Using Synthetic Biology as a Tool for the Early Diagnosis of ADHD

Group 30
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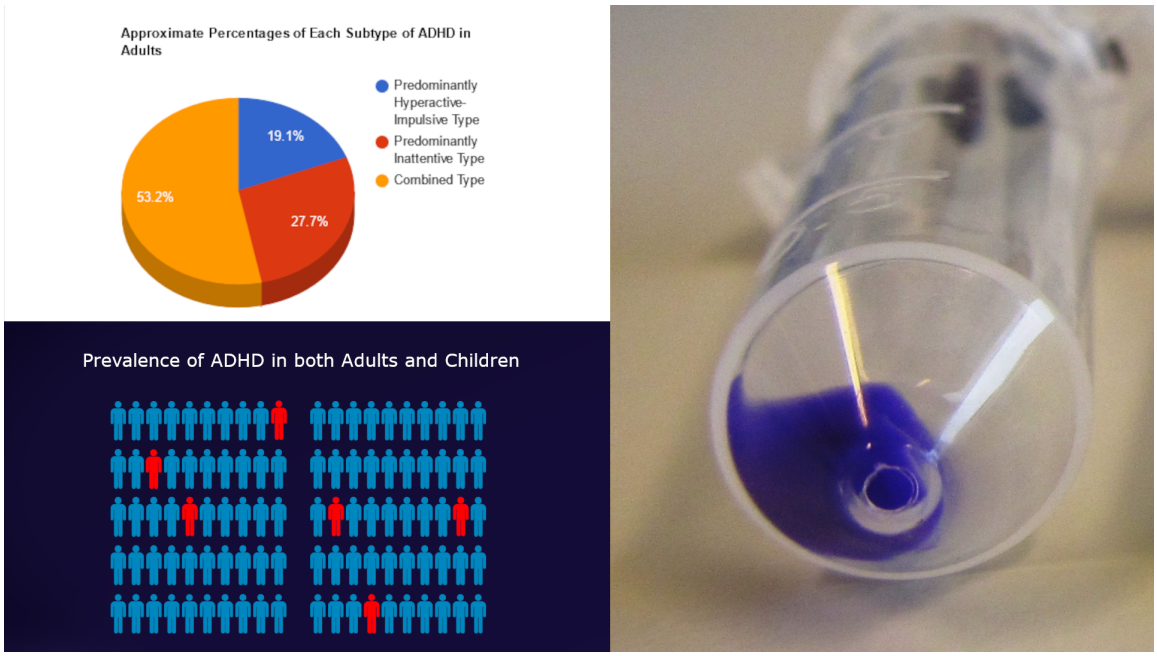

Figure 1: Bottom figure shows the approximate prevalence of ADHD in both adults and children [1]. Right figure shows color produced by amilCP [2]. Top figure shows a chart of approximate percentages for each type of ADHD in adults [3].

Abstract

Attention Deficit Hyperactivity Disorder (ADHD) is a childhood-onset condition that can persist into adult life. Studies have shown ADHD to be linked to both genetic and environmental factors such as nutritional deficiencies. Specifically, mutations in Dopamine Receptor D4, ADRA2A, and deficiencies in Iron and Zinc have been linked to an increased risk of ADHD. The issue with diagnosing ADHD today lies in the fact that there are different subtypes that each have different clinical presentations. Men present more as the hyperactive subtype while women statistically present as the inattentive type, leading to discrepancies in the diagnostic process. By examining both the genetic and environmental factors associated with the disorder, we have found a way to use synthetic biology to allow for early detection of ADHD by producing a coloured output when the individual displays iron and zinc deficiencies, as well as mutations in the DRD4 and ADRA2A genes.

