iGEM Project Proposal - Current Topics in Synthetic Biology

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Developing Portable Rapid Nipah Virus Detection Toolkit using Cell Free Systems

Motivation for the Project

Nipah has been a deadly disease that has claimed many lives in south Asian and southeast Asian countries. As of 2019, there is no approved treatment for Nipah virus infection [1]. Nipah Infection is the highly contagious and best method to prevent the spread is isolating the suspected cases. Several promising antibodies have been developed and tested in animal models, yet these medicines are still in clinical trials. India, Malaysia, Singapore and Bangladesh have seen several Nipah outbreaks with highest lives claimed in Bangladesh during the series of outbreaks from 2003 - 2005.

Nipah Virus is a retrovirus (the genome is encoded in the RNA). The approved laboratory diagnosis method of NiV (Nipah Virus Infection) is done by using reverse-transcriptase polymerase chain reaction (RT-PCR). For correctness, IgG and IgM antibodies detection is done after recovery. Most of the outbreaks have started in rural areas, while the laboratories with these facilities are limited. This extends the confirmation time and allows the infection to spread further in the population. This can be shown using a suitable agent-based or network-based model.

Accelerating the process by replacing the RT-PCR step with an on-site, rapid, paper-based cell-free method, would help in containing the infection. Further, this method can also be used for Nipah Virus surveillance that would come handy to scientists. This could further prevent possible cases of Nipah to prevent severe outbreaks.

Similar Previous Works

iGEM 2019 EPFL team has worked on developing an easy to detect platforms for phytoplasma-borne disease Flavescence Dorée and Bois Noir that infect grapevines of Europe. Low cost, rapid detection toolkit have been developed for Zika virus, Norovirus and water contaminants [2], [3], [4].

Usually, the amount of target should be high enough to get a detectable change in the biosensors. This is generally not the case in raw samples. Hence, there is a need to amplify the target, which could either be RNA or DNA, RT-PCR is mainly used for this purpose, however, the components are non-lyophilizable and the process is non-isothermal. This requires special lab types of equipment the prolongs the detection process due to logistic reasons. Here, I propose to use alternative isothermal

nucleic acid amplification methods. One such method that suits our purpose is Nucleic Acid Sequence Based Amplification (NASBA). Further, the toehold switch can be used to check the strain. Toehold switch can be deployed on paper-based cell-free systems which also circumvents biosafety issues.

Methodology

This work has three main components.

1. A Model that asserts the importance of rapid, on-site detection system in case of an epidemic
2. Amplification strategy to increase the concentration of specific RNA (viral RNA) in the mixture
3. Sequence-specific check the device to ascertain the strain
4. Demonstrate that the product works

Susceptible-Infected-Isolated/Dead/Recovered Model

Network-based SIR model has been extensively used for studying effects of various policies on population. It has been extremely useful in developing better government policies [5], [6]. Susceptible-Infected-Recovered/Isolated/Dead network-based or agent-based model can be used to show that early detection and constant surveillance might reduce lethal cases.

Developing amplification toolkit specific for Nipah Virus (with a model)

The genome of Nipah Virus is available on the NCBI genome database. The genome is about approx. 18 kbp long. Specific parts that do not code for a lethal toxin and is unique to the virus can be targeted and amplified. The amplified target can then be tested using Cell-free paper-based toehold switch.

Choosing an appropriate method for genomic RNA amplification is extremely important. Traditionally Reverse Transcriptase has been used for this purpose, however, thermocyclers cannot be used outside the lab. It is important to use an isothermal, RNA targetting method. NASBA (Nucleic acid sequence-based amplification) targets amplification of RNA only and any nonspecific amplification of other DNA material is prevented. This specificity is not even seen in RT-PCR.

The reagents required for NASBA (RNAse H, Reverse Transcriptase, T7 RNA polymerase) can be individually obtained by either overexpressing them in *S. cerevisiae* or *E. coli* and purifying from by using column purification method. Pardee et al. have shown that these reagents can be lyophilized and still be used [2]. This step is routinely done in almost all labs. These enzymes can also be obtained from Collins Lab [2].

