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Research Report

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Title of Research Report

Design of a Proximity-Initiated CRISPR Cas 12A Detection Scheme and Evaluation of Potential Isothermal Amplification Techniques for Sensitive Protein Detection

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Design of a Proximity-Initiated CRISPR Cas 12A Detection Scheme and Evaluation of Potential Isothermal Amplification Techniques for Sensitive Protein Detection

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Abstract

Timely detection of disease biomarkers is a crucial step in disease management. The development of point-of-care (POC) diagnostics has become crucial as the COVID-19 pandemic places a strain on healthcare system and increases the demand for diagnostic procedures accessible to rural areas. Unlike laboratory tests, these kits must be easy-to-use, fast and cost-effective. Existing POC tests typically detect protein biomarkers but lack in analytical sensitivity [1]. While several isothermal amplification techniques have been developed recently, they are limited to nucleic acid detection [2]. Herein, we developed a novel protein detection system based on the concept of proximity-initiated CRISPR-Cas12A amplification. We utilized a split proximity circuit (SPC) concept to translate the recognition of a target protein into a generic DNA barcode [3]. The CRISPR-Cas12A was designed to read this barcode and generate an amplified fluorescence signal. We designed four DNA barcode strands and assessed the effectiveness of our SPC-CRISPR-Cas12A system in detecting a model split target (ST), a DNA proxy for the target protein in this feasibility study. Correspondingly, the SPC we tested herein included a DNA strand complementary to the ST, instead of an antibody, to ensure the feasibility of this system. Our preliminary assessments with ST revealed that one of our SPC-CRISPR-Cas12A designs could achieve an appreciable signal-to-background ratio of 1.83. Additionally, we also assessed the pure CRISPR technique to be a more sensitive isothermal amplification method than the other enzyme-free technique known as hybridization chain reaction (HCR). With a high sensitivity of the CRISPR module used in our protein detection system and ease of use for POC diagnostics due to the use of a master mix for its reaction, our assessments provide a promising foundation for further research on protein detection and POC diagnostics at large.

Keywords: Split proximity circuit (SPC), Hybridization Chain Reaction (HCR), CRISPR

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Commitments on Academic Honesty and Integrity

We hereby declare that we

1. are fully committed to the principle of honesty, integrity and fair play throughout the competition.
2. actually perform the research work ourselves and thus truly understand the content of the work.
3. observe the common standard of academic integrity adopted by most journals and degree theses.
4. have declared all the assistance and contribution we have received from any personnel, agency, institution, etc. for the research work.
5. undertake to avoid getting in touch with assessment panel members in a way that may lead to direct or indirect conflict of interest.
6. undertake to avoid any interaction with assessment panel members that would undermine the neutrality of the panel member and fairness of the assessment process.
7. observe the safety regulations of the laboratory(ies) where the we conduct the experiment(s), if applicable.
8. observe all rules and regulations of the competition.
9. agree that the decision of YHSA(Asia) is final in all matters related to the competition.

We understand and agree that failure to honour the above commitments may lead to disqualification from the competition and/or removal of reward, if applicable; that any unethical deeds, if found, will be disclosed to the school principal of team member(s) and relevant parties if deemed necessary; and that the decision of YHSA(Asia) is final and no appeal will be accepted.

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Table of Contents

Introduction

Disease biomarkers are the central figures of diagnostic procedures [1]. Many biomolecules can serve as biomarkers. For example, low-density lipoproteins could suggest a risk of cardiovascular diseases [2], while accumulating beta-amyloid plaques in the brain often indicate the progression of Alzheimer's disease [3]. In recent years, protein biomarkers are receiving much attention because of their involvement in many common diseases. Many techniques have been developed to detect protein biomarkers. These include enzyme-linked immunosorbent assay (ELISA), immunoblotting and mass spectrometry [4]-[7]. However, these assays are complicated and require much time and resources to perform, rendering them inaccessible to most rural areas. This provides the impetus to develop a simple yet effective tool to detect protein biomarkers.

The recently established split proximity circuit (SPC) is one such detection system that can be adapted for proteins. The SPC has a distinct on and off mechanism to detect protein presence. Thus, it can be used as a molecular switch. The SPC is based on the principle of proximity induced strand displacement and can be modified to detect protein in three steps: **(1)** Initiator 1 (I1) and initiator 2 (I2) are probed with antibodies. **(2)** The presence of a specific antigen then initiates the binding of I1 and I2. **(3)** The proximity of I1 and I2 kinetically favours the formation of a complete trigger strand (c* b*) which is stabilized by a DNA association region (a a*) [8]. The SPC detects the target protein and converts it into generic DNA barcode, which can then be amplified to signal the protein's presence. (Fig. 1). In our study, split target - a linear target DNA strand and I1I2 are used, which subsequently can be replaced with suitable protein and antibody respectively.