Introduction

ADHD is among the most common childhood neurodevelopmental disorders. It often lasts into adulthood and affects an estimated 8.4% of children aged 12-17, and 2.5% of adults, with males being more commonly diagnosed than females[1]. ADHD includes a combination of persistent symptoms such as inattention (difficulty keeping focus), hyperactivity (excess movement that does not fit the setting), and impulsivity (quick acts that occur without thought). These symptoms often have a significant effect on work, home, and student life [2]. However these symptoms can vary greatly from person to person. In fact, ADHD has been divided into three different types: predominantly hyperactive-impulsive, predominantly inattentive, and combined. As seen in Figure 6, the most common type is combined, affecting approximately 53.2% of adults diagnosed with ADHD[3][A6]. Moreover, this wide range of symptoms makes ADHD difficult to diagnose. Currently, ADHD is diagnosed through subjective assessments and because of this, early recognition of the disorder can be very challenging [4]. Specifically, ADHD is gravely underdiagnosed in young females as they often present with the inattentive type whereas men present with the hyperactive type. This difference in the presentation of symptoms can make it difficult for women to get diagnosed since they may not be hyperactive or impulsive as is usually assumed with ADHD [5]. There isn't one key biomarker that can be used to diagnose ADHD, but a combination of biomarkers can be used to help decrease the heterogeneity and identify homogeneous subtypes of ADHD. Both genetic and non-genetic factors can be considered when it comes to the diagnosis of ADHD[6].

Regarding non-genetic risk factors, iron and zinc are revealed as two potential biomarkers. It has been noted that iron deficiency leads to inattention, hyperactivity, and impulsivity in children whereas zinc is associated with behavioral problems in children with ADHD [7]. The DRD4 gene is another biomarker of interest for the early diagnosis of ADHD and is the most studied gene associated with ADHD, which many believe has a correlation with the novelty seeking trait in ADHD individuals. The DRD4 gene codes for the Dopamine receptor D4, which can be activated by dopamine, and has been linked to many behavioral traits associated with ADHD. It has been shown the DRD4 gene is susceptible to different polymorphisms that can lead to a decline in promoter activation and thus lower mRNA levels that have been noted in individuals with ADHD [8]. Alpha-2A-adrenergic receptor (ADRA2A) is another key gene related to ADHD. Studies have shown that individuals with homozygous G alleles (ADRA2A polymorphism) had higher scores in a test of ADHD symptoms. Particularly, it was observed that those

with homozygous G alleles scored significantly higher for symptoms of inattentive ADHD compared to those with other genotypes. This gene is important for solving our problem as we established that many females are underdiagnosed for ADHD due to their symptoms presenting as inattentive rather than hyper-active[9].

The recent emergence of synthetic biology entailed new possibilities for ADHD. The combination of these different biomarkers can allow us to differentiate between inattentive type ADHD and hyperactive type ADHD without the immediate presentation of symptoms. To detect ADHD, our synthetic biology circuit will utilize Iron and DRD4 in a NOR gate and Zinc and ADRA2A in another NOR gate. Our 2 NOR gates will then result in an AND gate to produce our coloured output via amilCP chromoprotein, as seen in Figure 7[A7]. In the presence of low iron, low zinc, mutated DRD4 and mutated ADRA2A, the circuit will produce amilCP to indicate that the individual has ADHD.

Materials

Part	Type	Obtained from
BBa_J23100	Consecutive promoter for DRD4 and ADRA2A	iGEM [10]
BBa_I765000	Iron promoter	iGEM [11]
BBa_K190016	Zinc Promoter	iGEM [12]
BBa_R0040	Intermediate 1 promoter	iGEM [13]
BBa_R0051	Intermediate 2 promoter	iGEM [14]
BBa_B0034	Ribosome binding site (RBS)	iGEM [15]
TetR	PCS for DRD4 and iron logic gate	iGEM [16]
cl repressor	PCS for ADRA2A and zinc logic gate	iGEM [17]
luxR	PCS for Intermediate 1	iGEM [18]
lasR	PCS for Intermediate 2	iGEM [19]
BBa_K091117	lasR Promoter	iGEM [20]
BBa_I1051	luxR promoter	iGEM [21]
amilCP	Output PCS	iGEM [22]
BBa_B0010	terminator	iGEM [23]
pUC19	Plasmid backbone	Addgene [24]
E. coli DH5α	Host Bacteria	Thermofisher [25]
BbsI	Restriction enzyme	NEB [26]
PureLink PCR purification Kit	-	Thermofisher [27]
PCR materials as listed in protocol	-	Thermofisher [28]
Restriction digestion materials listed in protocol	-	NEB [29]
Ligation materials as listed in protocol	-	Thermofisher [30]
Blood agar base	-	Thermofisher [31]

