Measuring DNA tension effects on *in vitro* levels of gene expression

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Abstract

By controlling the DNA tension on magnetic bead-DNA molecules immobilized onto a surface, it may be possible to regulate the transcription process. In this study we focus on the generation of a method for artificial mechanical control of gene expression. We carry out a step-by-step procedure for the immobilization of a synthesized 8kb DNA onto a surface used for the quantification of RNA transcripts. We use polymerase chain reaction for designing a 8kb DNA template having a digoxigenin label at one end and a biotin label at the other end. We calculate the theoretical production of RNA and evaluate the possible methods of detection.

We present the final experiment consisting of the transcription reaction within the flow cell and discuss the initial studies on the effect of magnetic force upon tethered DNA-bead system.

1 Introduction

RNA polymerase has an essential role in the process to produce RNA from DNA, *transcription* and in the subsequent synthesis of proteins, *translation*. G*ene expression* consists of both transcription and translation processes.

RNA is synthesized in the 5` to 3`direction from the *template strand*. The other strand is the *non-template strand* having the same sequence as the new transcript but uracil is replaced by thymine. A polymerase binds to the 3' end of a gene or promoter on the DNA template strand and travels toward the 5' end.

Transcription can be divided into 3 stages: *initiation*, *elongation* and *termination.*

Initiation: in bacteria, transcription begins with the binding of RNA polymerase to the promoter in DNA forming a complex which is opened to initiate the transcription.

Elongation: one strand of DNA, the *template strand* is used as a template for RNA synthesis. As transcription proceeds, RNA polymerase traverses the template strand and uses base pairing with the DNA template to create an RNA copy.

Termination: It involves disassembly of the elongation complex and release of new transcript.Transcription is highly regulated and the initiation stage of the transcription has been show to be controlled by DNA tension.[1]

In vitro transcription and translation could be used to synthesize protein from *synthetic gene brushes*.[2] Gene brush assembly consists of dsDNAs attached at one end to a biochip surface. A DNA template was used encoding firefly luciferase as a gene reporter under control of a T7 promoter. At concentrated packing in a solvent genes turn out in a extended conformation. Hence, steric and electrostatic

forces affect the packing of the gene brushes. Gene expression and transcription reactions were carried out at different brush configurations by controlling the density of the genes. Buxboim et al[2] found that both the transcription/translation and transcription reactions were highly sensitive to brush density, conformation and orientation.

Our first motivation is to establish artificial mechanical control over *in vitro* gene expression. Hence, by controlling DNA tension on magnetic bead-DNA molecules immobilized onto a surface the transcription process could be regulated (see Fig. **1**). Our main interest is to create a method for artificial control of gene expression.

In this study, a step-by-step procedure of the DNA-bead tethering system used for quantification of RNA transcript is presented.

First, the design of the 8 kb DNA template for transcription using polymerase chain reaction (PCR) is discussed. Subsequently, this 8 kb DNA is labeled with a digoxygenin label at one end and a biotin label at the other end. Subsequently, a theoretical quantification of RNA production is calculated and the possible means of detection are evaluated and discussed. The feasibility of this DNA-bead tethering system in the flow cell is evaluated. Finally, the experiment consisting of the transcription reaction within the flow cell is discussed and the initial studies of the effect of magnetic force upon tethered gene brushes are discussed.

A more ambitious goal could be the invention of e.g. an artificial pancreas where the insulin gene can be switched on or off by regulation of the transcription

reaction using magnetic forces. This artificial pancreas would comprise a magnetic bead–DNA tethered system which has an insulin reporter gene.[3]

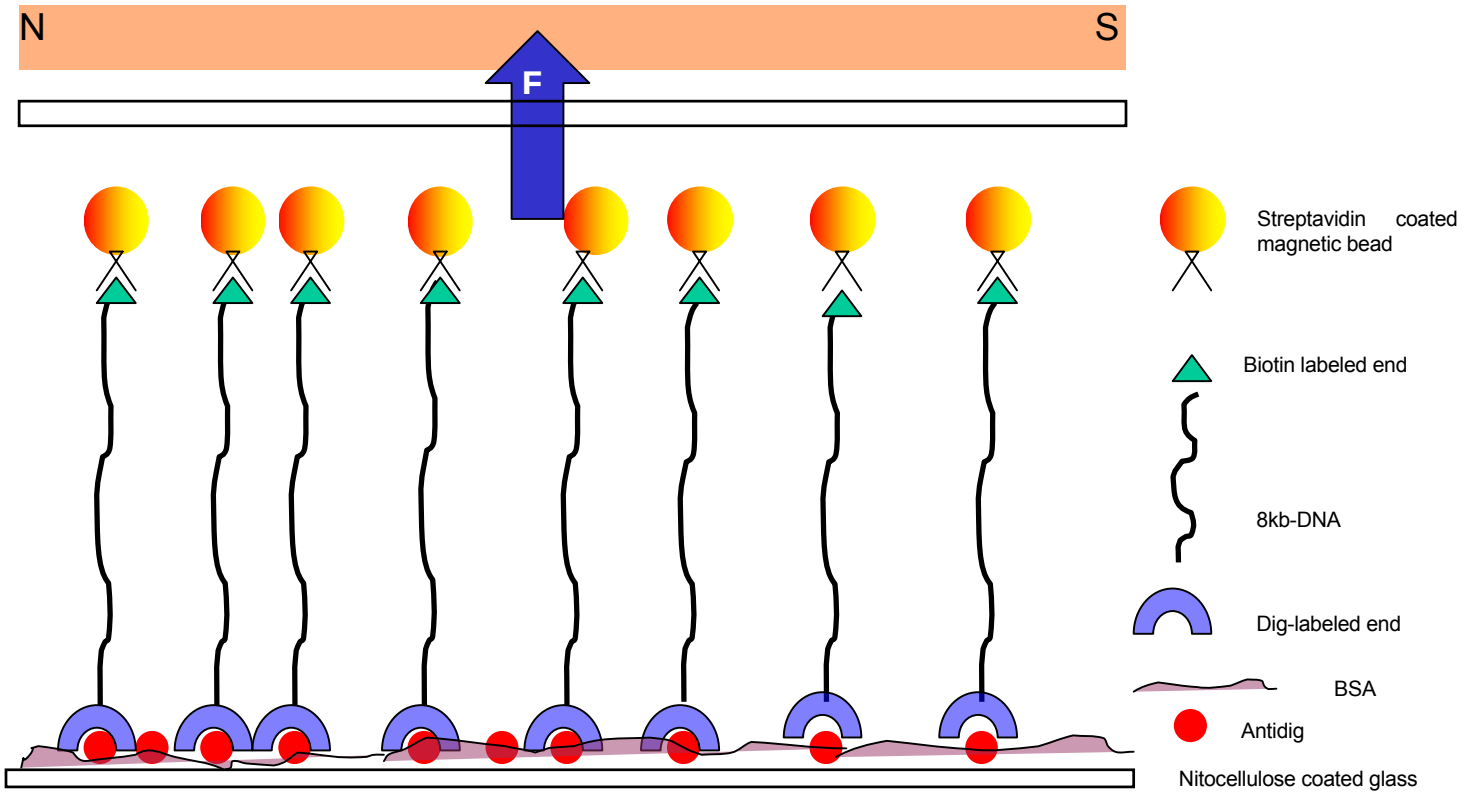

Fig. 1: Schematic representation of the set-up used

2 Materials and methods

2.1 Materials

The following compounds were used as received:

Phusion HF DNA polymerase and1x Phusion HF buffer (Finnzymes, Finland)

100% Dimethyl sulphoxide (Stratagene, The Netherlands)

Forward primer (Biolegio BV, The Netherlands)

Reverse primer (Peron Biotechnologies, Germany)

T7 DNA template (Sibgene)

PCR nucleotide mix (10mM Promega, The Netherlands)

Antidig (Roche)

BSA (Sigma)

Dynabeads ® M-280 Streptavidin (Dynal Biotech, Norway)

Trichloroacetic acid TCA (Sigma, The Netherlands)

ChromaTide. Alexa Fluor® 546-14-UTP (Invitrogen, The Netherlands)

Cy3-aminoallyl-UTP (Cy3-UTP) and Cy5 aminoallyl-UTP (Cy5-UTP) (GE Healthcare Europe, Belgium)

Buffers:

Phosphate buffer saline (pH 7.4)

Tris acetate EDTA buffer (pH 8.0)

MOPS-EDTA-sodium acetate (MESA) (1x MESA Buffer containing 40 mM MOPS, 10 mM sodium acetate and 1 mM EDTA, pH 8.3).

10 mM Tris EDTA buffer (pH 8.0), 200 mM NaCL (TE/NaCL Buffer).

2.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was used for the amplification of a T7 DNA template having a *Φ*13 promoter (Class III promoters) for T7 RNA polymerase in the nucleotide 27,273.[4] Thus, the transcription start site (the promoter sequence Φ13) was in the middle of the DNA template. The 8 kb PCR product was designed so as to have a biotin label on the 5' end of one strand and a digoxigenin (dig) label on the 5' end of the other strand (see Fig. 2).

Fig. 2: DNA construct designed by PCR

2.2.1 Primer Design

Table 1: Primer for PCR

Primer	***Seq 5' to 3'***	***5' Label***	***Length /nt***	***Tm/ºC***
Forward primer	Biotin-CACACCGAGAAGGTAATCAC	Biotin	20	60
Reverse primer	Dig-AGCGACTGAGTTGGAGAG	Dig	18	60

2.2.2 PCR program

The short program shown in Table 2 was chosen as a optimal for the production of a 8 kb PCR product using a standard Thermal Cycler (BioRad).

Table 2: Thermal cycle program for PCR

Stage	***Temp (C)***	***Time (mm:ss)***	***Reps***
1	98	00:30	
2	98	00:10	
3	58	00:10	
4	72	04:00	
5	Go to 2		29
6	72	08:00	
7	20	00:04	
End			

2.2.3 PCR mixture

The PCR mix shown in Table 3 was prepared in the order presented and mixed by pipetting several times. Phusion HF polymerase was added at the end and the mixture was again gentling mixed by pipetting several times (an ice bath was used to keep the enzyme at low temperature).

Table 3: PCR mix in order

Compound	*Volume(µl)*
MQ water	30
5x Phusion HF Buffer	10
10 mM dNTPs	1
Forward primer	1
Reverse primer	1
1 ng/µl T7 DNA	5
DMSO	1.5
Phusion DNA polymerase[a]	0.5

a: Polymerase is the last component to be added

The tube was centrifuged for 5 s at low rpm to sediment the whole mixture and thus the tube were ready to be put in the PCR machine.

The PCR program was run. After finishing the program, the tubes were centrifuge before cleaning up.

2.2.4 Cleaning Up

The PCR products obtained before were cleaned up by using the protocol for direct purification of the Nucleospin® Extract II kit, (Bioké).

2.2.5 Analysis

5 µl of the sample was analyzed by 0.7% agarose gel electrophoresis using a 2-log ladder (Biolabs).

2.3 Gel electrophoresis

5 µl of PCR samples and 1 µl of loading dye (Promega) per sample were analyzed by electrophoresis in 0.7% agarose gel containing 0.5 µg/ml ethidium bromide, in TAE buffer (pH 7.4). The gels were exposed to UV light for visualization of the

DNA present in the gels. A DNA marker of 10 kb (2-log ladder, Biolabs) was used as a control.

Five microliters RNA transcripts were analyzed by electrophoresis using 0.7 % agarose in 1xMESA / 1% formaldehyde as follows: One mililiter of formaldehyde was added to the agarose gel mixture in MESA buffer. The gel was poured into the electrophoresis vessel and allowed to cool.

The RNA samples and ladder (ssRNA ladder, Biolabs) sample were prepared by mixing 5 µl of RNA sample and 1 µl of RNA sample loading buffer. This mixture was heated at 65ºC for 10 minutes. The sample was centrifuged for 5s at 2000 rpm and left to cool on ice. The samples were loaded in the gel using MESA as a running buffer at 75 mA.

2.4 Nanodrop and microarray

A Nanodrop ND-1000 spectrophotometer instrument was used to measure the concentration of DNA or RNA (detection limit: 2 ng/µl).

The MicroArray module of Nanodrop was used to measure the absorbance of a fluorescent dye, allowing the determination of the dye concentration (detection limit: 0.2 pmol/µl). This equipment was kindly provided by Mr. Wilfred van Ijcken, EMC Rotterdam.

2.5 DNA tethering

Streptavidin magnetic microspheres (Dynabeads® M-280 Streptavidin) were tethered to a glass slide by 8 kb DNA having a biotin label in one end of one strand and a dig label at the other end of the other strand. The tethering procedure was as follows.[5]

A glass slide was covered with 0.1% of nitrocellulose in amyl acetate as follows: 5 µl of 0.1% nitrocellulose in amyl acetate was sprayed using a pipette tip onto the whole cover slip surface and left to air-dry.

A 50 x3 mm (150 x 10^6 μm^2) mould was used to prepare the parafilm flow cell. Three layers of parafilm were used to fabricate the flow cell and put in as a spacer between the 0.1% nitrocellulose bottom glass slide and a non-coated glass slide.

The following compounds were added in the below order (Table 4) into the flow cell and incubated for 15 min. Following addition of the Dynabeads the flowcell was incubated for a further 30 minutes. Fig. 1 shows schematically the layers on the cover slip.

Table 4: Flow cell tethering

Compound	***Concentration***
Reference beads solution	1 µl of tosylactivated polystyrene beads in 1 ml of PBS (pH 7.4)
Antidig	0.1 mg/ml PBS (pH 7.4)
BSA	1 mg/ml PBS (pH 7.4)
DNA solution	5 –0.5x10^{-3} ng / µl
Dynabeads ® M-280 Streptavidin	10µl of magnetic beads/100 µl of TE/NaCl buffer[a]

a: Magnetic beads were previously three times washed in 100 µl of PBS (pH 7.4).