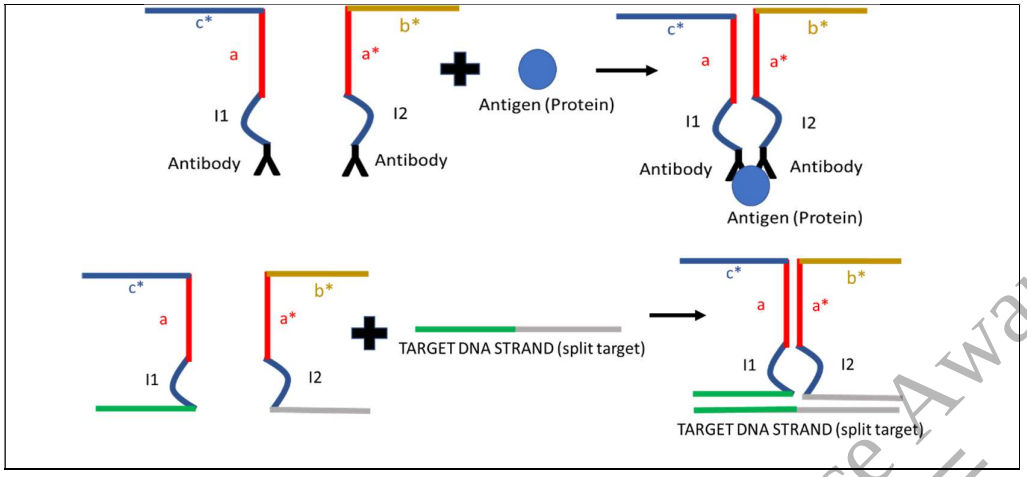

Fig. 1: Split Proximity Circuit Detection Mechanism

Signal amplification is necessary for detecting low concentrations of protein biomarkers. Conventional methods for amplifying the DNA barcode presented, such as polymerase chain reaction (PCR) requires expensive instruments, a long executing time, and are not suitable heat-labilebile protein targets due to its thermal cycling process. While many isothermal amplification methods such as nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), and helicase-dependent amplification (HDA) have recently been developed for nucleic acids detection, they typically involve the use of high temperatures (40 – 65 °C), making them unsuitable for protein detection [9]-[18] (Appendix, Table 1).

Thus, we propose a protein detection assay based on the clustered regularly interspaced short palindromic repeats (CRISPR) system, containing the endonuclease Cas12a and a guide RNA (gRNA) complementary to the generic DNA barcode mentioned above. In the presence of the target protein, the gRNA will hybridise with the DNA barcode presented by the SPC. This will trigger Cas12A to cleave many reporter molecules, which can then be detected. In effect, this system would detect the protein and produce an amplified signal of its presence [19]-[22]. We assess our CRISPR-based system by comparing its results with an established hybridisation chain reaction (HCR) signal amplifier. The HCR involves two hairpin nucleotide sequences (H1, H2) tagged with fluorophores and quenchers. These sequences hybridise with the target DNA and produce a fluorescence signal, which intensity is proportional to the amount of amplified DNA [23]-[24]. (Fig. 2)

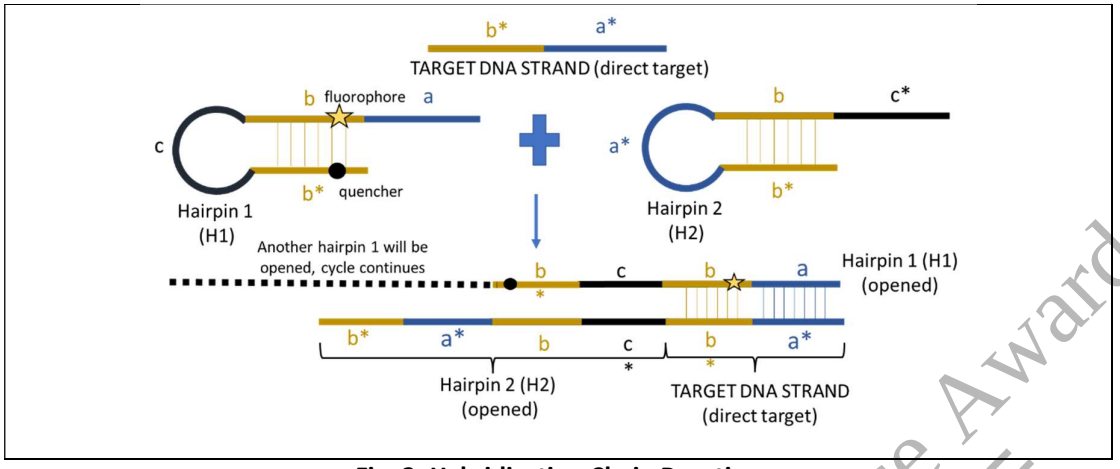

Fig. 2: Hybridisation Chain Reaction

Herein, we design a CRISPR-Cas12A system that complements the DNA barcode on the SPC. We sample and test various barcode sequences to enhance SPC-CRISPR complementarity. We also assess signal translation and amplification capability of the novel combination of SPC and CRISPR-Cas12A by comparing it with an SPC-HCR system [25].