Methods

Constructing the biological circuit

Due to the inability to conduct a true live experiment, Simbiology simulation is needed to confirm that the relationships between the molecules and genes would give the expected results. For the sake of simplicity, certain assumptions had to be made, such as RNase concentration being constant throughout the whole reaction, protein decay is neglected, and mRNA dilution is neglected. The equations used for the rate of change of mRNA molecules (left) and proteins molecules (right), are:

$$\frac{d[mRNA]}{dt} = \frac{k_{tr}\left(\frac{W^n}{K^n}\right)^{\mu}}{1+\left(\frac{W^n}{K^n}\right)} - dm \cdot [mRNA] \qquad \frac{d[P]}{dt} = k_{tl}[mRNA] - d_p \cdot [P]$$

To amplify the sequences of DNA required to construct our biological circuit, a PCR reaction will be performed following the outlined procedure [32]. The specific primer sequence for each genetic component is listed in Table 1, in Appendix B [B1].

The plasmid will undergo restriction digestion to allow for the ligation of the biological circuit following the protocol outlined by Addgene [33]. This means that the backbone plasmid will be cut at two restriction sites with the restriction enzyme BbsI. This enzyme combined with a reaction buffer, and water will cause the plasmid to separate after a one hour incubation. Gel electrophoresis can be conducted to ensure the digestion is successful. Following digestion, the results will be purified following the protocol outlined in the PureLink PCR Purification kit [34]

Golden Gate Assembly will be used to compile the different circuit components [36]. In this assembly, type IIS restriction enzymes will be used, which do not cut at the site of recognition, meaning the sticky ends can be customized for each joint. BbsI cuts 4 base pairs starting two base pairs downstream from the recognition site. The primers included the 6 base long recognition site, 2 bases which will be cleaved, 4 bases which will be the sticky ends that would stick to the adjacent segment's primer, and finally around 20 bases to stick to the parent segment [36].

After the ligation of the biological circuit into the plasmid as outlined in the protocol by Addgene, heat shock transformation is used to introduce the plasmid into the bacteria[36]. Calcium chloride is added to the bacteria to make them chemically competent so that when the bacteria is heated, its pores widen and the plasmid can enter the cell. When the heat subsides, the cell membrane will recover to its original state. Following this transformation, the bacteria will be plated on a blood agar plate for cultivation [37].

In Vitro Experiments to test the Efficacy of the Biological Circuit

Transforming DRD4 and ADRA2A Genes into a Bacterial Cell

Our circuit recognizes mutations in DRD4 and ADRA2A and uses these mutations to repress our promoters. To replicate this environment *in vitro*, mutated DRD4 and ADRA2A genes will be transformed into a bacterial cell following the same procedure outlined above except we will use PCR to amplify the DRD4 and ADRA2A genes only and have those genes inserted into the plasmid backbone and then have the plasmid containing the 2 genes transformed into the *E. coli.* To replicate the normal condition, the unmutated genes will be used.

