Last Update: April 10, 2023

Rolling Circle Amplification
(RCA)

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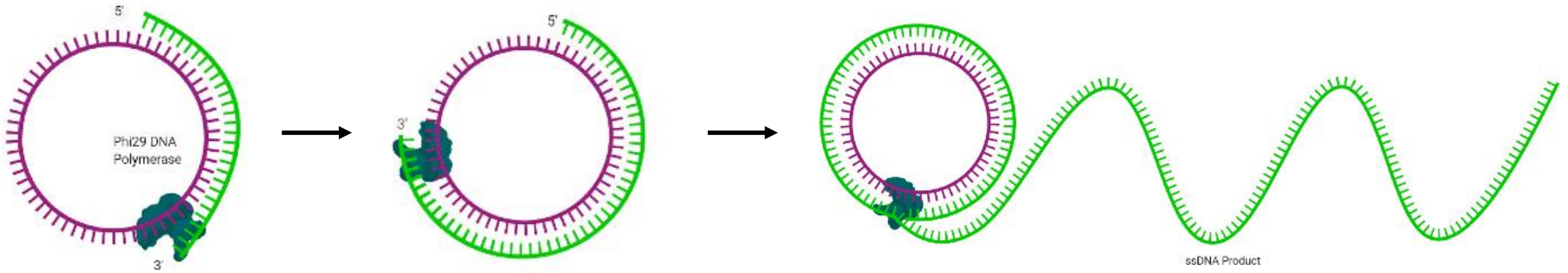
RCA Concept

Introduction:

- RCA stands for Rolling Circle Amplification which is a process of generating copies on DNA that are complementary to the template.
- This is similar to a PCR reaction in which amplification of DNA is done using a template, staple in the presence of free bases and an enzyme which does the polymerization.
- There can be many cross polymerizations that might occur in PCR reaction. These might lead to undesired DNA strands being generated than desired.
- RCA employs a circular template which avoids most of the chance of cross polymerizations.



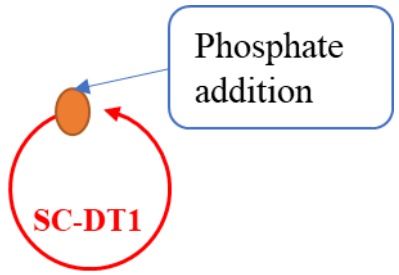
Experiment in Bulk solution

Initial Materials:

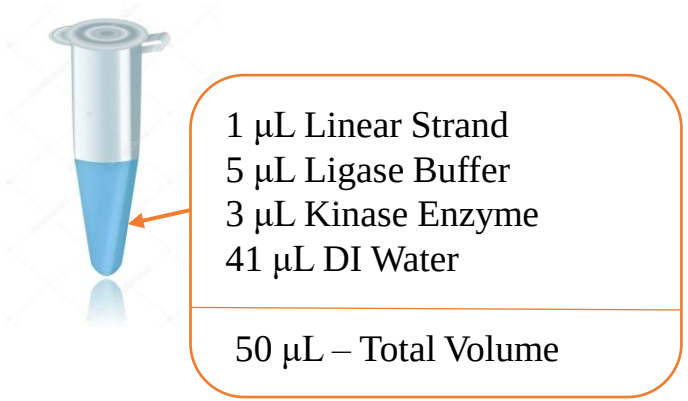
SC-DT1, 46nt: GCCTTCCCTAACCCTAACCCTAACCCTAACCCTAACCCTAATGACT (Template Strand)
SC-DP1, 18nt: AGGGAAGGC AGTCATTAG (Splint Strand)

Step 1: Phosphorylation of 5' end

- Can be done by using Kinase Enzyme.
- Kinase enzyme should be used with the Ligase buffer.
- Linear Strand is introduced such that it is 20 µM in the total volume (50µL).
- Anneal it at 37^0C for 2 hr.