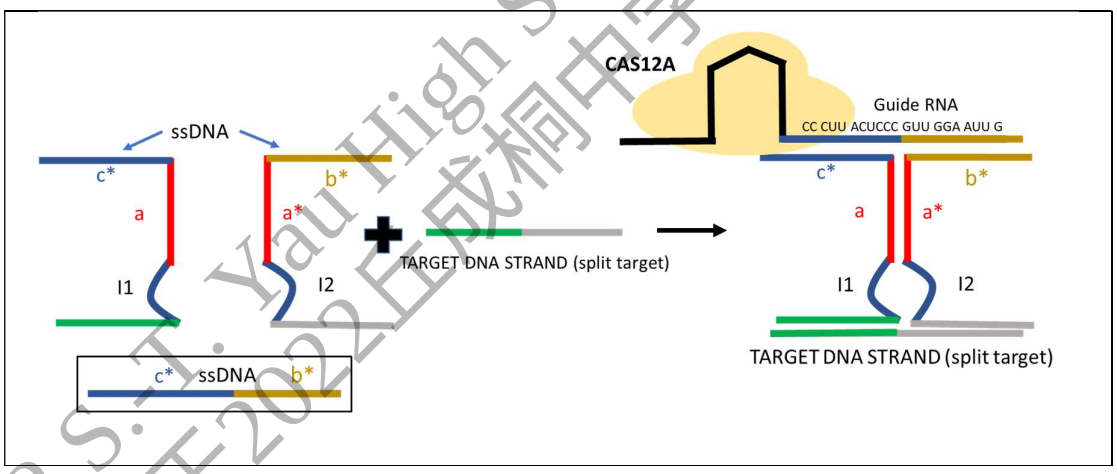

Fig. 3: SPC-CRISPR

Materials and Methods

Oligonucleotide Strand Design: NUPACK web server was used for the design and analysis of nucleic acid structures and systems. NUPACK analysis was carried out for n number of interacting DNA species at 37◦C (RTP) to form a maximum complex size of n strands. (Appendix, Schematics)

HCR: HCR protocol was adapted from a study done by Ang et al. [24] (Appendix, Protocol 1). A solution of 2X HCR master mix and DT was prepared. Solution with DT was then serial diluted by 2x, from a starting concentration of 10nM, for 8 samples. A control tube with 5uL of SPC buffer was also prepared. One pot addition of the 2X HCR master mix to the target samples (including the control) in equal volume was performed.

SPC: SPC protocol was adapted from a study done by Ang et al. [23] 2X SPC master mix and of ST was prepared (Appendix, Protocol 1). Solution with ST was then serial diluted by 2x from a starting concentration of 10nM, for 8 samples. A control tube with 5uL of SPC buffer was also prepared. One pot addition of the 2X SPC master mix to the target samples (including the control) in equal volume was performed.

CRISPR CAS12A: CRISPR CAS12A protocol was adapted from a study done by Li et al. [25] (Appendix, Protocol 2). 2XCRISPR master mix and CRISPR target was prepared (refer to CRISPR protocol). Sample solutions of CRISPR target was then serial diluted by 4x, from a starting concentration of 8nM, for 10 samples. A control of 5uL of DNase free H2O was also prepared. One pot addition of the 2X CRISPR Cas12a master mix to the target and control samples in equal volume was conducted.

Determining Limit of Detection (LOD): CRISPR CAS12A protocol was adapted from a study done by Li et al. [25] 2xCRISPR master mix with 3.125nM I1 and I2 and ST was prepared (refer to CRISPR protocol). ST was then serial diluted by 2x starting from 8nM, for 9 samples. One pot addition of the 2X SPC-CRISPR Cas12a master mix to the target samples of ST (including the control) in equal volume was conducted.

Fluorescence Readout*:* Fluorescence measurement was performed on Tecan Spark 10M microplate reader. Reactions were carried out at room temperature for HCR and SPC, and 37°C for CRISPR Cas12a. A reaction volume of 10 microlitres are used in the 384-well black plate. For kinetics study of HCR and SPC, a mFluor™ Blue 630 SE dye with an excitation wavelength of 495nm and an emission wavelength of 630nm was used. Fluorescence measurement was performed every 1 min for a total analysis time

of 1h at room temperature. For kinetics study of CRISPR Cas12a, the fluorescence measurement will be performed every 1.5 min for a total analysis time of 1h at 37°C.

RESULTS AND DISCUSSION

1. CRISPR Cas12A produces signals with higher signal to background ratio than HCR

Signal grows as time elapse; different concentration of target DNA will result in different rate of signal growth. The rate of signal growth decreases overtime. In addition to that, the lower the concentration of target DNA, the lower the rate of signal growth. As shown in figure 4a & 4b below, after 30 minutes (1800 sec) the signal growth for targets of lower concentration started to plateau off (no significant increase in intensity).