Both before addition of the anti-dig solution and BSA the flow cell was washed with 450 µl of PBS to wash away the excess of reference beads and the unbound anti-dig, respectively. Before the addition of DNA and magnetic beads the flow cell was

washed with 450 µl of TE/NaCl to remove free BSA and free DNA, respectively. Free DNA should be removed to prevent sticking together of the magnetic beads. After incubation with magnetic beads the flow cell was washed with aprox. 1 ml of TE/NaCl buffer to remove free magnetic beads.

2.6 Magnetic Tweezers

Once the DNA is tethered within the flow cell, the DNA molecules were kept under tension by the field gradient of two small permanent magnets place above the sample in a custom-built magnetic tweezers apparatus[6] The magnetic tweezers were used to determine the number of tethers per flow cell in the flow cell against the concentration of DNA and the force-extension curve of the DNA. After preparing the DNA-bead tethered system as described in the previous section at DNA concentrations of 0.5, 0.05, 0.005 and 0.0005 ng/µl, the tethers were counted on 10 random screens at the same magnet position (0.2 mm of the surface). The pixel resolution of the screen was 484 x 648 and the cell size of 0.09 µm x 0.09 µm. So, there are circa 2,500 μm^2 per screen. The tether's number was the average of the 10 screens for each DNA concentration.

For the final experiment, 6 rectangular magnets (40x25x10) were kept above the flow cell during the transcription reaction to generate a high magnetic force along the whole flow cell as shown in Fig. 1.

2.7 Transcription reaction

The following reagents were added to a 1.5 ml eppendorf tube (RiboMAX™ Large Scale RNA production systems-T7 kit from Promega):

Table 5: Transcription reaction mix (in order)

Compound	*Volume (µl)*
5xTranscription Buffer	5
DNA template	5
10 mM rNTPs	2.5
T7 RNA polymerase	2.5
Nuclease-free water	10

The reaction mix was gently mixed by pipetting and incubated at 37ºC for 2 hours. After the transcription reaction was completed, 1 µl DNase was added and incubated for 15 min at 37ºC. The whole reaction was purified using the Qiagen kit for RNA clean up.

2.8 Transcription and Labelling of RNA

2.8.1 Transcription reaction in bulk

The following reagents were added to a 1.5 ml eppendorf tube (Megascript T7 kit from Ambion):

Table 6: Transcription mix for labeling

Compound	*Volume(µl)*
25 mM of rNTPs (ATP/CTP/GTP)	3
50 mM UTP	1
10xTranscription Buffer	1
Nuclease-free water	2
Cy3-UTP(GE healthcare)	3
DNA template	2.5
RNA polymerase (Enzyme mix)	1.25

These reagents were gently mixed and incubated at 37ºC for 2. After the transcription time was run, 1µl of Dnase/10 µl of reaction was added and incubated at 37ºC for 15 min.

The whole aliquot was purified using the Qiagen kit for RNA clean up.

2.8.2 Transcription reaction in flow cell

The flow cell was made following the instructions detailed in section 2.5.

For reactions conducted within the flow-cell a larger master-mix was made with the same relative proportions of reagents shown in Table 6 and stored on ice until use. When required, 1.25 µl of T7 RNAP mix/10 µl of master mix was included in the reaction prior to introduction into the flow cell.

The entire flow cell was incubated at room temperature for 2 hours. After recovery of the flow-cell contents, 1µl of Dnase/10 µl of reaction was added and incubated at 37ºC for 15 min. An aliquot of 20 µl was purified using the Qiagen kit for RNA clean up

2.8.2.1 Potential conditions to increase the RNA production in the flow cell

The DNA concentration for incubation was increased to ~5 ng/µl, in order to increase the total number of template molecules, thereby, in theory, increasing the amount of RNA product produced.

RNase inhibitor was added to the master mix (SUPERase•In™, Ambion), 1 µl per 50 µl of transcription reaction in order to reduce the chance that RNA degradation by RNAse was responsible for possible negative results.

A new flow cell was used for each transcription reaction. This was done in order to eliminate the possibility that repeated flushings of the flow-cell may be removing the tethered DNA from the flow-cell surfaces.

The transcription reaction was incubated instead of at room temperature at 37ºC in a Merck incubator in the RNAlab and for 4 hr. The increased temperature and reaction time should also result in an increased production of RNA products.

2.9 Recovery and purification of the product from the flow cell

2.9.1 Direct Recovery and fluorimetric analysis of labelled RNA

Before using the pump and tubing for running a transcription reaction, RnaseZap (Ambion) was flushed through for Rnase decontamination. The tubing was subsequently washed afterwards with MQ water.

A peristaltic pump with changeable pump directions was used for the recovery approach as follows: 50 µl of transcription reaction was pumped into the flow cell using a small adapter as a reservoir. Once the transcription reaction was pumped, a female to male adapter with tubing was inserted in this reservoir (some water added in the reservoir to form an outward meniscus to prevent air bubbles entering into the flow cell) and the direction of the flow was changed using the pump. Since water is added during the insertion of the adapter, a subsequent dilution of the transcription samples is expected during the recovery stage. MQ water was used to push the transcription product out of the flow cell. Thus, 100 µl of product was recovered after 2 hours of incubation.

1µl of Dnase/10 µl of reaction was added and incubated at 37ºC for 15 min. An aliquot of 20 µl was purified using the Qiagen kit for RNA clean up

A 1xbromophenol blue dye solution (0.01% bromophenol blue, 0.25% EDTA, 50% glycerol) was used to determine the factor of dilution of the recovered solution (see Appendix 1).

2.9.2 Trichloroacetic-Acid precipitation, filtering and fluorescence scanning

Both the entire flow cell contents and the washing solutions of the flow cell with 500 µl of MQ water and 500 µl of TCA were collected and added to 5 ml cold TCA. 50 µl of BSA were added to enhance the precipitation of the polymers.

The entire solution was passed through a glass filtration apparatus (Sigma) having Whatman 3MM filter paper and a fritted glass as filter support.

The precipitated material was retained on the filter paper which was then washed twice with 10% TCA, once with 50% ethanol: 50% diethyl ether and once with 100% diethyl ether to displace any remaining aqueous solution before air-drying of the filter papers. The filter papers were then analyzed using a Typhoon fluorescent scanner for measuring the presence of fluorescent labeled RNA. A series of bulk transcription reactions at different DNA concentration were run and TCA precipitated in filter paper for control and quantification purposes.

2.10 Typhoon and microarray scanner

Typhoon fluorescent scanner can detect fluorescent images from fluorescent dyes containing samples. The light is emitted after excitation from the fluorescent-labeled sample in proportion to the amount of labeled molecule or dye in the sample. The emitted light is collected and converted to electrical signal. This electrical signal is digitalized for image display. So that Typhoon was used to detect the fluorescence of the RNA transcript. The detection limit is approx. 200 amol of fluorescent dye in the nucleotide.[7]

The scan Array Express HT (Microarray scanner, Perkin Elmer) use confocal optics to enhance the quality of the imaging. This microarray scanner was used to visualize the samples spotted with buffer on a chip. The detection limit of the Miroarray scanner is less than 0.1 fluorescent molecule per um^2.