- T4 Kinase Enzyme: 10000 units/mL

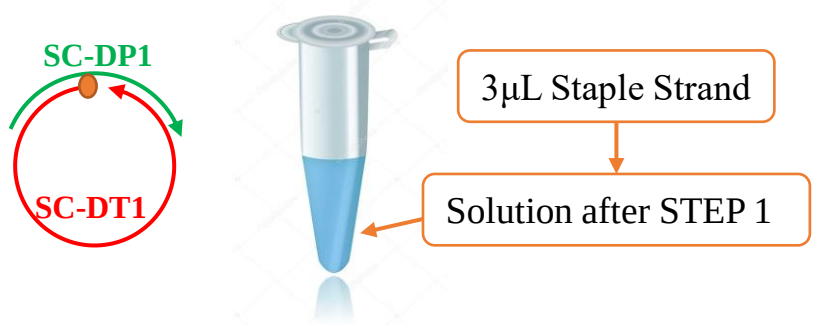


Note: Using Ligase Buffer from first step instead of kinase buffer is recommended. Ligase buffer contains ATPs and DTT's. Kinetion and Ligation can happen in Ligase buffer but, Ligation cannot occur in Kinase buffer alone.

Step 2: Binding Splint Strand with Circular Strand

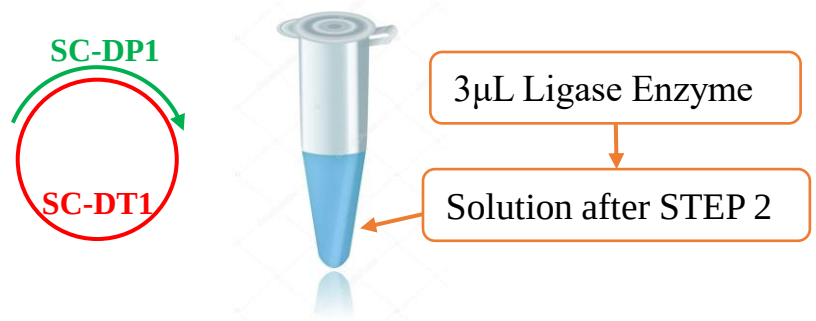
- Introduce the Staple strand (1.2 times molar ratio of Linear Strand)
- Put it in a annealing cycle

  95^0C for 5 min
  65^0C for 30 min
  50^0C for 30 min
  37^0C for 30 min
  22^0C for 20 min
- Staple strand will bind to the Phosphorylated Linear strand to form a circular shape.



Step 3: Ligating The Circular Strand

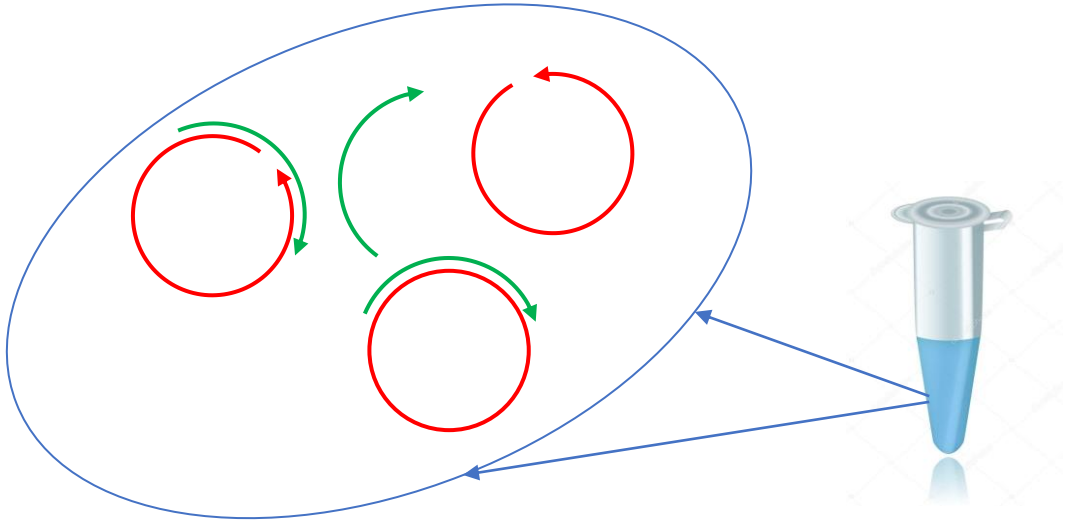
- This step bonds the phosphorylated 5’ end to the 3’ end and makes the circular strand to form a complete circular template.
- Ligase Enzyme is used for this job.
- Add Ligase Enzyme in required amount and leave the sample in a dark place for 16hrs.



- Ligase: 400000 units/mL

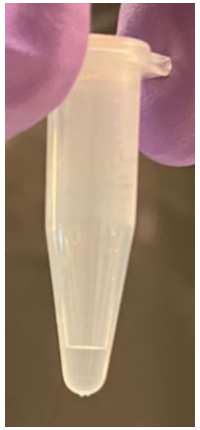
What could possibly be in the final solution?