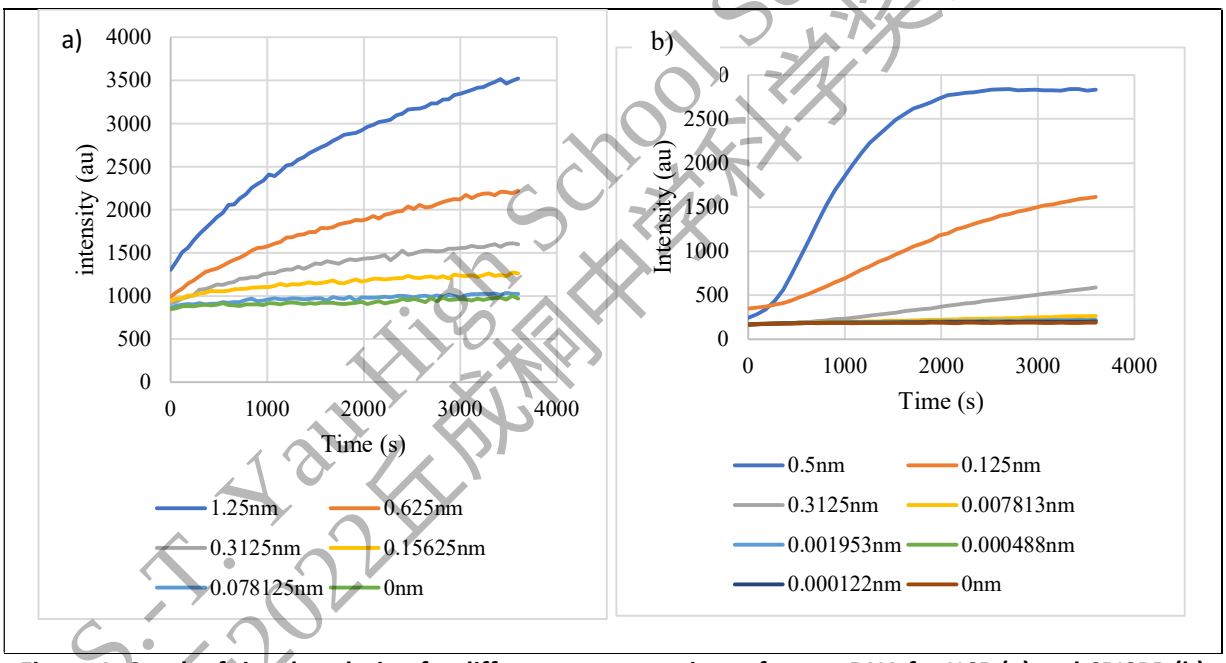

Figure 4: Graph of signal evolution for different concentrations of target DNA for HCR (a) and CRISPR (b)

By plotting intensity against concentration (a calibration curve), we will determine the limit of detection by looking at 3 standard deviations above a negative set up (absence of target strand), by finding the intercept between the 2 lines shown on the figures 5a & 5b, we can then estimate the limit of detection.

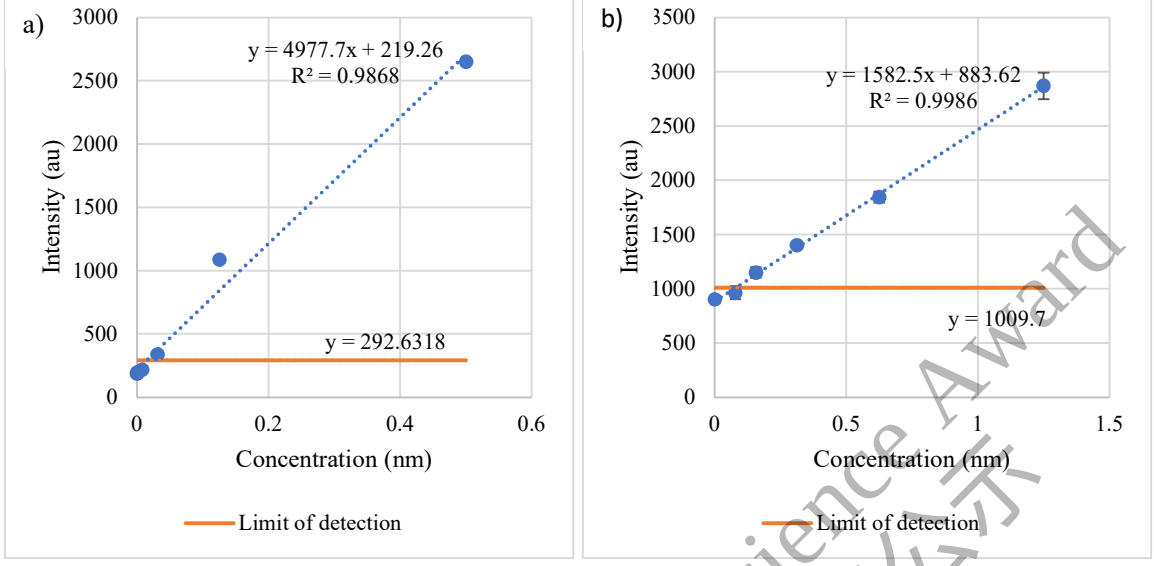

Figure 5a: Calibration curve (CRISPR); Figure 5b: Calibration curve (HCR); a) Limit of detection: 0.01474nm; sensitivity: 4977.7 au/nm; **b)** Limit of detection: 0.07096nm; sensitivity: 1582.5 au/nm

These results suggest that CRISPR is a more sensitive method as compared to HCR.

2. SPC appears to be effective at detecting proteins

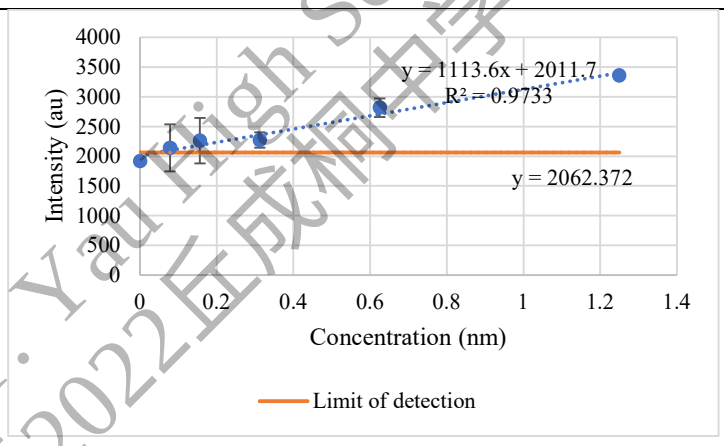

Figure 6: Calibration curve for SPC initiated HCR;
Limit of detection = 0.045503nm; Sensitivity = 1113.6 au/nm

The sensitivity for SPC initiated HCR – 1113.6 au/nm (detection using split target) is lower than HCR – 1582.5au/nm (detection using direct target), possibly because direct target is easier to be detected as they are independent of interactions between the different strands of DNA (I1-I2 complex required to be form for detection using SPC).

3. Hairpin trigger for CRISPR Cas 12a

The results assure us that the initiator strands (I1,I2) of SPC can act as trigger when they come together, forming a structure similar to the hairpin trigger.