This equipment was kindly provided by Mr. Wilfred van Ijcken, EMC Rotterdam. I would like to thank Mr. Wilfred van Ijcken for his much appreciated assistance in fluorescence scanning measurements

3 Results and discussion

3.1 Production of eight kb DNA template for transcription by PCR

As already mentioned in the introduction our main goal is to invent a method for artificially controlling gene expression. One of the means to achieve this goal is to investigate the effects of the *force* on the production of *RNA* by stretching a *DNA* molecule which is tethered to a glass surface via dig-antidig bond and to a microsphere *via* a biotin-streptavidin bond.

Thus, a DNA construct needed to be designed for this purpose (see Fig. 1 and Fig. **2**). For this reason, PCR was used to amplify a T7 DNA template having a *Φ*13 promoter in the middle of the construct. A biotin labeled forward primer and a dig-labeled reverse primer were used in PCR. Bacteriophage T7 DNA was chosen because of consisting of only a single subunit and the knowledge of its complete sequence.[4]

Fig. 3 shows an agarose gel electrophoresis of the product obtained by PCR. The lane PCR shows clearly a 8 kb DNA fragment which has a biotin label on the 5' end of one strand and digoxigenin (dig) label on the 5' end of the other strand.

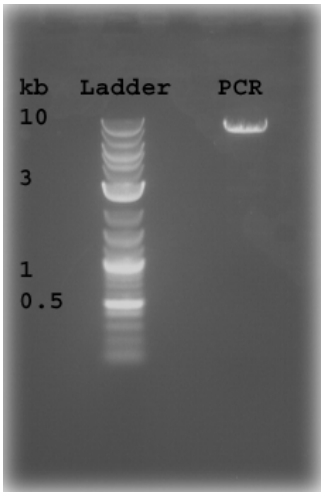

Fig. 3: Agarose gel electrophoresis of the PCR product.
The lane PCR shows a 8kb-PCR product.

3.2 Testing of production of 4.0 kb RNA transcript in bulk transcription reaction

Once the DNA construct was synthesized by PCR, the next step was to transcribe DNA into RNA in bulk. As expected, a 4 kb transcript can be observed in the lane T of Fig. 4. Therefore, a 8 kb PCR product having a promoter in the middle of the construct produced a 4 kb transcript in a transcription reaction in bulk.

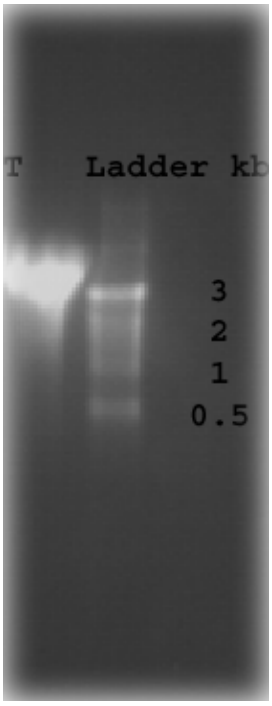

Fig. 4 RNA denaturing agarose gel electrophoresis
The lane T shows a 4kb transcript

3.3 Theoretical quantification of RNA production

The transcription process produces 22 transcripts per DNA molecule and per minute. This value is calculated based on the assumption that one DNA molecule produces one RNA molecule every 3 seconds.[1]

Let´s calculate the RNA concentration necessary to be detected by Typhoon Scanner, assuming that only one fluorescent nucleotide is present per 4 kb and 30 min of transcription reaction time.

$$\text{RNA molecules produced in 30 min per DNA molecule} = \frac{1\,\text{RNA molecule}}{\text{DNA molecule x } 3s} x \frac{60s}{\text{min}} x30\,\text{min} = 600$$

$$\text{RNA molecules needed to be detectable by Typhoon scanners} = 200x10^{-18}\,\text{mol of RNA}\,\frac{N_A}{\text{mol of RNA}} = 120\text{x}10^{6}$$

where N_A is Avogadro´s number

$$\text{DNA molecules needed in the flow cell} = \frac{120x10^{6}\text{RNA molecules}}{600\ \text{RNA molecules}} x\ \text{DNA molecules} = 200{,}000\ \text{DNA molecules}$$

$$\text{Mol of RNA needed to be detectable} = 200000\ \text{DNA molecules x} \frac{600\ \text{RNA molecules}}{\text{DNA molecule}} x \frac{1\ \text{RNA mol}}{N_A} x$$

$$\frac{1}{50\mu l\ \text{of solution}} x \frac{1\ ul}{1x10^{-6} l}$$

$$= 4\ \text{pM}$$

where N_A is Avogadro´s number

Hence, we expected a RNA concentration of about 4 pM.

A gel electrophoresis of serial dilutions of 4 kb transcripts obtained in bulk is shown in Fig. 5. By a normal ethidium bromide staining, the RNA detection limit is about 800 pM, very far away, circa 100 orders of magnitude from the expected value of 4 pM. Thus, gel electrophoresis with ethidium bromide staining can not be used for the determination of the amount of RNA produced in our flow cell.

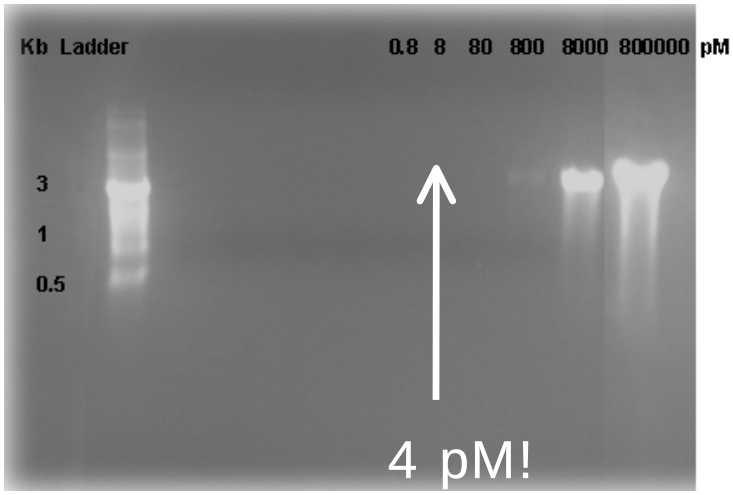

Fig. 5: RNA denaturing agarose gel electrophoresis,
The lanes indicate 4-kb RNA product at different concentrations. From right to left the concentrations are 800000, 8000, 800, 80, 8 and 0.8 pM. The arrow indicates the localization of the expected RNA product.