- ✓ Free DNA (Linear and Splint Strands)
- ✓ Non-Ligated Circular Template
- ✓ Secondary Structures
- ✓ Ligated Circular Template (Desired)



Step 4: Extracting Ligated Circular Template

- Use dPAGE to extract the circular template.
- Follow the dPAGE protocol and electrodialysis for recovery.
- Butanol concentration is preferred to extract the DNA from dialysis product.
- Dissolve the DNA salt in DI water and measure the concentration by absorption measurements.



RCA calculations:

- Look into the excel sheet for any change in new concentrations

Stock Solutions:

	Linear Strand (SC-DT1)	Staple Strand (SC-DT2)
OD	34.9	17
nmoles	83.9	89.6
mg	1.16	0.5
Mol. Wt. (g/mol)	13783	5612.7
Conc. (mM)	1	0.1
Conc. (ug/uL)	13.826	

Kinase	10000	units/mL
Ligase	400000	units/mL
Phi29	10000	units/mL
Buffers	10	X
dNTPs	10	mM

Phosphorylation:

	Volume (uL)	Conc. (uM)
Linear Strand	1	20
Kinase	3	
Ligase Buffer	5	1X
DI Water	41	
Tot Vol.	50	

Staple Strand Binding:

	Volume (uL)	Conc. (uM)
Staple Strand	15.78947368	24
Tot Vol.	65.78947368	

Ligation:

	Volume (uL)	Units
Ligase	3	1200
Tot Vol.	68.7894737	

After dPage:

DNA sol.	50	uL
UV dilution factor	30	
Absorbance measured	0.05535	
Exc. Coeff.	408303	
Circular Strand	4.06683272	uM

Staple Strand Binding:

	Volume (uL)	Conc. (uM)
Circular Strand	10	0.81336654
Staple Strand	0.488019926	0.97603985
Phi29 Buffer	5	1x
DI Water	34.51198007	
Tot Vol.	50	

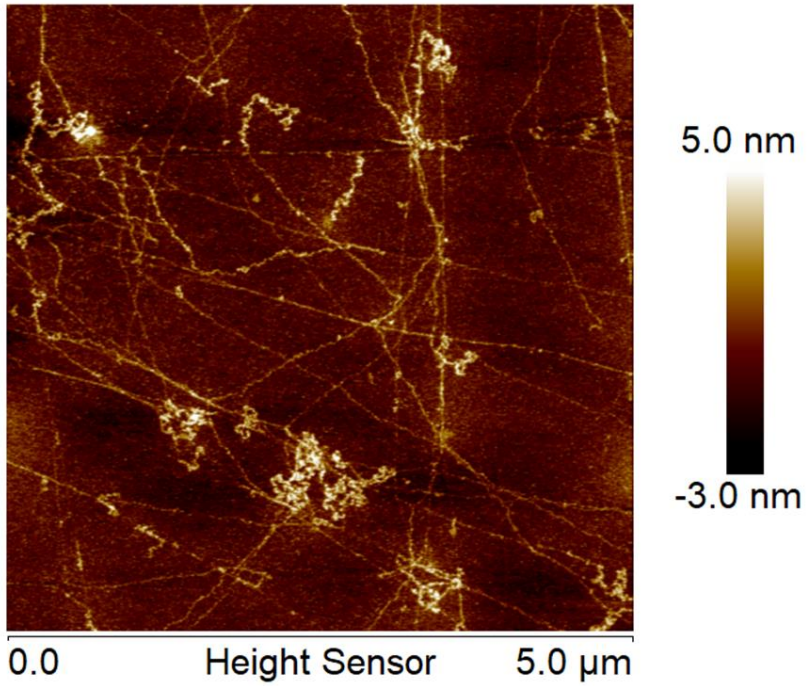
Polymerisation:

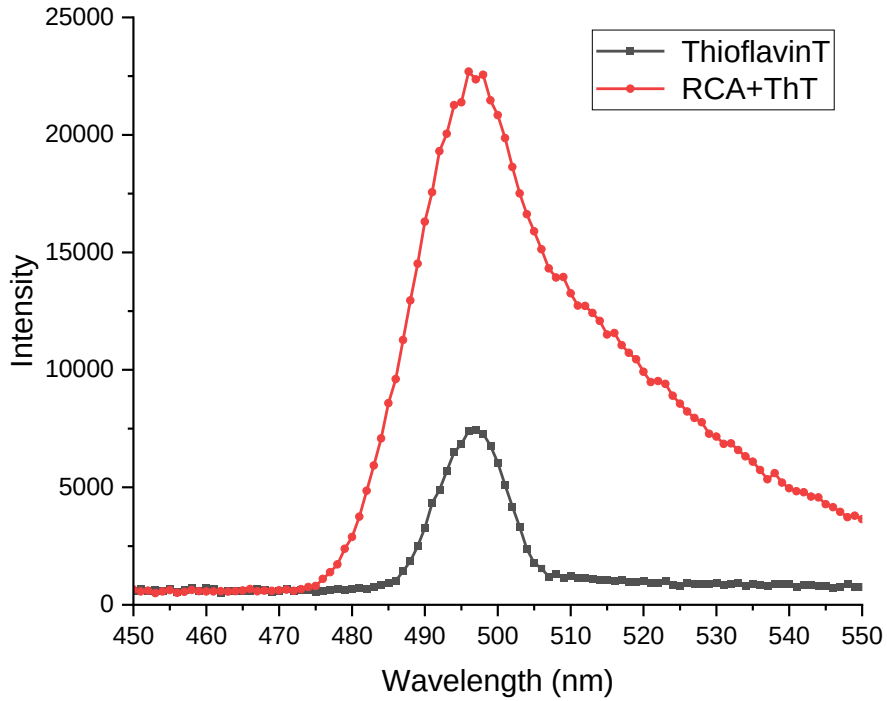
	Volume (uL)	Units
dNTPs	2	
Phi29	1.5	15

Characterization of RCA product

Characterization:

- The designed template complementary yields G-Quadruplex for every one cycle of amplification.
- ThioflavinT (ThT) a fluorescent organic molecule is used for the characterization of G-Quadruplex.
- ThT has an excitation of 425 nm and an emission at 495 nm.
- In the presence of G-Quad the fluorescence of ThT gets enhanced by a large order and hence can be used as a quantifying agent.