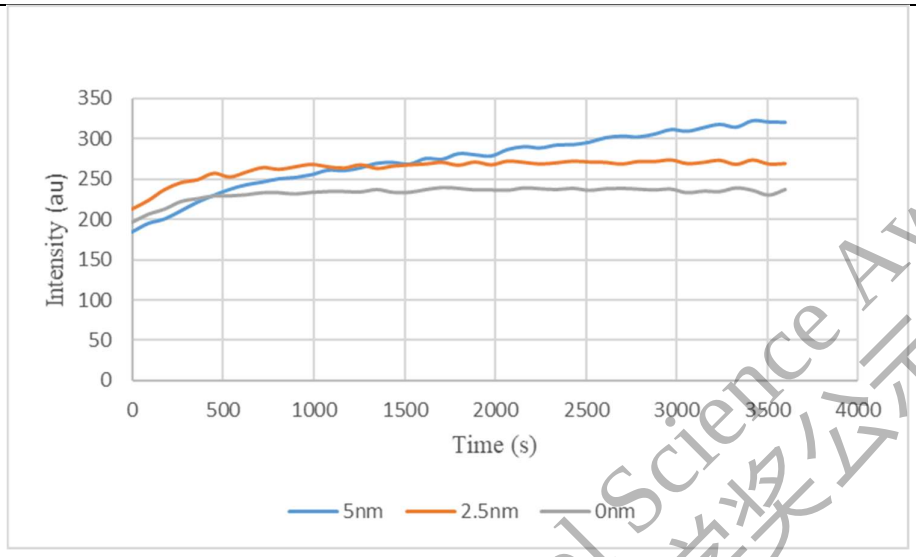

Figure 7: Graph of signal against time for various concentrations of hairpin trigger

4. Optimizing Design of Initiator Strands as Translator for CRISPR Cas12a Readout

From Table 1, the higher the concentration of I1I2, the higher the noise signal (intensity obtained without target present) with 3.125nM giving the best signal-to-background ratio. At higher concentrations, random interactions between the I1I2 strands due to chance increases which results in I1I2 complex forming and hence increased background signal. Although signal was expected to increase with increasing concentration of I1 and I2, our results suggest otherwise. A possible reason for this would be that the reaction mixture was concentrated with the various species (I1, I2, ST), complicating the interactions as it would be harder for the strands to move freely, causing them to work differently from what is expected.

Table 1: Finding Optimal (i.e., highest signal to noise) Concentration of I1I2 (using 4nt design)

Concentration of I1I2 (nM)	Signal (au)	Background (au)	Signal: Background Ratio
12.5	326.5	361.5	0.90318

6.25	544.5	334	1.63024
3.125	601	328	1.83232

Table 2: Comparing Signal: Background Ratio of 4 Different Designs

Strand	Signal (au)	Background - noise (au)	Signal: Background Ratio
I1I2 4nt association region	601	328	1.83232
I1I2 +7nt	294.333	271	1.086101
A46	528	366	1.435337
I1I2 6nt association region	3371.667	3023.667	1.115092

I1I2 4nt association region design was designed as a study done by Ang et. al [26] suggested that having 4nt at the association region would result in better signal: background ratio (among association regions with lengths ranging from 3nt-6nt). This design gave us the best signal: background ratio because the amount of ST-I1-I2 complex formed was more significant than I1-I2 complex formed as compared to the other designs.

I1I2 6nt association region design has high signal regardless of the presence of ST. This is due to higher rate of ST-I1-I2 (with ST)/ I1-I2 (without ST - noise) complex forming because the interactions (Hydrogen bonding) between 6 complementary nucleotides would be stronger (forming more stable complex) and hence there is higher signal for both cases as compared to the other designs. Due to the high leakage, the signal to background ratio is low.

A46 design contains hairpin loop where the region complementary to the guide RNA is enclosed in the loop and hence it potentially minimizes the background signal since it is less accessible and can only generate signal when the loop is opened. This design gave us the 2nd highest signal: background ratio. Possible reasons as to why the signal (with ST) received is lower than 4nt design might be due to slow opening of hairpin loop.

I1I2 +7nt has a 7 nucleotide long irrelevant sequence on both ends. A study done by Long T. Nguyen [27] suggests that having a 7-mer DNA extension to crRNA enhances sensitivity and specificity of nucleic acid detection, hence this des ign was tested out. However, this design resulted in low

signals and low signal: background ratio. It is possible that the 7 nt has a negative impact on the binding to the guide RNA, resulting in lower signal than the design without.