Experiment 1-Testing the sensitivity of the circuit

Experiment 1 is conducted to test whether the promoters are sensitive enough to detect abnormally low concentrations of biomarkers. A positive control-medium that contains cells with mutated DRD4 and ADRA2A genes, and no zinc and iron, will be used. This positive control is going to mimic the *in vivo* ADHD case. This positive control should produce a blue output since the absence of iron and zinc and the mutated DRD4 and ADRA2A will trigger the circuit and produce the required output. A negative control-circuit will include the circuit produced without any ligation enzyme during the construction of the circuit. Since there is no ligation enzyme, the circuit will not be produced properly and no output would be produced. Our tests would include plating the properly performed transformation reaction on a medium with either high or low levels of our biomarkers to test the sensitivity of our circuit. For the medium containing low levels of biomarkers(<0.6 mcg/mL for both iron and zinc [38] [39]) and mutated DRD4 and ADRA2A genes, a blue output is expected. This case mimics the *in vivo* environment of someone with ADHD. For the medium containing high levels of biomarkers(>1.7 mcg/mL for both iron and zinc [38] [39]) and the normal DRD4 and ADRA2A genes, we would expect there to be little to no output since this test will mimic the *in vivo* environment of someone who does not have ADHD.

Experiment 2- Determining the Optimal Temperature for Growth

Genetic circuits are fragile and perturbations to environmental conditions frequently alter their behavior. While experiment 1 will be run at room temperature (20°C), experiment 2 will run at different temperatures. Blood temperature is around 37°C, so we want to replicate this environment *in vitro* to see if there will be any discrepancies between incubating at different temperatures and to ensure our results are most representative of the results we would see if we were to test *in vivo*.[40][41] Our tests would be the properly produced biological circuit following the procedure outlined in the method section for constructing the biological circuit. The test, positive control and negative control would all be plated on a medium containing no biomarkers and the mutated DRD4 and ADRA2A. The positive and negative control will incubate at a temperature of 20°C, the first test at 37°C, and the second test at 10°C. The tests will incubate for a specified time and they will be analyzed. *E. coli* growth rate increases with increasing temperature, and so the test incubated at 37°C would be expected to have the greatest growth after the specified time [42].

Results

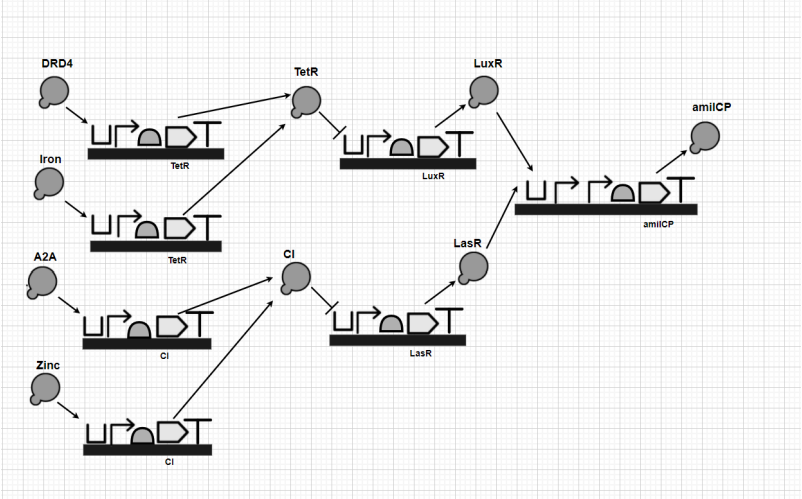

Figure 2: As a summary of the circuit, biomarkers like DRD4, iron, A2A, and zinc are used as the first layers of input. DRD4 forms a NOR gate with iron, and A2A forms another NOR gate with zinc. Under normal conditions, the outputs would be TetR and Cl repressor, they serve to inhibit the production of LuxR and LasR respectively. As a result, this is because only when LuxR and LasR are both present (AND gate), amilCP would be produced. Therefore, normal levels of the biomarkers would prevent the output of amilCP. On the other hand, under conditions where DRD4 and A2A are mutated, as well as iron and zinc deficiency, amilCP would be produced due to no TetR and Cl repression.

Simulation of the biological circuit is conducted in a Simbiology model. The levels of AmilCP are compared across various different scenarios. The following graphs show the results obtained from the simulation.