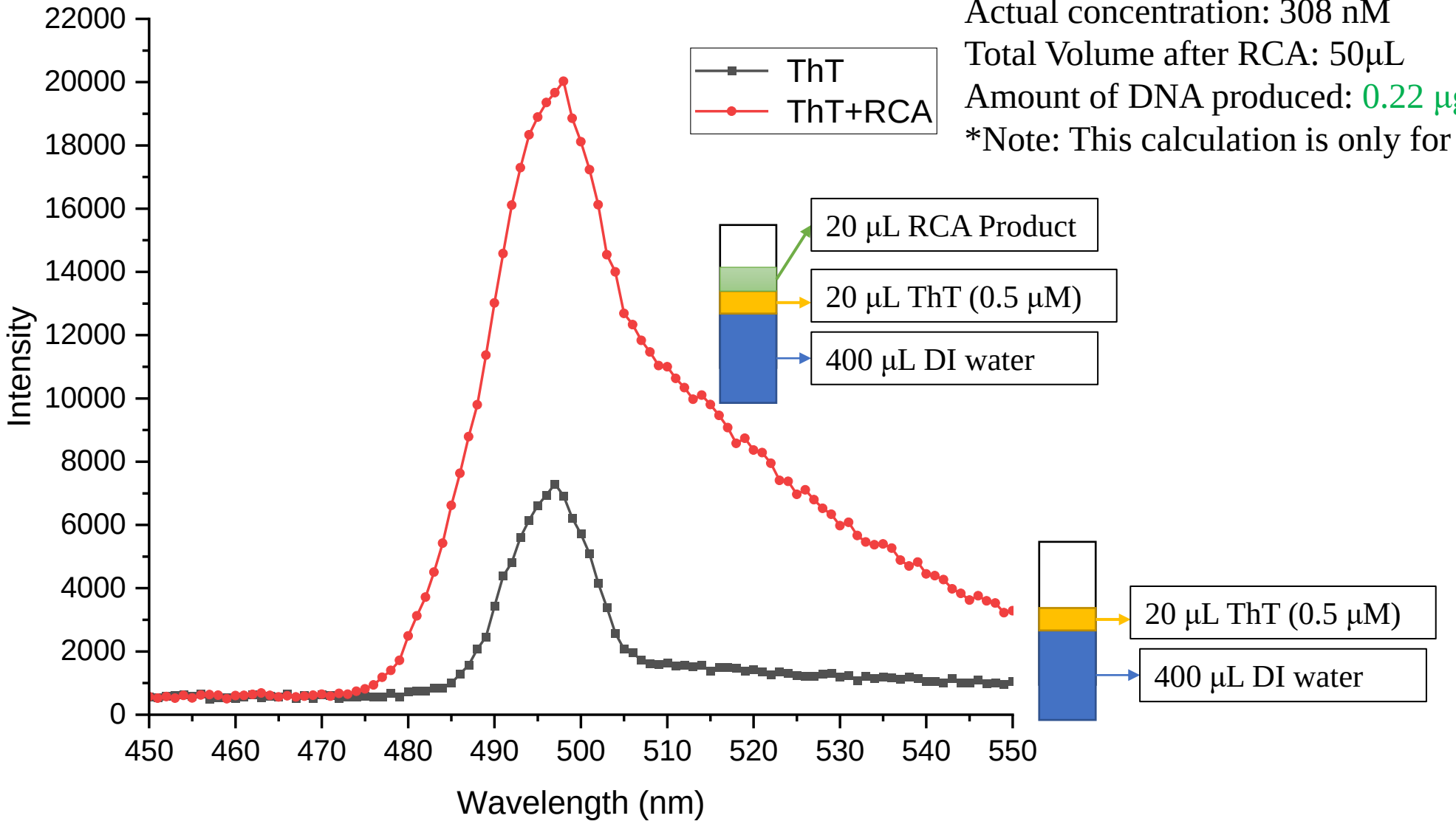




Spectrophotometer Results:

Excitation: 425 nm
Emission: 490 nm

Rough concentration estimate: 14 nM of DNA in cuvette
Actual concentration: 308 nM
Total Volume after RCA: 50µL
Amount of DNA produced: 0.22 µg
*Note: This calculation is only for G-Quad sequence

