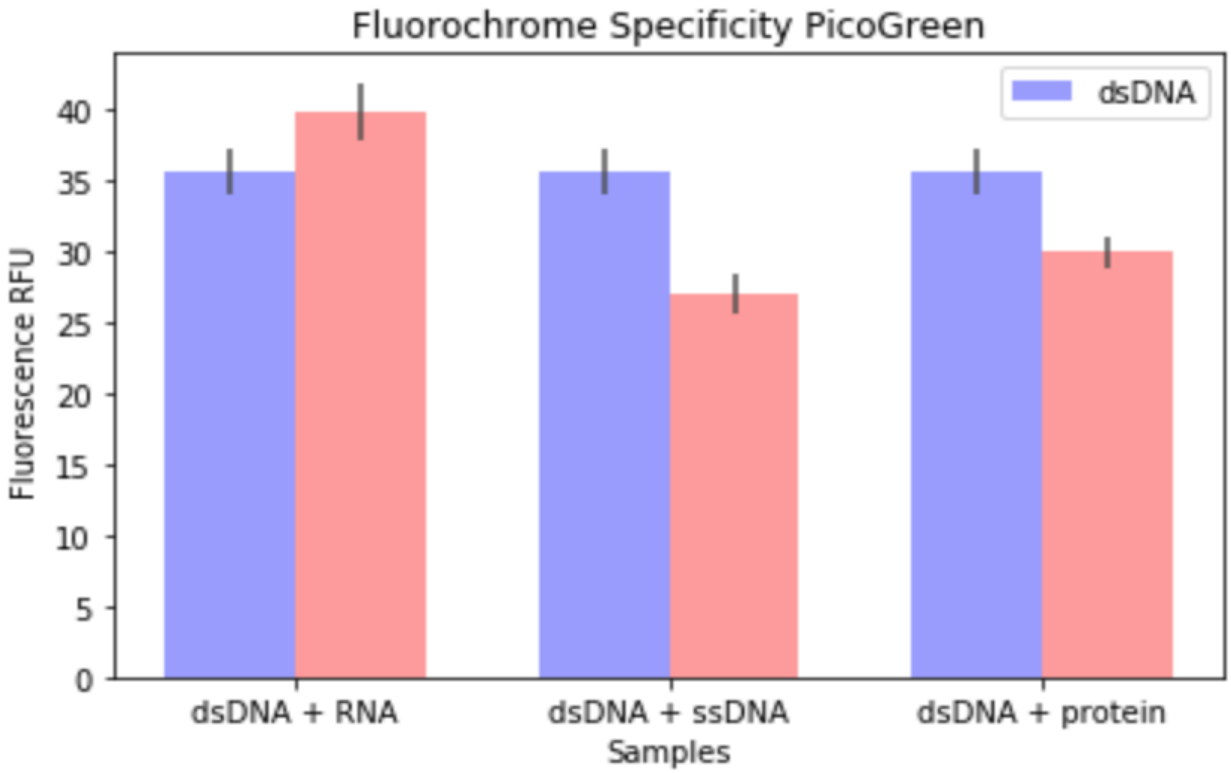
	average	cv	fluorescences	name	standard_deviation	unspecificity	specificity	fluorochrome
0	39.809333	0.049989	[39.195, 37.738, 42.495]	dsDNA + RNA	1.990028	11.742360	88.257640	PicoGreen
1	26.924667	0.053524	[28.496, 27.263, 25.015]	dsDNA + ssDNA	1.441109	24.424110	75.575890	PicoGreen
2	29.914333	0.035666	[31.322, 29.681, 28.74]	dsDNA + protein	1.066932	16.032299	83.967701	PicoGreen


Fluorochrome Specificity PicoGreen
dsDNA
Fluorescence RFU
0
5
10
15
20
25
30
35
40
dsDNA + RNA
dsDNA + ssDNA
dsDNA + protein
Samples