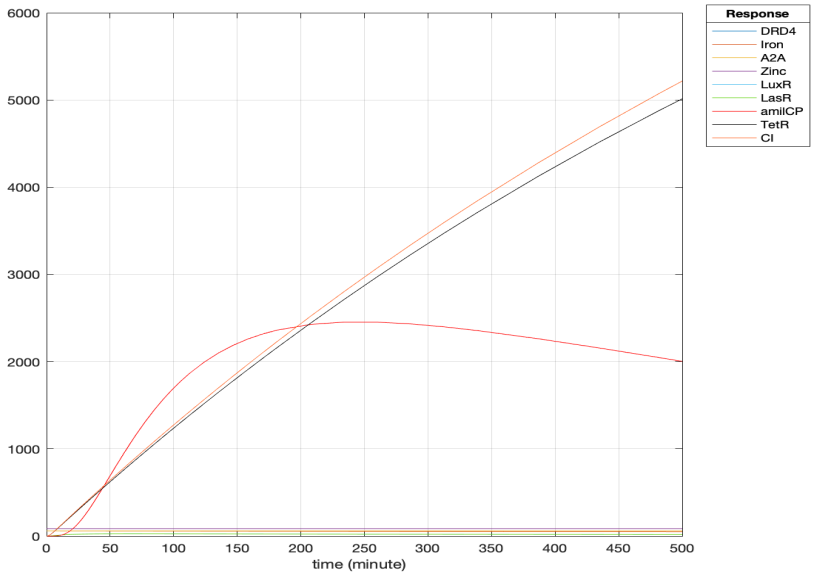

Figure 3: This graph shows the production of AmilCP when all biomarkers are present. A heightened level of TetR and Cl are observed. At the same time, LuxR and LasR levels are close to zero. The final levels of AmilCP after 500 minutes settle at around 2000 μM.

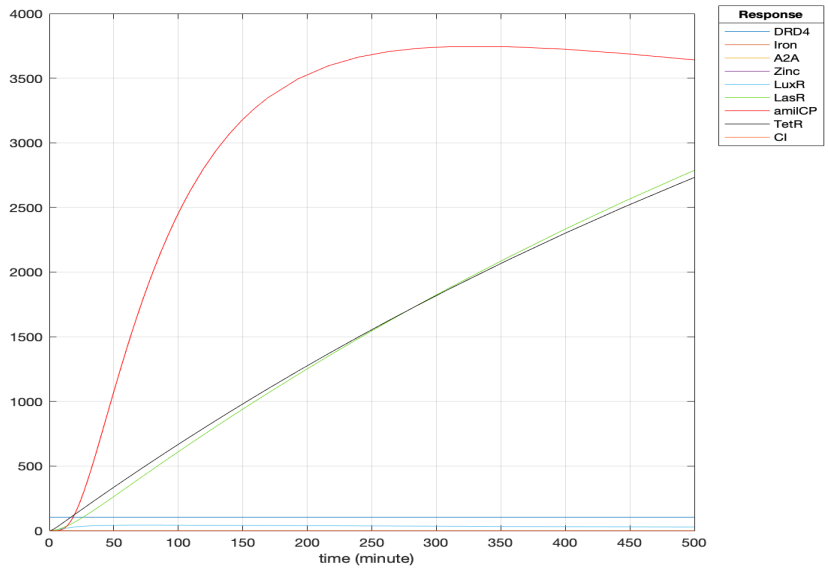

Figure 4: This graph shows the production of AmilCP when only DRD4 is present. A high level of Cl and LasR is observed, whereas no TetR and LuxR is shown. The final levels of AmilCP after 500 minutes settle at around 3600 µM.