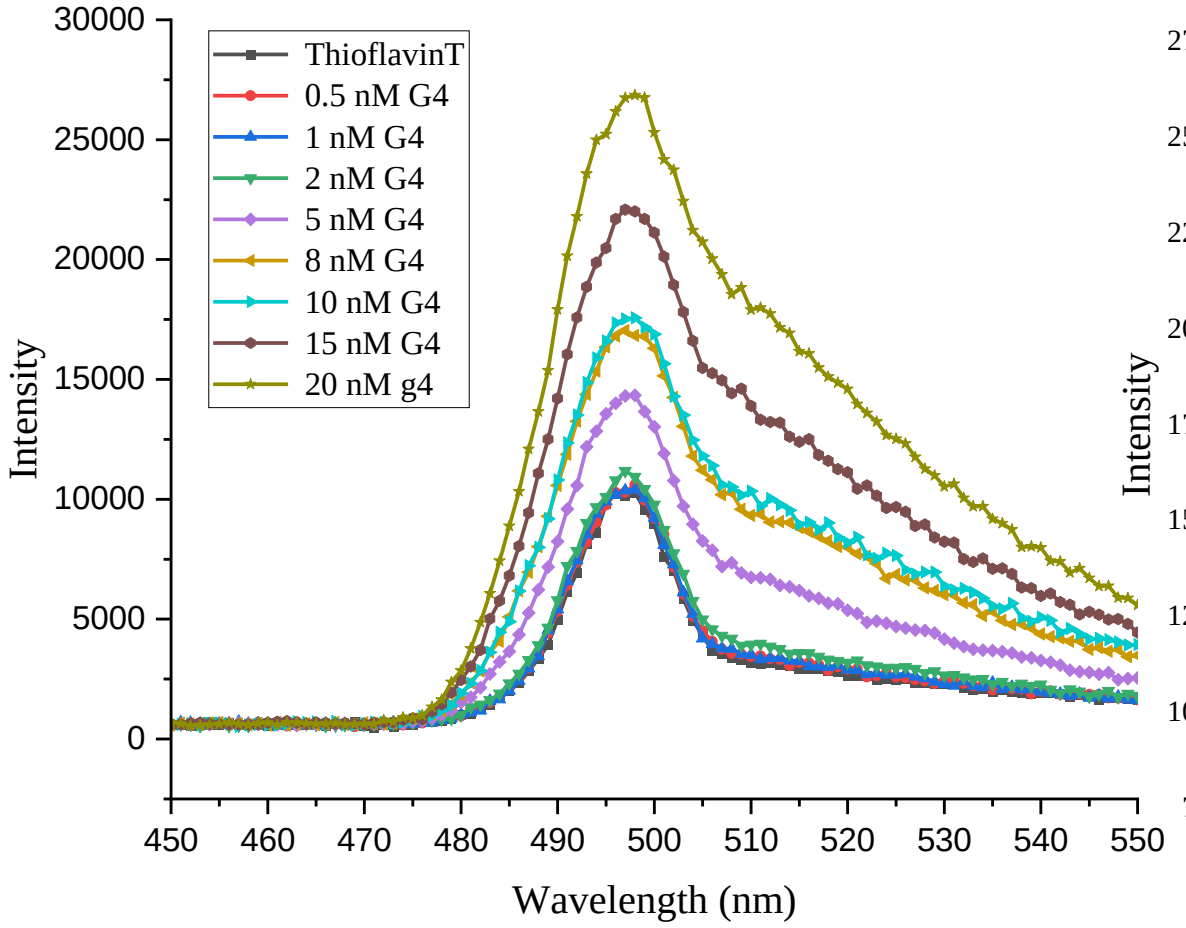


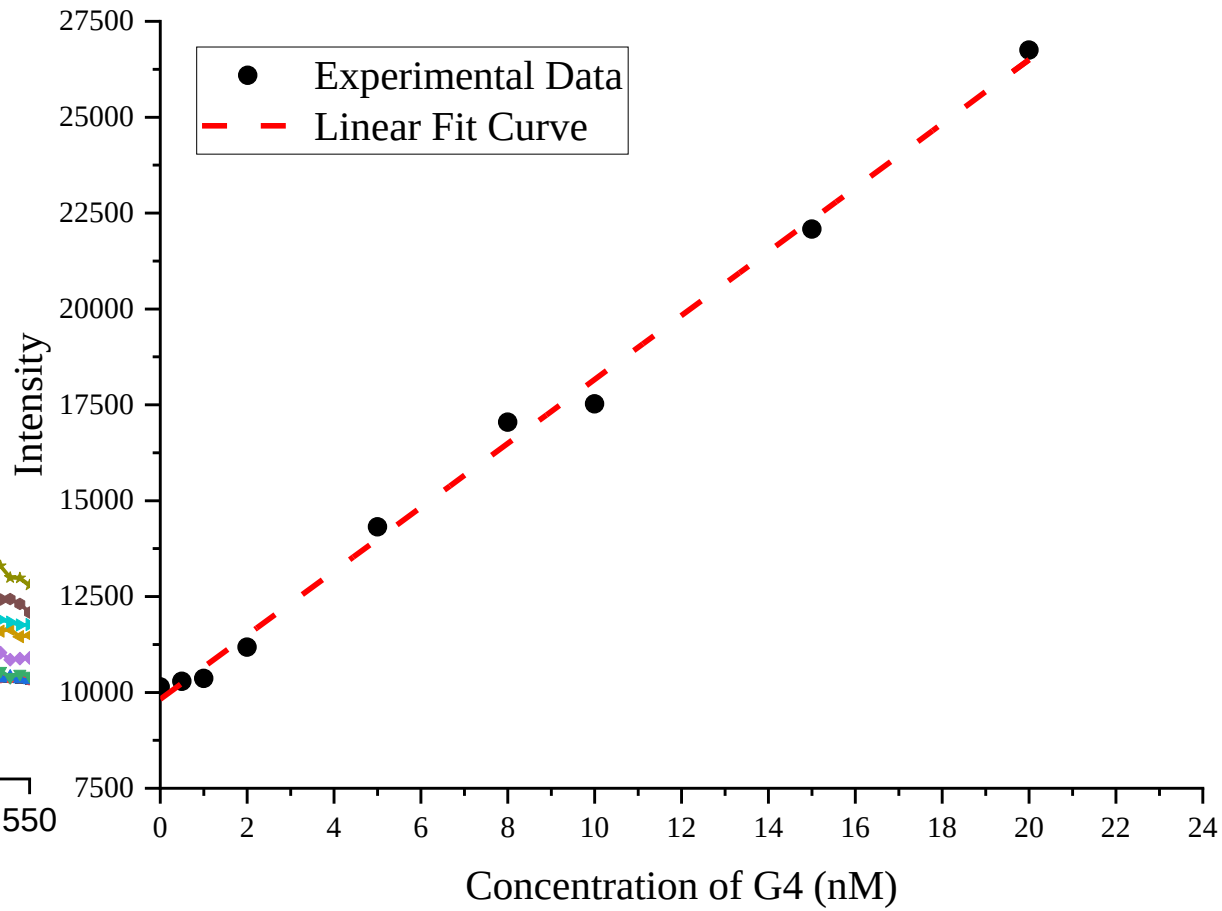
Fluorescence measurements on G-quadruplex DNA