Since ethidium bromide staining can no be used to detect the presence of RNA in our system, another kind of labeling needs to be considered. Fluorescence labeling was the alternative approach to label the RNA transcripts in order to be used in the fluorescence scanners

3.4 Fluorescence labeling of RNA in bulk

Once we had identified the need to incorporate fluorescent nucleotides in the RNA, we investigated using the following commercially available nucleotide analogs, that are compatible with the Typhoon imager: ChromaTide Alexa Fluor® 546-14-UTP, Cy3-aminoallyl-UTP (Cy3-UTP) and Cy5 aminoallyl-UTP(Cy5-UTP). After several transcription tests with these three fluorescence nucleotides, Cy3- UTP, was chosen for further labeling because its higher incorporation efficiency presumably due to its higher hydrophilicity. Cy-dyes have also higher sensitivity[8] that make them more suitable to be used in different kind of fluorescent scanners.

Base/dye ratio for Cy3 labeled RNA

The ratio of bases to dye incorporated in the RNA molecule can be determined by using the values of the absorbance obtained from the Nanodrop-microarray spectrophotometric analysis:

Table 7: Spectrophotometric values for Cy3 dye and ssRNA

Dye	***Wavelength at maximum absorbance / nm***	***Extinction Coefficient ε / $cm^{-1}M^{-1}$***	***Dye correction factor CF***	***MW_{base}***
Cy3	550	150000	0.08	1296
ssRNA	260	8250	-	320

Table 8: Values obtained from spectrophotometric measurements of 10-fold diluted fluorescent RNA at different Cy3-UTP concentrations

Sample	***Cy3-UTP stock solution/ mM***	***A_{260}***	***A_{dye}***	***Concentration / ng/µl***	***Dye concentration/ pmol/ µl***
1	5.0	0.985 ± 0.003	2.195 ± 0.003	373	146 ± 0.02[b]
2	1.5	1.102 ± 0.003	0.182 ± 0.003	441	12 ± 0.02
3	0.15	1.605 ± 0.003	0.157 ± 0.003	640	10 ± 0.02
4	0.015	2.039 ± 0.003	0.047 ± 0.003	814	3 ± 0.2
5	0.0015	1.608 ± 0.003	0.031 ± 0.003	642	2 ± 0.2
Control 1[a]	1.5	0	0	0	0

a: A transcription reaction without template was used as a control of the reaction.
b: This concentration was above the upper detection limit of the Nanodrop (100 pmol/ µl) for Cy3 dyes.

The absorbance of the RNA can be calculated using the values from the Table 7 and Table 8, including the correction factor of the dye for its absorbance at 260 nm, in the following equation[9]

$$A_{base} = A_{260} - A_{dye}\, CF_{260}$$

Thus, the base to dye ratio can be calculated using the following equation:

$$\frac{base}{dye} = \frac{A_{base}\, \varepsilon_{dye}}{A_{dye}\, \varepsilon_{base}}$$

Using a 5 mM stock solution of Cy3-UTP as purchased, the base to dye ratio of the transcript produced, sample 1, was 7 (see Table 9). This ratio is more than 2 order of magnitude higher than the assumed value of 4000 bases per dye for theoretical quantification of detectable concentration of RNA by fluorescent

scanners and so the concentration of Cy3-UTP in the reaction was reduced. Serial transcription reactions at different Cy3-UTP concentrations ranging from 5 to 0.0015 mM were prepared. The spectrophotometric data obtained is shown in Table 8.

From the starting site of RNA (nucleotide 27,273) to the end nucleotide of the RNA (nucleotide 31,323) of the coding strand of the T7 genome, 909 thymine nucleotides which are substituted for uracil in RNA, were counted from 4050 bases. Hence, the minimal base to dye ratio assuming that all of the UTP were Cy3-UTP is 4050/909 = 4. Thus, the base to dye ratios of the Table 9 are in agreement of the expected values.

Table 9: Base to dye for Cy3-labeled RNA

Sample	***Cy3-UTP stock solution/ mM***	***Base/dye***
1	5.0	7
2	**1.5**	**109**
3	0.15	190
4	0.015	787
5	0.0015	941
Control 1	1.5	-

Reaction 2 and 3 were selected as optimal conditions for the incorporation of fluorescence nucleotides into the RNA transcript due to their high base to dye

ratios. We decided to use the reaction conditions number 2 for our subsequent experiments.

In order to determine the detection limit of the microarray scanner, serial dilution of the fluorescent nucleotide at 5mM Cy3-UTP were make and spotted onto a glass slide. Fig. 6 shows that a microarray scanner can be used to determine the concentration of the transcripts produced within the flow cell. A microarray scanner can easily detect the minimal concentration of transcripts expect in the flow cell.

The concentration of the 10 fold diluted sample 2 in pM was calculated as follows:

$$pM \text{ of Cy3-labeled RNA} = \frac{\approx 400\ ng}{\mu l} x \frac{1x10^{-9}\ g}{1\ ng} x \frac{1\ pg}{1x10^{-12}\ g} x \frac{1\ pMol}{320\ pg} x \frac{1}{4000\ b} x \frac{1\ \mu l}{1x10^{-6}\ l} \approx 300$$