A specific region RNA genome of NiV must be selected first. Primers containing a site for T7 promoter must be included. Other specification must be considered as given in supplementary material of Pardee et al.[2].

For NASBA amplification, the reaction mixture needs to remain at 41-42^{o}C for about 90-120 minutes. On-field, this can be provided by constant rubbing or keeping the vial in lukewarm water. Body heat is another alternative for providing the required heat [7]. This makes the methods extremely useful for onsite amplification purpose.

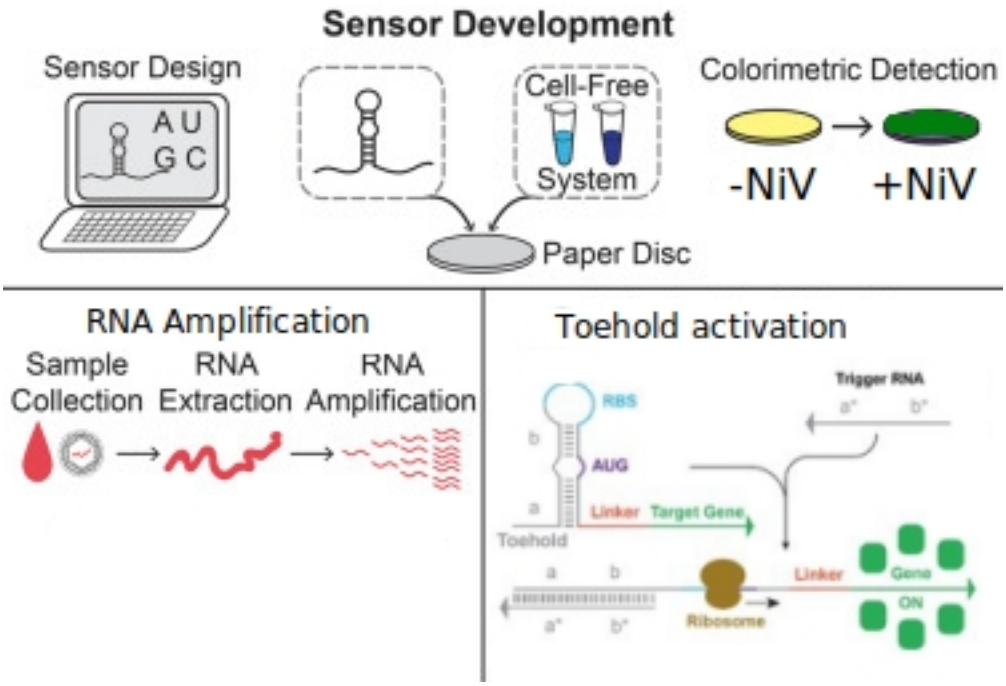

Figure 1: **Development of complete switch** Adopted from Pardee et al. [2]

Building on-field detection device (Cell-Free Toehold sensor)

One of the amplification is completed, the reaction mixture would also have a lot of single-stranded RNA. The presence of the single-stranded RNA can be detected by using a toehold switch. In this case, the target RNA would be the specific sequence that lies between the two primers in the genomic RNA of Nipah Virus. mRNA coding for GFP would be downstream of the toehold switch. Once the target RNA matches the toehold mRNA, translation of GFP would begin and the GFP would be formed.

Toehold switch containing mRNA and Ribosome can be prepared and isolated [8]. Sandeep Kumar Panda (PhD. student in Prof. Guhan's lab) also has experience designing toehold switches. A detailed protocol of sequence considerations is given in Green et al [8]. The isolated mRNA and ribosomes can be lyophilized on sterilized paper. The amplification mixture can be then added on paper and a positive result would result in fluorescence that can be visualized. Note the since, our reagents are in the cell-free state, all the reagents are usable outside the lab use without much biosafety concerns. RNAfold would be very useful to determine the structure of the RNA based toehold switch. Gregorio et al. would be extremely useful for procuring protocols for Cell-free systems [9].