SybrSafe

dsDNA

'average': 33.06166666666667,
'cv': 0.013103438746895445,
'fluorescences': [32.919, 32.617, 33.649],
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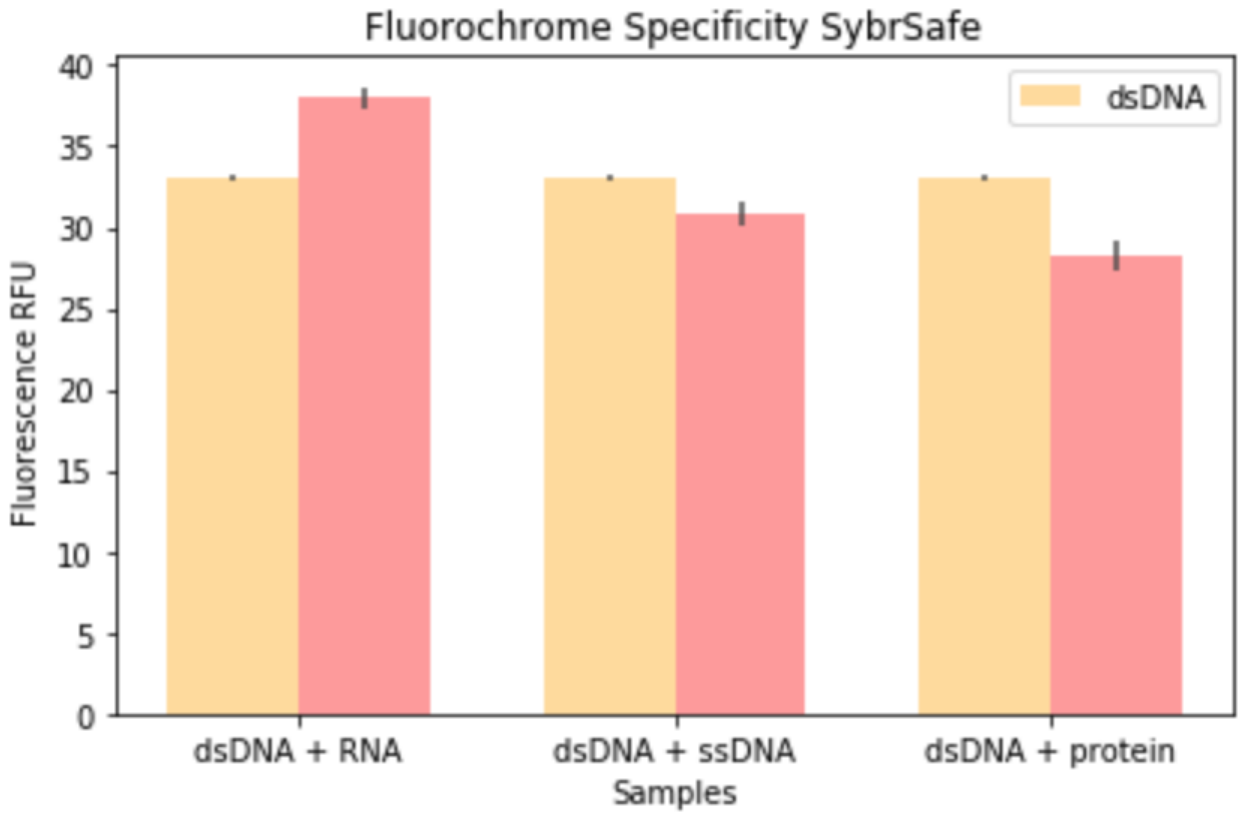
:

	average	cv	fluorescences	name	standard_deviation	unspecificity	specificity	fluorochrome
0	38.014667	0.032483	[36.691, 39.663, 37.69]	dsDNA + RNA	1.234842	14.981096	85.018904	SybrSafe
1	30.820667	0.049567	[28.692, 32.205, 31.565]	dsDNA + ssDNA	1.527703	6.778243	93.221757	SybrSafe
2	28.205000	0.067691	[29.192, 29.888, 25.535]	dsDNA + protein	1.909237	14.689721	85.310279	SybrSafe