5. 4nt association region design is more sensitive than A46 design

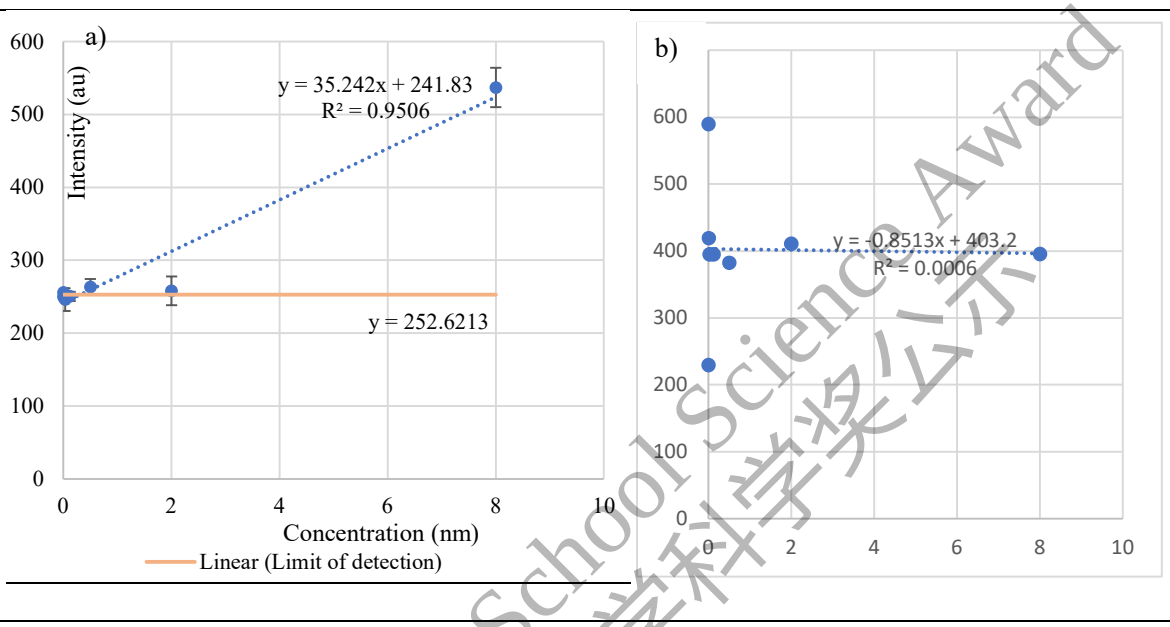

Figure 8a: Calibration curve for 4 nt association region design – Limit of detection: 0.306206nm ; sensitivity: 35.242 au/nm

Figure 8b: Calibration curve for A46 design

(Fig. 8a) Low sensitivity is obtained for this design, likely because complex ST-I1-I2 has low formation rate (as the concentration of target DNA increase, the increase in complex (ST-I1-I2) formation is not very significant), a possible reason for that would be that the 4nt region was too little for ST-I1-I2 complex to form and instead other complexes that do not contribute to the signal (like ST-I1/ST-I2) is formed.

(Fig. 8b) The calibration graph was not as expected, with results all over the place and there seem to be no trend at all. This design possibly doesn't work well for detection. Some possible reasons for this might be due to low binding rate between the guide RNA and ST-I1-I2 complex which was not significantly improved when even when the concentration of ST-I1-I2 complex is increase. It is possible that the hairpin loop did not open even when the complex if formed and hence the sequence could not be accessed by the guide RNA.

6. Evaluation of the Assessed Proximity-Initiated Signal Amplification Methods

SPC-HCR method is more sensitive (1113.6 au/nm > 35.242 au/nm) and has lower limit of detection (0.045503nm < 0.306206nm) as compared to SPC-CRISPR method (with best design as of now). It seems like SPC-HCR method is better than SPC-CRISPR, but this is likely because the design for the DNA strands involved in SPC-CRISPR has not been fully optimized yet. We believe that with a better optimized initiator strand design, SPC-CRISPR will have better performance than SPC-HCR due to potential in the CRISPR method as compared to HCR.

CONCLUSIONS

After a theoretical evaluation of potential isothermal amplification techniques and an experimental comparison of CRISPR Cas 12A and HCR, we identify CRISPR Cas 12A as the most-suitable tool for protein detection. Our novel proximity-initiated CRISPR Cas 12A detection scheme promises to be more effective than a proximity-initiated HCR system. Additionally, our analysis of potential designs of the SPC's generic DNA barcode provide the foundation for further research on engineering a sensitive protein detection tool.

FUTURE WORK

We could analyse our strand designs further and assess various others to integrate SPC with CRISPR Cas 12A more effectively and provide more thorough guidelines on using this novel system. Furthermore, we could improve the best design we have currently, 4nt assoc., to achieve a greater signal to background ratio. Since we tested our SPC-CRISPR system using the split target DNA strand, we could also repeat our tests using proteins and thereby, bring our system closer to meeting its intended goal.

Declaration of Previous Submission

Name of Competition	Date	Awards Won
Singapore Science and Engineering Fair	Jan – Mar 2022	NIL

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ANNEX

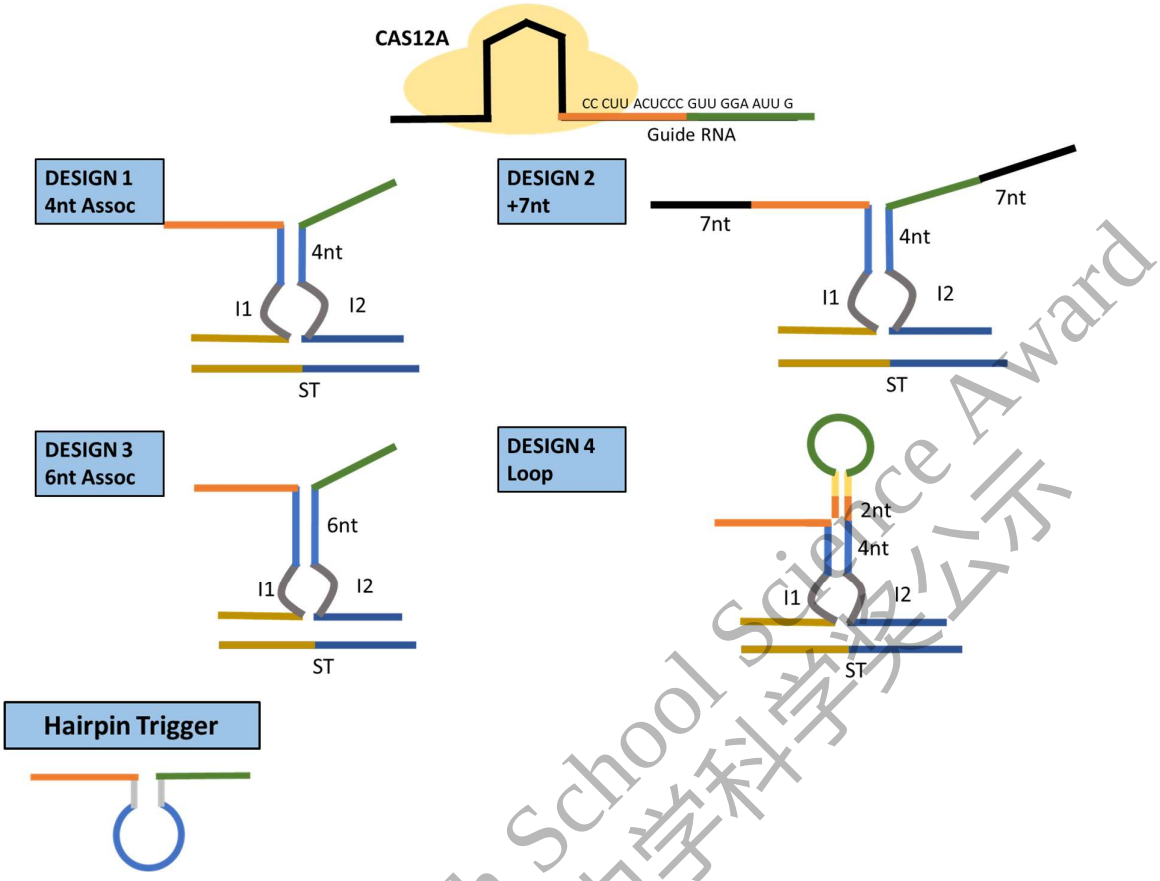
Table 1:

Isothermal Method	Enzyme used	Reaction Temp.	Detection Methods	Why not suited for protein detection applications
Nucleic acid sequence-based amplification (NASBA)	Avian myeloblastosis virus reverse transcriptase T7 DNA-dependent RNA polymerase (DdRp) RNase H	41°C (Need pre-heating, 65°C for RNA & 95°C for DNA)	Gel electrophoresis Real time monitor Elisa	Pre-heating step that denatures protein RNA that has an unstable nature
Loop-mediated isothermal amplification (LAMP)	*Bst* DNA polymerase	60–65 °C	Gel electrophoresis Turbidity Real time monitor Visual	High temperature that denatures proteins. Complex design that requires two or more sets of primers, making protein detection more challenging
Helicase-dependent amplification (HDA)	Bst DNA polymerase Helicase	37 °C	Lateral flow Gel electrophoresis Elisa	slow reaction rate, taking up to an hour Expensive reagents
Recombinase polymerase amplification (RPA)	Bsu DNA polymerase Recombinase	37-42 °C	Lateral flow Fluorescence	Highly prone to false positives as it tolerates primer mismatches Effect of mismatches is hardly predictable
Strand displacement amplification (SDA)	Nt.BstNBI Nicking enzyme Bst DNA polymerase	37 °C (Need pre-heating)	Gel electrophoresis Fluorescence Real time monitor	Pre-heating step that denatures protein Prone to unspecific amplification, limiting sensitivity levels of detection
Nicking extension amplification reaction (NEAR)	Nicking enzyme Strand displacing DNA polymerase	37 °C (Depends on nicking enzyme and polymerase used)	Lateral flow Fluorescence	Prone to unspecific amplification, limiting sensitivity levels of detection

Table 2:

Strand	Sequence
HCR	
HP1-9129	GTT GGA ATT GGG AGT AAG GGC TCT TAC TTT GCC CTT ACT CCC
HP2-9129	GCC CTT ACT CCC AAT TCC AAC GGG AGT AAG GGC AAA GTA AGA
DT_9129	GCC CTT ACT CCC AAT TCC AAC
SPC	
I1	TAC CTT CAC CTT TTT TTT TTT TTT TTT GTGC CC TT CAATTCCAAC AAGG
I2	GCCCTTACTCC GCC GCAC TTT TTT TTT TTT TTT CAC CAT CCA CTC
STI2I2	GAG TGG ATG GTG AAG GTG AAG GTA
CISPR-Cas12A	
ST18_18	GAG TGG AGT GTA GAA GTG GAT AAA GCA GAA ACG AAA
I1_cas12a (4nt assoc)	TTT CGT TTC TGC TTT ATC TTT TTT TTT GTGC GAG TAA GGG
I2_cas12a (4nt assoc)	CAA TTC CAA CGG TT GCAC TTT TTT TTT CAC TTC TAC ACT CCA CTC
I1_cas12a (+7nt)	TTT CGT TTC TGC TTT ATC TTT TTT TTT GTGC GAG TAA GGG TATTATT
I2_cas12a (+7nt)	TATTATT CAA TTC CAA CGG TT GCAC TTT TTT TTT CAC TTC TAC ACT CCA CTC
I1_cas12a (6nt assoc)	TTT CGT TTC TGC TTT ATC TTT TTT TTT GTGC GG GAG TAA GGG
I2_cas12a (6nt assoc)	CAA TTC CAA CGG TT CC GCAC TTT TTT TTT CAC TTC TAC ACT CCA CTC
I1_cas12a (Loop)	TTT CGT TTC TGC TTT ATC TTT TTT TTT GTGC GG GAG TAA GGG
I2_cas12a (Loop)	GGAA CAA TTC CAA CGG TT CC GCAC TTT TTT TTT CAC TTC TAC ACT CCA CTC

Schematics



Protocols

Protocol 1: SPC/HCR

Detection of DNA / RNA Targets (Fluorescence Readout)

Buffers

- Oligo storage buffer: 1X TE (pH 8.0)
- SPC reaction buffer: 10 mM phosphate buffer (pH 7.4) + 140 mM NaCl + 10 mM $MgCl_2$ + 0.005% T-20

Overall Reaction Scheme

Each SPC kit contains 4 separate reagent probes. Users are to prepare a 2X SPC mastermix prior each experimental run. The target samples and 2X SPC mastermix are then mixed in equivolume amount. For example, 10 µL reaction volume is recommended for 384-well plates, hence 5 µL of 2X SPC probe mastermix will be added to 5 µL of target samples in the well.