Excitation: 425 nm
Emission: 490 nm

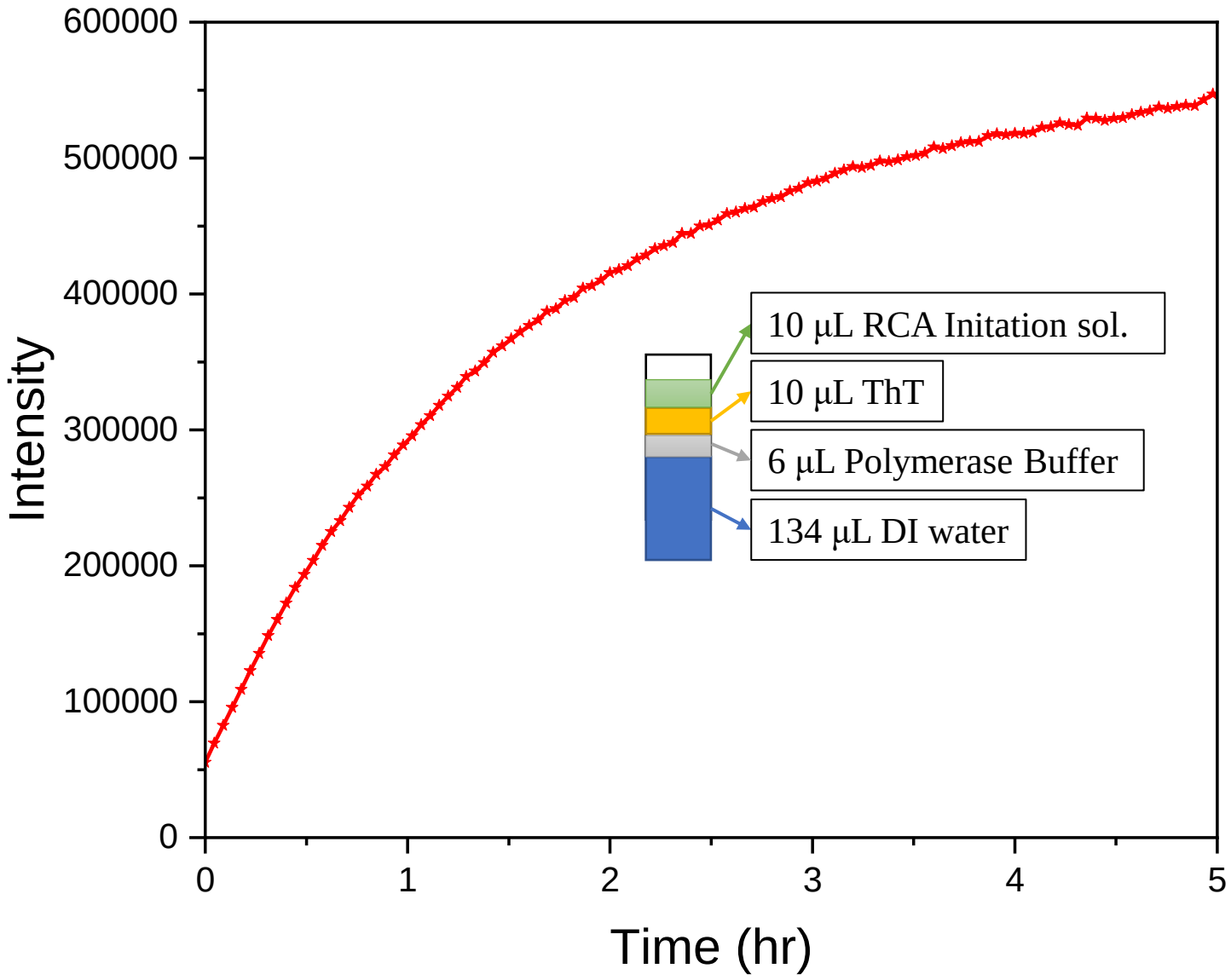
Concentration of ThioflavinT in all samples: 0.5µM
Concentration's of DNA used: 0.5nM, 1nM, 2nM, 5nM, 8nM, 10nM, 15nM and 20nM