A thermodynamic model can be made to optimize the sequence such that maximum toehold switches are released in the presence of target sequence and also avoid false positives. Statistical thermodynamic tools might come in handy in such case.

Demonstration

Since getting Nipah positive samples requires biosafety level 4 which is not available in our department, we can order sequence similar to that of a part of Nipah virus genome which does not code for a toxin but at the same time is unique to Nipah virus. This can be cloned into bacteria in two vectors. First in

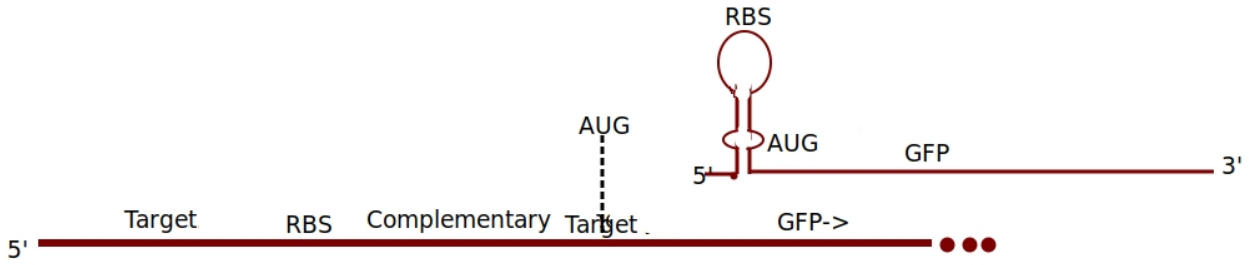

Figure 2: Toehold Switch Design

an expression vector (without RBS) and other in a cloning vector flanking strong terminator sites. First bacterial colonies can be used +NiV sample for testing. Second, a bacterial colony could serve as a test for lysogenic prophage and an empty bacterial colony as a negative control.

Then onfield conditions can be simulated in the lab by not making use of any lab types of equipment except pipettes and reagent grade water. NASBA reagent mixture is suspended. Isolated nucleic acids from the samples are added in the mixture and incubated for 1.5-2 hours at 41-42^{o}C, and added on the lyophilized toehold switch paper-based cell-free device. The fluorescence can be detected and reported. If this part works well then, it could be sent elsewhere to test on real-life samples.

Important facilities required

Available facilities

- Refridgerator (-80, -20, 4 ^oC),
- Centrifuge
- pH meter
- Laminar flow hood to cultivate test cultures
- Electrophoresis set up
- Ultrasonicator
- Incubators (Static and rotating)
- Eppendorfs, Tarson tubes, etc
- UV-Vis spectrophotometer
- Pippettes & tips
- Gel Doc
- Microwave
- Thermocycler (PCR for cloning)

Required facilities

- Purification Columns
- Lyophilizer (freeze drying) (need permission to use Prof G.K. Suraishkumar's lyophilizer)
- Fluorescence microscopy (if required then, need Prof. Mahalingam's help and permission)
- Electroporation (need permission from Prof Guhan or Prof Baskar)

Budgeting

Event

Assuming 1$ = 70 INR,

Sr. No	Events	Cost
1	Team Registration (Early Bird)	3,50,000
2	Attendee Registration (6)	4,17,200
3	Other Costs (Poster, Tshirt, Events, India Meet, Treats)	15,000
	Total	7,82,200

Reagents

Sr. No	Reagent/Reagent Kit	Cost
1	E. coli BL21 & Yeast	0
2	Reverse Transcriptase (100x20μl)	5000
3	Protein Purification kit (25 columns)	26000
4	Test, T7 pol, RNAse H, Reverse Transcriptase gblocks (custom)	0 (IDT free DNA)
5	Primers (test PCR amplification)	5600
6	Primers (for NASBA)	11,200
7	dNTPs and NTPs for NASBA (gbiosci 2x25μl of 25mM)	30,000
8	Expression Vector Plasmids	8400
9	Restriction Enzymes (EcoR1, Pst1)	10,000
10	Ligase	3000
11	RNase H (120 units)	53,330
12	T7 RNA polymerase (2500)	26,000
13	Other Consumables (Tips, Eppendorfs, Tarsons, chemicals, LB media, Agar etc)	35000 (upperlimit)
	Total	approx. 2,00,000

Note that many single time use common reagents are omitted assuming that those would be made available by UG lab.