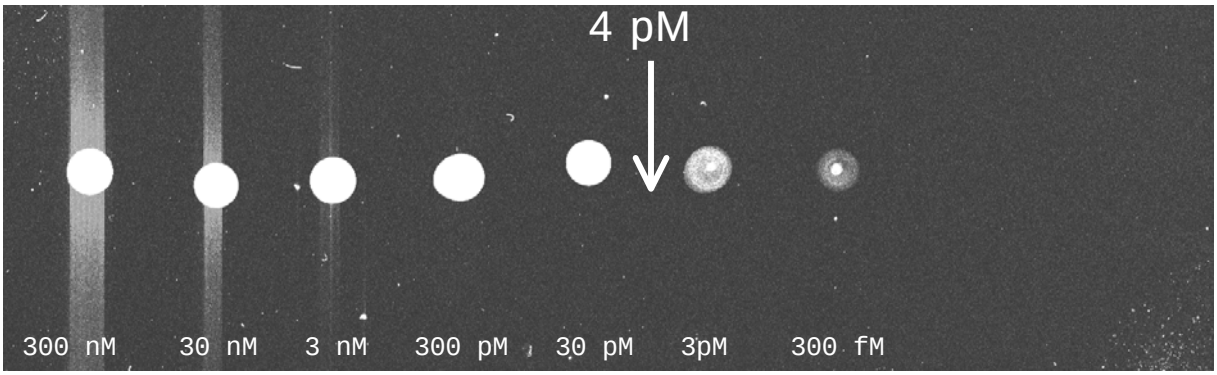

Fig. 6: Microarray scan of serial dilutions of Cy3-labeled RNA transcripts

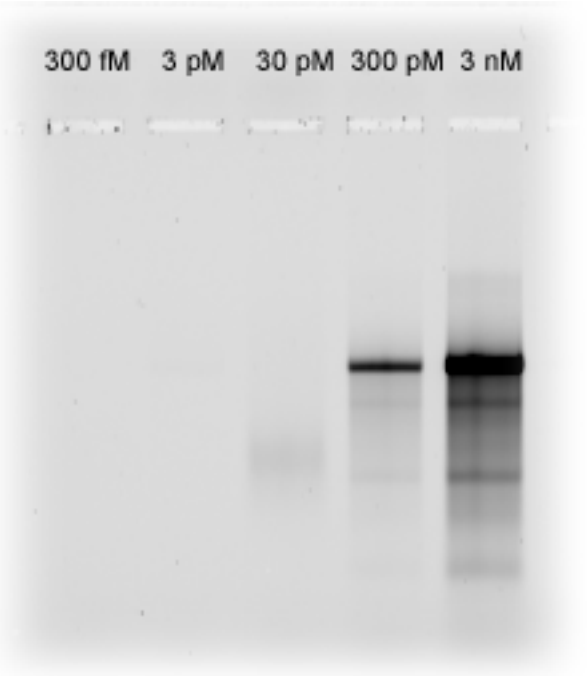

Fig. 7: Typhoon gel scan of the fluorescent RNA transcript at different concentrations
The lanes indicate Cy3-labeled RNA transcripts a different concentrations. From right to left the concentrations are 3nM, 300 pM, 30 pM, 3 pM, and 300 fM.

The Typhoon scanner can be used to scan agarose gels and so Fig. 7 shows a gel scan using typhoon scanner at serial dilutions of cy3-labeled RNA. This scan reveals that the RNA produced is indeed fluorescent and that using gels the detection limit of the typhoon scanner is around 300 pM of fluorescent RNA.

3.5 DNA- Bead tethering

Fig. 8 shows the plot of the tether's number per flow cell against the DNA concentration following the Nitrocellulose approach (see section 2.5) for DNA tethering within the flow cell. Taking into account the detection limit of the microarray scanner and the RNA being produced in the flow cell, we estimated

that at least 200,000 (see Section 3.3) promoters or DNA molecules in the flow cell are needed to be detectable by fluorescent scanners.

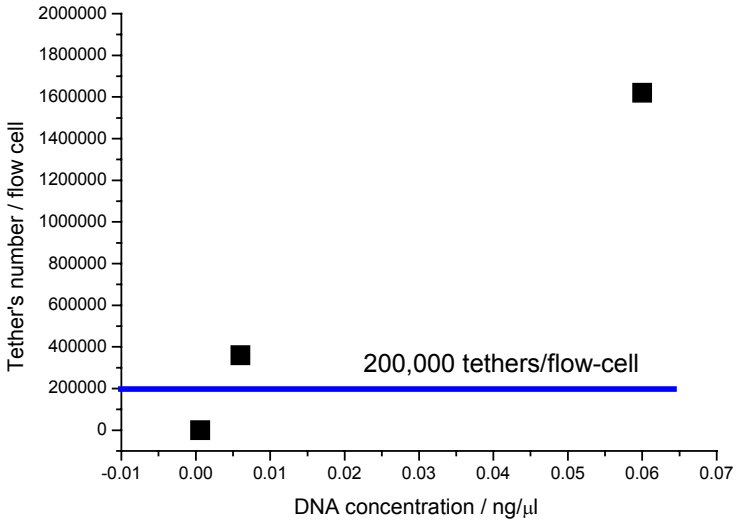

Fig. 8: Tether's number per flow cell against DNA concentration in the flow cell.

Hence, a DNA concentration of 0.005 ng/µl for DNA-tethering was found to be suitable for detection using the by fluorescence scanners.

3.6 Stretching of tethered DNA

The force-extension curve of the tethered 8 kb DNA (see Fig. 1) at 0.0005 ng/µl can be observed in Fig. 9.

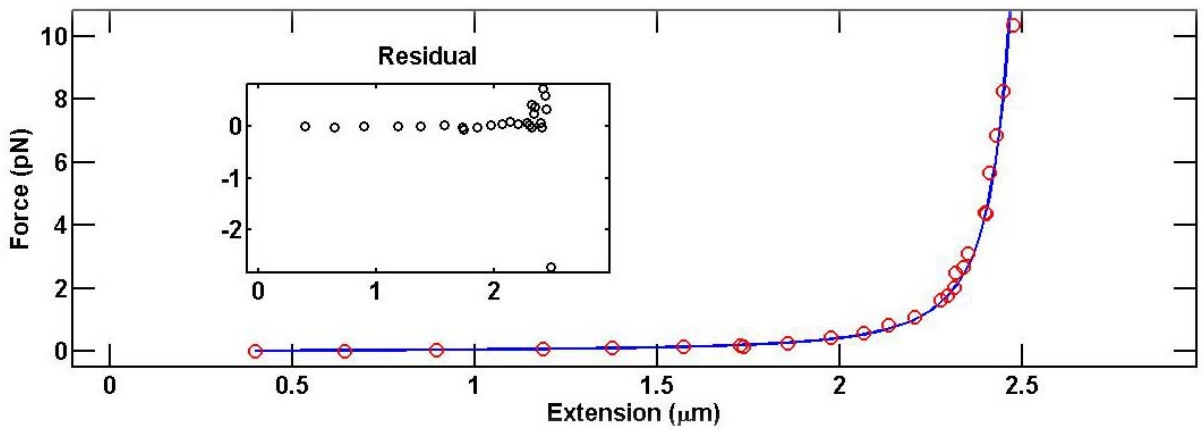

Fig. 9: Force-extension curve of 8 kb DNA

The expected contour length was 2.7 μm and the measured contour length was 2.58 ± 0.1 μm for 6 tethered DNAs. The average persistence length was 48.54 ± 6.71 nm.

3.7 Transcription reaction in the flow cell, initial studies of the effect of force upon tethered Gene-Brushes

The following tethering conditions within the flow cell for transcriptions reactions in the same flow cell were used:

Transcription reaction R1: No enzyme and no magnetic beads

Transcription reaction R2: Enzyme and no magnetic beads

Transcription reaction R3: Enzyme and magnetic beads

The concentration of DNA in each case was 0.005 ng/μl.

The *direct recovery* (see section 2.9.1) for the recovery and purification of the products was used and a microarray scanner was used to visualize the samples spotted on a chip.

No fluorescence spots were observed in these three samples and in particular no fluorescent RNA was observed in the transcription reactions 2 and 3. This implies that currently, in our flow cell, no RNA is produced or if RNA is produced, it remains contained inside the flow cell. A further possibility is that the amount of RNA in fact produced is below the detection limits of our current methods.

In order to address the apparent lack of RNA product during the first tests of the flow-cell experiment, a few possible options were explored such as increasing DNA concentration, using RNase inhibitor, using a new flow cell per experiment, increasing the temperature and increasing incubation time (for more details see section 2.8.2.1)

Additionally, we decided to explore an alternative method for collection of the fluorescent RNA products for subsequent analysis, trichloroacetic-acid precipitation (see section 2.9.2). This method is based on the principle that polymers of RNA will precipitate in a suitable acid, whereas unincorporated nucleotides remain soluble. This is a standard biochemical technique that has been used in the past to measure the incorporation of tritiated nucleotides by T7 RNAP.[10] In our case, we monitored for the incorporation of acid precipitable fluorescence, by fluorescence scanning.