Buffer Used: TAEM 6mM Mg





RCA reaction kinetics



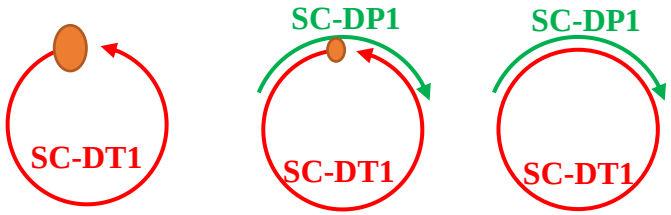
Details:

- RCA Initiation done in bulk and transferred to cuvette
- DNA amplification → Increase in G-quad → ThT binding to G-quad → Fl. increase

Excitation: 425 nm
Emission: 495 nm
Time monitored: 5 hrs
Instrument: Spectrofluorometer

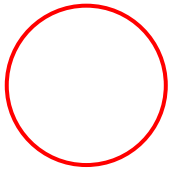
RCA Summary:

Phosphorylation of Linear Strand
Staple strand pairing
Ligating the circular strand



Initial Conc.: 20 µM

Purification of circular strand (dpage)
Ethanol concentration of DNA

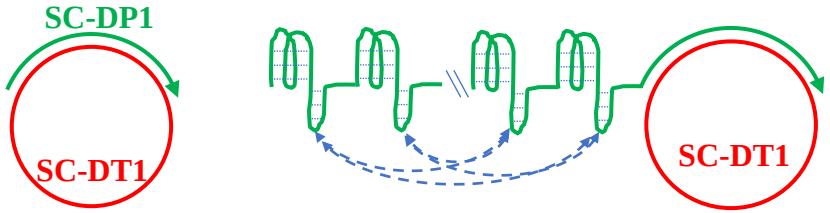


Critical Step:

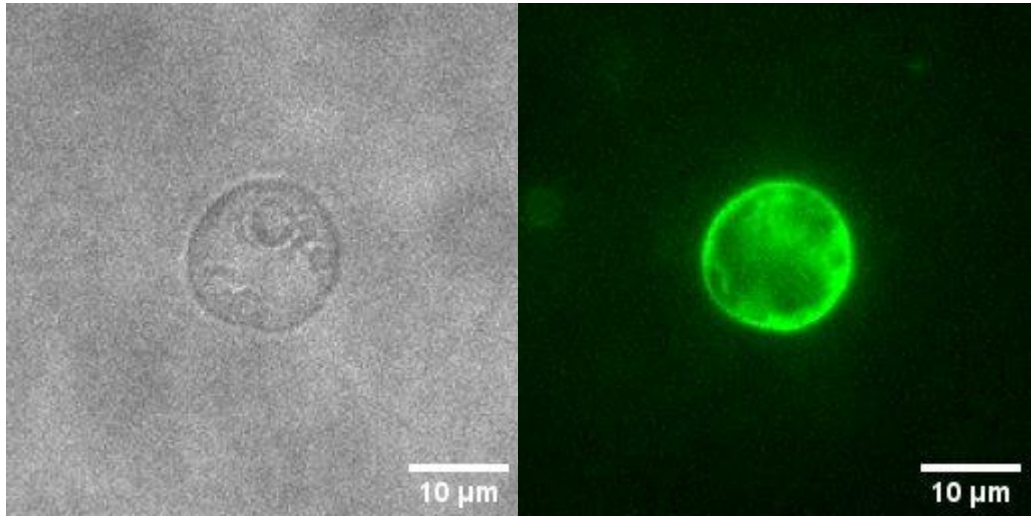
- Identifying band in gel.
- Extraction using electrodialysis.

Conc. after extraction: ~ 4.5 µM

Staple strand pairing
RCA



RCA in GUV



GUV Synthesis:

- Pipetting and centrifuging to make bilayer
- Time after reaction initiation = 10 min
- Flow in microchannel = 20 min