Reagents	Stock concentration	Working concentration	Reaction concentration
HP1	100 µM	1 µM	20 nM
HP2	100 µM	1 µM	20 nM
I1	100 µM	1 µM	20 nM
I2	100 µM	1 µM	20 nM

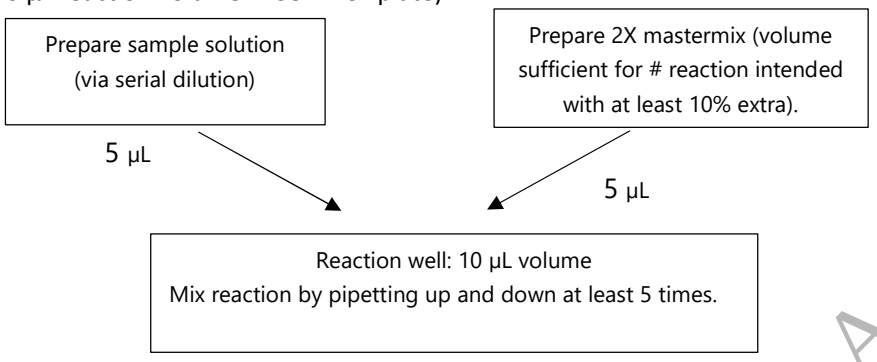
Preparation of SPC Reagents

Stock solution (100µM) (long-term storage) → 2X mastermix (40nM) (Prepare just before use) All components are added into a single tube → Working solution (1µM) (For use within the day) Prepare in separate tubes for each component.

**when performing HCR, only HP1 and HP2 needs to be prepared.*

Performing SPC / HCR reaction

(Assume 10 µL reaction volume in 384-well plate)



For the sample solution,

- Direct trigger (DT) is used as synthetic target for HCR
- Split target (ST) is used as synthetic target for SPC
- In protein system, the target / sample will be the protein of interest.

Protocol 2: CRISPR Cas12A

Protocol for Cas12a in vitro assay

Materials	
1	EnGen® Lba Cas12a (Cpf1) (100 µM) - M0653T
2	CrRNA (2nmole) Alt-R® l.b. Cas12a crRNA, 2 nmol
3	DNaseAlert substrate (25 x 50 pmol Fluorescent Substrate tubes)
4	1XPBS pH 7.4

Reagents	**Working concentration**	**Reaction concentration**
CrRNA	1 µM	12.5 nM
Cas12a	1 µM	12.5 nM
FQ probes	1 µM	50 nM

crRNA sequence	
Secondary structure/handler (fixed): 5’ UAAUU UCU ACU CUU GUA GAU	Protospacer: CC CUU ACUCCC GUU GGA AUU G 3’
DNA target sequence	
PAM strand hcrCas12aPAM2822	5’ CTTCA**TTTG** CCCTTACTCCCGTTGGAATTG CCTCATCTTATTATCCTTA 3’

Target strand hcrCas12aTar2822	5' TAAGGATAATAAGATGAGGCAATTCCAACGGGAGTAAGGGCAAATGAAG 3'

1. Preheat the microplate reader to 37°C at least 20mins before starting the experiment.
2. Prepare working solutions for Cas12a, and crRNA and FQ probes

Cas12a working solution (1 µM)- prepared fresh	
Cas12a (2 µM)	5µL
1X PBS	5µL
Total vol	10µL
crRNA working solution (1µM) - can be stored for 2weeks	
CrRNA (stock, 10 uM)	1µL
1XPBS	9µL
Total vol	10µL

3. Prepare working solutions for FQ probes.

Add 40 µL of nuclease free water to each DNaseAlert substrate tube. The final concentration of the substrate (FQ) is 1µM.

FQ probes (1µM) - can be stored for 2 weeks	
FQ probes stock	-
DNase Free H2O	40µL
Total vol	40µL

4. Combine Cas12a and crRNA to form the RNP complex in RNP tube (room temperature, 10-15mins)

RNP tube	1 Rxn (µL)	10 Rxn (µL)
Cas12a (1 µM)	0.125	1.25
crRNA (1 µM)	0.125	1.25
10X NEBuffer2	1	10
DNase Free H2O	1.25	12.5
Total vol	2.5	25

5. Dilute FQ probes for FQ probe tube

FQ probe tube	1 Rxn (µL)	10 Rxn (µL)
FQ (1 µM)	0.5	5
DNase Free H2O	2	20
Total vol	2.5	25

For preparation of FQ probes for LOD, ST is mixed into the FQ probe tube.

FQ probe tube	1 Rxn (µL)	10 Rxn (µL)
FQ (1 µM)	0.5	5
I1	0.03	0.3
I2	0.03	0.3
DNase Free H2O	2.44	24.4
Total vol	2.5	25

6. Prepare sample solutions via serial dilution

Combine RNP tube and FQ tube to form the mastermix. Add 5ul of mastermix into 5ul of each sample (e.g., HCR