Transcription reactions were then separately run at a DNA concentration of around 5 ng/uL, with 1 µl of inhibitor per 50 µl of transcription reaction, and at 37ºC for 4 h. The transcription product was recovered and purified according with the trichloroacetic-acid precipitation approach (see section 2.9.2). Fig. 10 shows a scan picture of the bulk transcription reaction at DNA concentrations ranging from 50 to 0.0005 ng/ µl. The samples A and B show clearly decrease of the fluorescence by decreasing the DNA concentration.

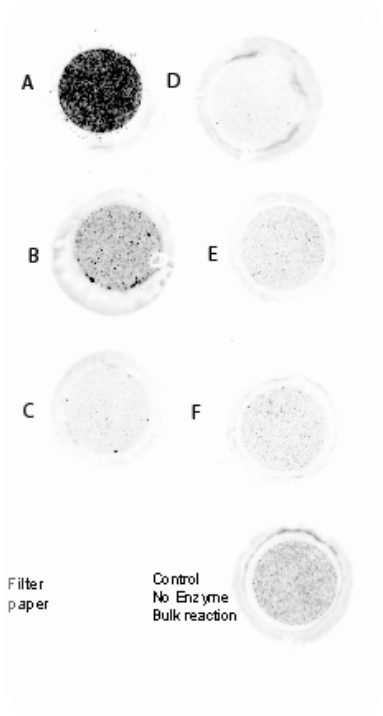

Fig. 10: Typhoon scan picture of bulk transcription reactions
Sample A: Bulk transcription reaction with a DNA concentration of 50 ng/ µl
Sample B: Bulk transcription reaction with a DNA concentration of 5 ng/ µl
Sample C: Bulk transcription reaction with a DNA concentration of 0.5 ng/ µl
Sample D: Bulk transcription reaction with a DNA concentration of 0.05 ng/ µl
Sample E: Bulk transcription reaction with a DNA concentration of 0.005 ng/ µl
Sample F: Bulk transcription reaction with a DNA concentration of 0.0005 ng/ µl
Control: Bulk transcription reaction without adding enzyme

The typhoon scans of the samples R1, R2, and R3 can be observed in Fig. 11. Transcription reactions R1, R2, and R3 were run within the same flow cell, washing out the DNA with 10 mM TE/ 200 mM NaCl and/or 100 µl of transcription buffer (if the transcription reaction is the following step) before adding the following compounds. The reaction without the addition of RNA polymerase was taken as a negative control for the transcription. No significant difference can be seen between the reaction R1 and the negative control reaction.

One of the possible explanations of having a negative control which yields positive results could be due to the leakage loss of the sample in the filter apparatus used as it can be observed in the most of the filters of the Fig. 10 and Fig. 11. Because the sample is passing out of the filter during the filtration, the vacuum did not pull the liquid through the funnel and so the sample is air dried onto the filter surface. This drawback happens when thick filters are used as in our case as Millipore claims. Millipore recommends to use Durapore or Teflon membrane filters for solvent applications. [11] In the future, both filters should be tested for being used as filters for TCA precipitation. Besides, perhaps the vacuum needed to filter the samples after the precipitation by TCA could be produced using a vacuum pump instead of a water trap. Ethanol/diethylether and diethylether rinsing can be done in a beaker using tweezers.

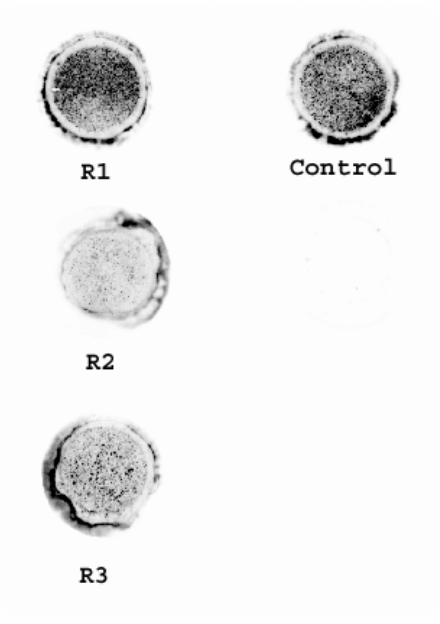

Fig. 11. Typhoon scan pictures of the transcription reaction in the flow cell
R1: Transcription reaction within the flow cell with 4 ng/µl of DNA
R2: Transcription reaction within the Flow cell with 4 ng/µl of DNA and magnetic beads
R3: Transcription reaction within the Flow cell with 4 ng/µl of DNA magnetic beads and magnetic force
Control: Control transcription within the flow cell with 4 ng/µl of DNA and no addition of enzyme.

On the other hand one of the possible reasons for the low or any amount of RNA produced is the direction of the moving of the transcripts in the designed construct relative to the nitrocellulose-coated surface.

If we consider in more detail the DNA molecule used for tethering in the flow cell as shown in Fig. 1 and Fig. 2, having the promoter Φ13 in the coding strand of the DNA, we discovered perhaps that the DNA tether within the flow cell could be oriented in such a way as to severely restrict the production of surface-released mRNA. The RNA start site is in the nucleotide 27,273. The RNA is synthesized from Biotin-5´-end to 3´-end. Hence, the transcripts are synthesized in the direction

of the bottom of the flow cell, which could create a barrier for the production of the new transcripts once the first one is attached to the bottom as shown schematically in Fig. 12. Although using TCA to flush the remnant transcripts in the flow cell, the production of RNAs could have been affected by the orientation of the promoter in the construct. Most of the experiments realized so far for transcription reaction in single molecule using biotin-labeled reverse primers where the promoter is top-oriented relative to the surface of the flow cell.[12] Buxboim et al showed in gene brushes marked differences between biosynthesis done at different gene localization and orientation. They claimed that brush structure is important for the expression efficiency of genes. They suggested that these marked differences between bottom and top configurations are due to changes in the local concentration of RNA polymerase near the surface and at the top or interface with the solution.

Nord et al[13] investigated gene expression in PCR products anchored to Streptavidin-coated microspheres. They found that the efficiency of gene expression was dependent of the directionality of the transcription/translation machinery relative to the bead surface. The configuration with the movement towards the bead was the most favorable one. They did not discuss clearly the reason for this finding but they did point out the relevance of that directionality and laid the debate on the table.

Therefore, the question about the relevance of the orientation of the genes in the configuration is not a trivial issue.

One way to demonstrate if this hypothesis is correct is to repeat the same procedure of transcription in the flow cell using a DNA produced by PCR using the same sequence for the primers as shown in Table 1 but with contrary labels. Therefore, a digoxigenined label forward primer and a biotinylated reverse primer would be used as a primer for the PCR.