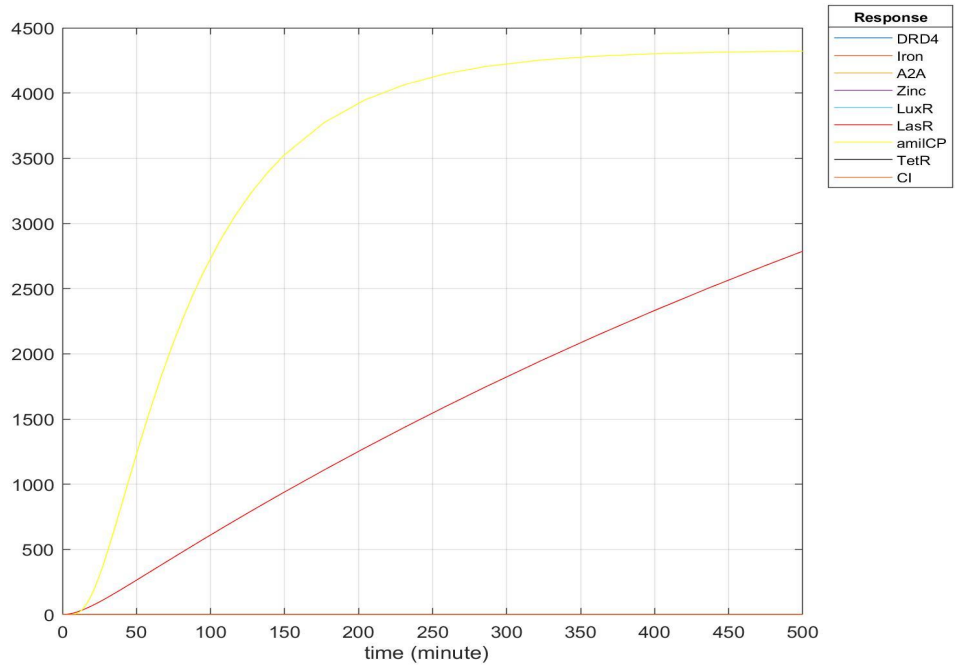

Figure 5: This graph shows the production of AmilCP when no biomarkers are present. A high level of LuxR and LasR is observed, whereas no TetR and Cl is shown. The final levels of AmilCP after 500 minutes settle at around 4300 µM.

Discussion

The results show that our biological circuit can successfully detect the presence of ADHD biomarkers. Our findings extend the work of Alejandro et al, as they discovered the connection between low iron and zinc levels and ADHD, and Myers et al who analyzed DNA variants within DRD4 and ADRA2A and presentended the possibility of their link to ADHD[43][44]. The fact that our circuit was able to detect the presence of these biomarkers, signifies its potential as an efficient and non-subjective diagnostic tool.

Specifically, using amilCP as an indicator, we tested our circuit at various levels of ADHD biomarkers. Key findings were that amilCP gets produced in all cases, however, the amount varies based on the presence of biomarkers. AmilCP levels reached 2000μM when mimicking biomarker levels of a healthy individual - 1.7 mcg/mL of iron and zinc, and non-mutated ADRA2A and DRD4 genes. However, in an environment where all biomarker levels are abnormal except for DRD4, the results show elevated levels of AmilCP at 3600 μM. In order for such a result to occur, there must be a mutation in the ADRA2A gene. This is significant, as while it did not meet the requirement for a positive diagnosis, this genetic variation may signify that the person is at risk for developing ADHD later in life[45]. Lastly, in the case that no biomarkers are present, the final levels of amilCP reach 4300μM. This results signifies that the circuit is successful in detecting biomarkers of ADHD and produces high levels of amilCP(4300 μM) as a result. Moreover, as amilCP production results in a bright fluorescence, these results can be observed visually without the need for special equipment. To help differentiate between the different levels of amilCP (ex. 3600 vs 4300μM), we will create a reference chart. However, in cases where the difference is not clear, a fluorometer can be used to measure the fluorescence produced from the amilCP. More research is needed to determine exact fluorescence values, and ultimately determine a fluorescence threshold for a positive ADHD diagnosis.