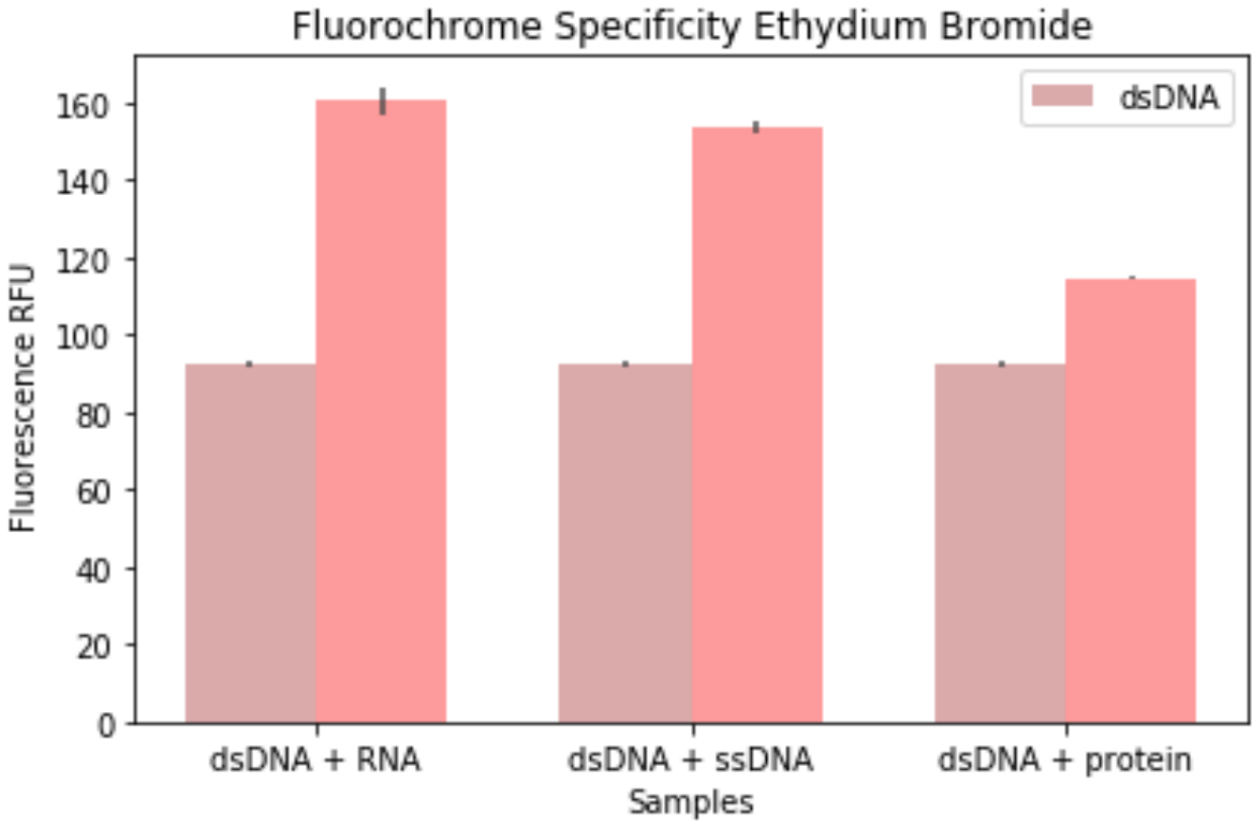


Ethydium Bromide

dsDNA

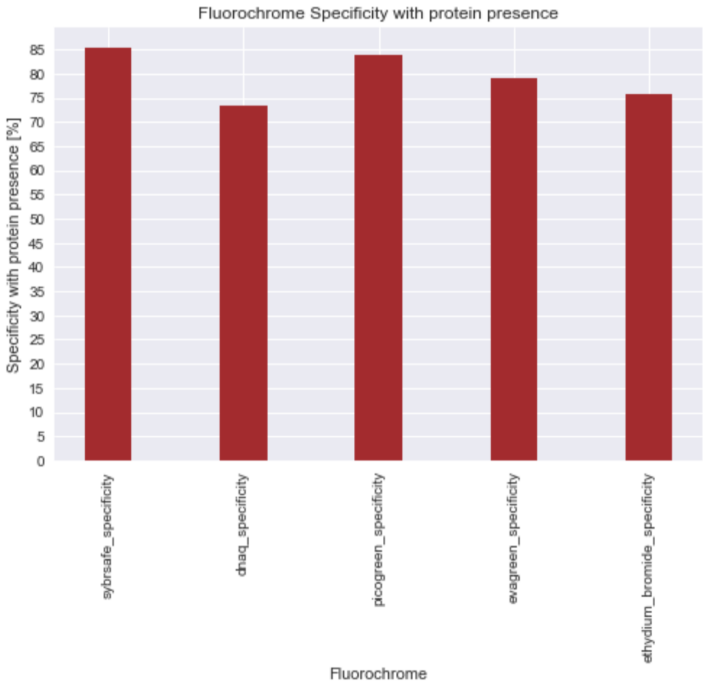
average': 92.25900000000001,
'cv': 0.020334885750596044,
'fluorescences': [94.81, 91.615, 90.352],
'name': 'dsDNA',
'standard_deviation': 1.8760762244642408