Risk Involved

- Financial Risk

- Care to be taken while selecting appropriate target DNA sequences that code for non-toxins. The target sequence should not have a Ribosome binding site and coding frame otherwise, there is a possibility of toxin polypeptides to be produced.
- Due to time constraints, the author is unable is to find out the procedure for approval from the institute ethics committee is required to work the sequence of NiV.
- Note that all the product is cell-free and hence, provides a safe platform.

Anticipated outcomes and Possible breakthroughs

The project vertical of the team can be divided into three parts, first standardising the lab protocol for NASBA and Network-based SIR model. Second, designing test sequences and seeking approval from the institute ethics committee. Third, designing a toehold switch and standardizing lab protocol for Cell-free toehold switch along with Ribosomes. This protocol is easy to develop given the amount of literature that exists. Moreover, Lavikova and Maerkl have developed a new cheap method to get the expression of proteins in cell-free systems [10].

Once, the institute ethics committee approves, the order can be placed to IDT for the toehold switch and the test parts. Meanwhile, the SIR model can be made and other protocols can be standardised. After the sequences arrive from IDT, test strains and toehold switches can be prepared. A test can be carried out to demonstrate that the system works when different parts are deployed in lyophilized form and reconstituted.

One possibility is that the institute ethics committee might not approve the use of Nipah virus sequences. This is easy to overcome by selecting another retrovirus with which institute ethic committee is okay.

Worst possible outcomes of this project could be there would be a lot of logistic delays, as many reagents unique are required in this project. This might end up in a logistic nightmare. To overcome this, we might require a dedicated logistic team to coordinate with ICSR and the vendors to get the reagents and other deliveries on time. Another worst-case scenario would be the lack of expertise with cell-free systems or unfamiliarity of the students in the lab.

Best case is everything works well or we end up with some parts of the project working well which is still a big step, given the experience of the people working in the lab. If everything works well, then this technology can be extended further to encompass any retroviral disease diagnosis and can be translated into a startup.

With a better understanding of the system, we might even be able to develop better cost-effective techniques what would be of great help to humankind. According to Pardee et al, their cost of testing was 1$ per test, which would be our target for now.

Timeline

This is a large project, but given that iGEM is a team project, we can parallelize large portions of the work. The work can be broadly divided into the following:

1. SIR/D/I model
2. Seeking permission from Institute ethic committee

3. Standardizing protocol for NASBA
4. standardizing protocol for cell free paper based protein expression
5. Desigining Toehold switch

To maximize the chance of getting a desired result, we can select three target regions.

Month	SIR model	IEC	NASBA	CFS Protein Expr.	Toehold switch
1	Start	Initiate the procedure	Decide on Strategy to procure reagents	Lit. Survey OnePot [10]	Lit. Survey
2	Get Results	Get approval/ New retrovirus	Place order for Reagents Design primers&PO	Fix method, Design primers (test)& PO	Select the target sequence(3)
3	-	-	Start tinkering Fix a protocol	Start tinkering Fix a protocol	Thermodynamic model; PO: Opt. (3) seq.
4	-	-	PR: amplification	PR: show *in vitro* expression	(3) toehold switches buffer
5	-	-	Buffer	Buffer	Toehold Switch arrive; Cloning
6	-	-	Test amplification of targets	Test Expression *in vitro*	Test *in vivo*
7	-	-	Assemble (lyophilized check)	Assemble (lyophilized check)	Assemble (lyophilized check)

PO: Place Order; PR: Preliminary Results
NOTE: Does not take into account Quizzes, Endsems, etc.

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