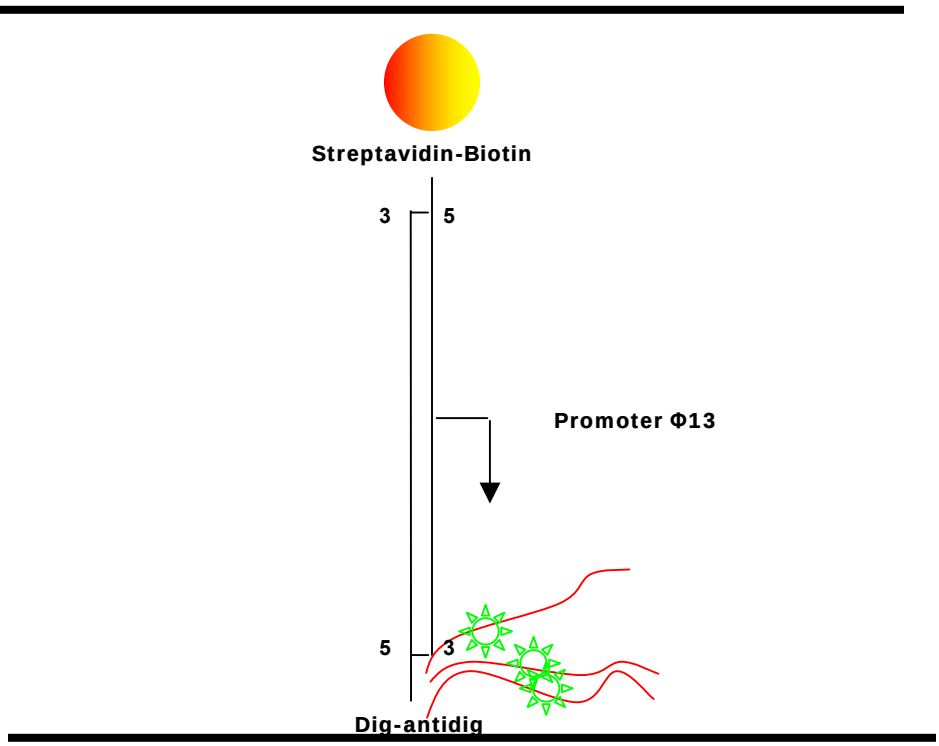

Fig. 12.: Scheme of the DNA tether
The DNA construct used in this study is represented. The promoter is bottom-oriented

4 Conclusions and Outlook

In this six-month research study it was shown the synthesis by PCR, tethering within the flow cell and stretching by magnetic tweezers of dig and biotin label 8 kb PCR product.

This dig and biotin 8 kb DNA construct was used as a template for the fluorescent labeling transcription in bulk. So Cy3-labeled 4kb transcripts were produced in bulk containing a fluorescent dye every 109 bases.

Microarray scanners were chosen as a suitable detection technique for the calculated low concentration of RNA product because of high resolution scanning.

The results obtained so far are satisfying in view of the limited time frame of this internship. Although the final aim of this study, the invention of a mechanically controlling gene expression device or method, was not achieved a sequential procedure was carried out to enhance the chances of success in the achievement of the goal. Besides, alternative approaches for future work were suggested such as the review of the filter used for the recovery of the transcripts and the design of a new DNA construct having a top-oriented promoter.

APPENDIX 1

In brief, the concentration of a compound is usually determined by measuring the absorbance (A) of the compound at a wavelength using the Beer-Lambert law:

The Beer-Lambert law[14] is:

$$A_\lambda = \varepsilon l C \qquad \text{Eq. 1}$$

where ε is the molar absorption coefficient or molar extinction coefficient ($M^{-1}cm^{-1}$), l is the light path length (cm) and C is the compound concentration (M).

The Beer-Lambert law establishes that the absorbance of a compound dissolved in a given solvent and measured at a given wavelength is proportional to the path length and the concentration of the compound. The constant of proportionality is the molar extinction coefficient.

In our strategy to recovery the product of the transcription within the flow cell, we were interested in the degree of dilution of the assay mix during the flow-cell recovery process. For this reason, a solution of bromophenol blue dye was used to monitor the recovery of 50 µl volume in the flow cell.

A peristaltic pump with changeable pump directions was used for the recovery approach as follows: 50 µl of dye solution with an absorbance of 4.973 at a wavelength of 590 nm was pumped into the flow cell using a small adapter as a reservoir. Once the dye was pumped, a female to male adapter with tubing was inserted in this reservoir (some water is added in the reservoir to form an outward meniscus to avoid air bubbles coming into the flow cell) and the direction of the flow was changed in the pump. Since water is added during the insertion of the adapter, a dilution is expected in the recovery approach. MQ water were used to

push the dye solution out of the flow cell. Thus, 100 ul of dye solution was recovered and its absorbance was measured. The procedure was repeated ten times.

The absorbance of the dye before it goes into the flow cell was always the same, around 5 and we measured the absorbance afterwards to see the decrease of the dilution factor since we were diluting the dye solution by using water in the insertion of the adapter. It is to clarify that we are going to use 50 µl transcription reaction.. The Cy3-UTP dye is very expensive and we have already begun running transcription reactions with 70% less than the recommended concentration of fluorescent nucleotides.

Serial dilutions of bromophenol blue with a dilution factor of 0.2 were made and their absorbance at max absorbance wavelength (590 nm) were measured in the nanodrop.

As expected, a linear fit is observed (see Fig. 13).

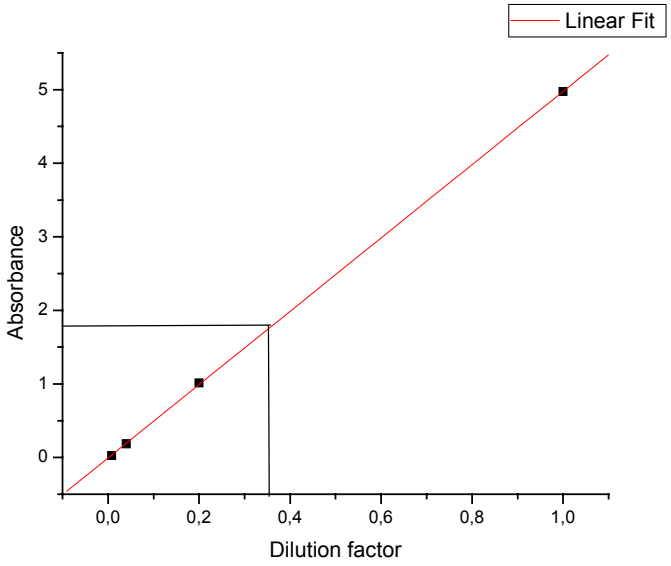

Fig. 13: Absorbence vs dilution factor using serial dilution with a dilution factor of 0.2

The average absorbance value of the dye solutions after recovery is 1.7856 (see Table 10)

Table 10: Absorbance of the dye solution after recovery

Output	***Absorbance***
1	1.543
2	1.626
3	1.694
4	1.844
5	1.554
6	2.103
7	1.687
8	1.891
9	1.782
10	2.132

This absorbance corresponds to a dilution factor of 0.36x.

The standard deviation is 0.198.

The relative standard deviation is 11.1 %.

In conclusion, with this recovery strategy, an increase in the dilution factor of 30 % will be expected.

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