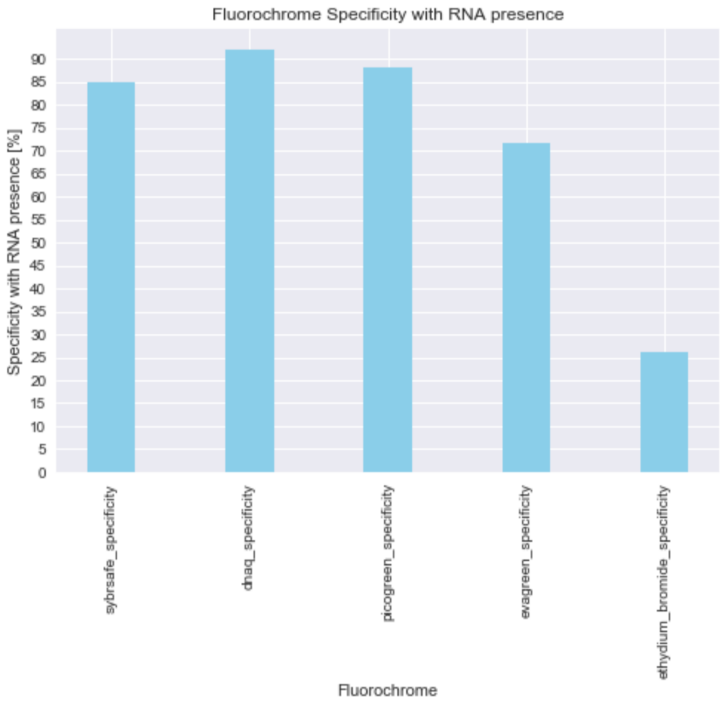
	average	cv	fluorescences	name	standard_deviation	unspecificity	specificity	fluorochrome
0	160.490000	0.042999	[152.53, 159.58, 169.36]	dsDNA + RNA	6.900884	73.955928	26.044072	Ethydium Bromide
1	153.583333	0.024453	[148.87, 153.82, 158.06]	dsDNA + ssDNA	3.755532	66.469757	33.530243	Ethydium Bromide
2	114.723333	0.012456	[116.67, 113.28, 114.22]	dsDNA + protein	1.428993	24.349205	75.650795	Ethydium Bromide

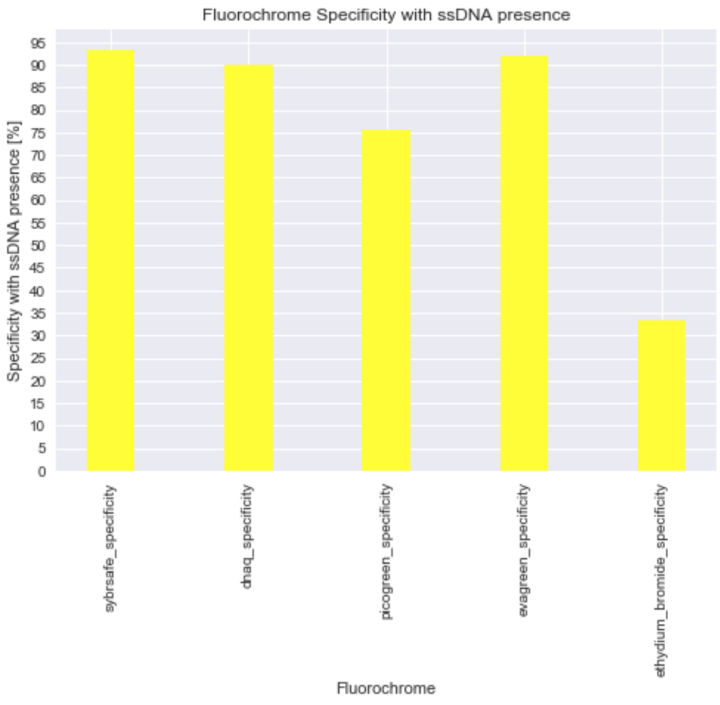


Comparison

Specificity with potein presence	specificity with RNA presence	specificity with ssDNA presence	specificity_data_names
85.310279	85.018904	93.221757	sybrsafe_specificity
73.412890	91.942114	90.003001	dnaq_specificity
83.967701	88.257640	75.575890	picogreen_specificity
79.219529	71.501786	92.053540	evagreen_specificity
75.650795	26.044072	33.530243	ethydium_bromide_specificity







Discussion - DNA fluorochromes

Among all fluorochromes the worst specificity has Ethydium Bromide.

Interesting thing is that specificity is prompt because fluorochrome binds to different molecule imiting or not emitting fluorescence. For example: DNAQ with protein presence has lower fluoresence – fluorochrome binds to protein and doesn't emit fluorescence, in opposite to RNA presence. It’s important to have this knowledge during fluorochrome assay to find explanation to received results. It’s impossible to precise compare concentration of pure DNA sample with DNA sample with protein presence.

In comparison to different popular technique – spectrophotometry, spectrofluorometry has vary high specificity. Spectrophotometry specificity is 0%, in comparison to spectrofluorometry essay: between 71% and 93%.

If there is no spectrofluorometer, spectrofluorometry assay can be performed in qPCR machine thermocycler which has proper channel of excitation and emmision. All fluorochromes assay should be performed in room temperature.

RNA specific fluorochromes comparison

Comparison is not available because it is only one fluorochrome on the market

RNA Assay Kit Quant – TT\

"standard name" : "TRIZOL RNA from mouse brain",

"concentration": [128000, 64000, 32000, 16000, 8000, 4000,2000, 1000, 500, 250, 2.5, 0.025, 0.00],

"fluorescence [RFU]" : [10.794, 12.641, 13.596, 9.545,5.955, 3.411, 3.423, 1.640,

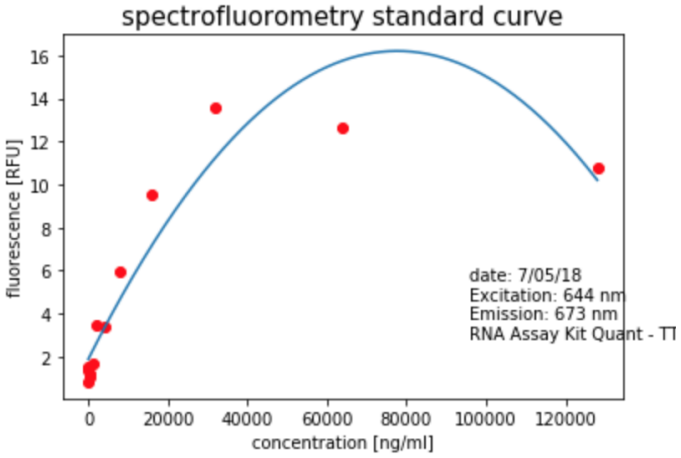
1.183, 1.044, 1.347, 1.479, 0.8196666666666667],

"Excitation" : "644 nm",

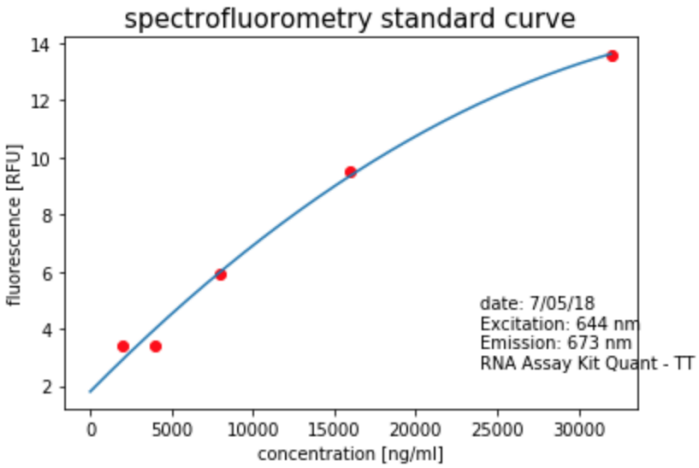
"Emission" : "673 nm",

"fluorochrome" : "RNA Assay Kit Quant - TT",

"blank" : [1.327, 0.455, 0.677]




```
blank standard deviation: 0.3700102100693379
blank average: 0.8196666666666667
detection limit: 1.9296972968746804
```



Observations:

-this experiment requieres repeat, because producent's protocol is to combine 190 ul of fluorochrome working solution and 10 ul of sample. This ratio 190:10 doesn't allow to proper dilution and have high mistake. Next experiment should be applied with ration 1:1 between working solution and sample, the same that was used in the rest of fluorochromes.

Protein specific fluorochromes comparison

During this experiment some problem appeared:

- Excitation and Emission Wave length wasn’t exactly known thus spectrum was checked using the highest concentration of protein with fluorochrome working solution
    - In every measurement peak with emission didn’t appear
    - the same spectrum pattern was in the empty wells (!) -

To check if this problem is only in protein fluorochromes (NileRed and NanoOrange) I used Ethydium Bromide with Lambda DNA (concentration 142 000 ng/ml):

- Emission peak was observed in expected wave length for Ethydium Bromide (590 nm)
- The same spectrum with the same peak was observed in empty wells, without any fluorochrome and any DNA standard.

Questions:

- Why not emission peak is visible in protein fluorochromes spectrum?
- Why empty wells in 96-well plate had the same spectrum that wells with fluorochrome working solution and sample?
- Why some substances allows to see emission peak in spectrum but some not?