The success of our circuit in these initial tests indicates promise for similar biological circuits being engineered for other mental illnesses that currently have no non-subjective methods of diagnosis. Specifically, research has shown elevated levels of cortisol, deletion of the GABRA3 gene and mutations in the 5-HTT gene are all potential biomarkers for depression[46]. This information can be used to create a biological circuit similar to ours that can detect these biomarkers.

This solution is feasible, however, there are limitations and assumptions made that may have negative effects on the proposed circuit. An assumption was made that there are only 2 implicated genes associated with ADHD, when in reality, there are numerous to focus on. We only focused on DRD4 , which is one of the most studied genes for ADHD, and ADRA2A, a receptor that can be directly stimulated by certain ADHD drugs[47][48]. Some limitations include the use of zinc and iron in our circuit. Deficiencies in both of these are associated with an increased risk of ADHD, but the location in which we access the levels of biomarkers may not be optimal and can interfere with part function. For instance, only trace amounts of zinc are present in the blood and a zinc deficiency is difficult to identify from a blood sample, which can affect the zinc promoter[7]. Another interference is that iron is the most common nutritional deficiency, limiting its accuracy as an indicator of ADHD[49]. This would have an impact on the iron promoter, as low iron may not necessarily correlate to ADHD.

Conclusion

The proposed circuit that produces an coloured output in the presence of low iron, low zinc, mutated DRD4 and mutated ADRA2A, displayed its feasibility through the SimBiology Model. Our threshold for amilCP can be set to around 4300 μM to indicate that the individual has ADHD. To further our model, more *in vitro* testing would need to be done to determine where we can find optimal levels of our biomarkers to ensure the most accurate results, as well as determining the threshold levels of our biomarkers to identify a more accurate threshold for amilCP. *In vivo* testing would need to be done to determine the efficacy of our circuit using these newer threshold values and ensure we get the expected output. Eventually we would like to market our circuit and include freeze-dried versions of our synthetic gene circuits can be used to create an extremely low cost and highly portable diagnostic tool for ADHD.This tool would allow us to diagnose women and younger children sooner than they would otherwise be diagnosed to allow for enough time to develop techniques to cope with symptoms of ADHD that may make school, relationships and life more challenging.

Appendix A

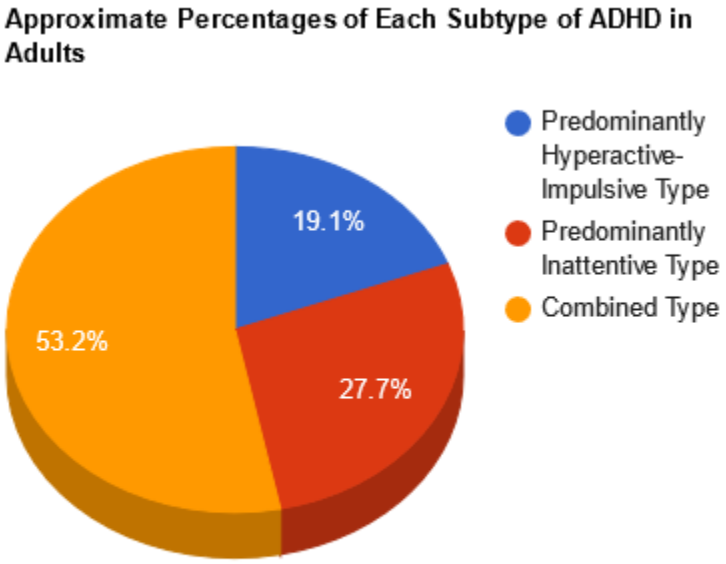

Figure 6: The approximate percentages of the three different subtypes of ADHD.

DRD4	Iron	Intermediate 1	ADRA 2A	Zinc	Intermediate 2	Intermediate 1	Intermediate 2	Output
0	0	1	0	0	1	0	0	0
1	0	0	1	0	0	1	0	0
0	1	0	0	1	0	0	1	0
1	1	0	1	1	0	1	1	1

Figure 7: Logic gates for the biological circuit. The 2 NOR gates highlighted in orange and the one AND gate in blue.

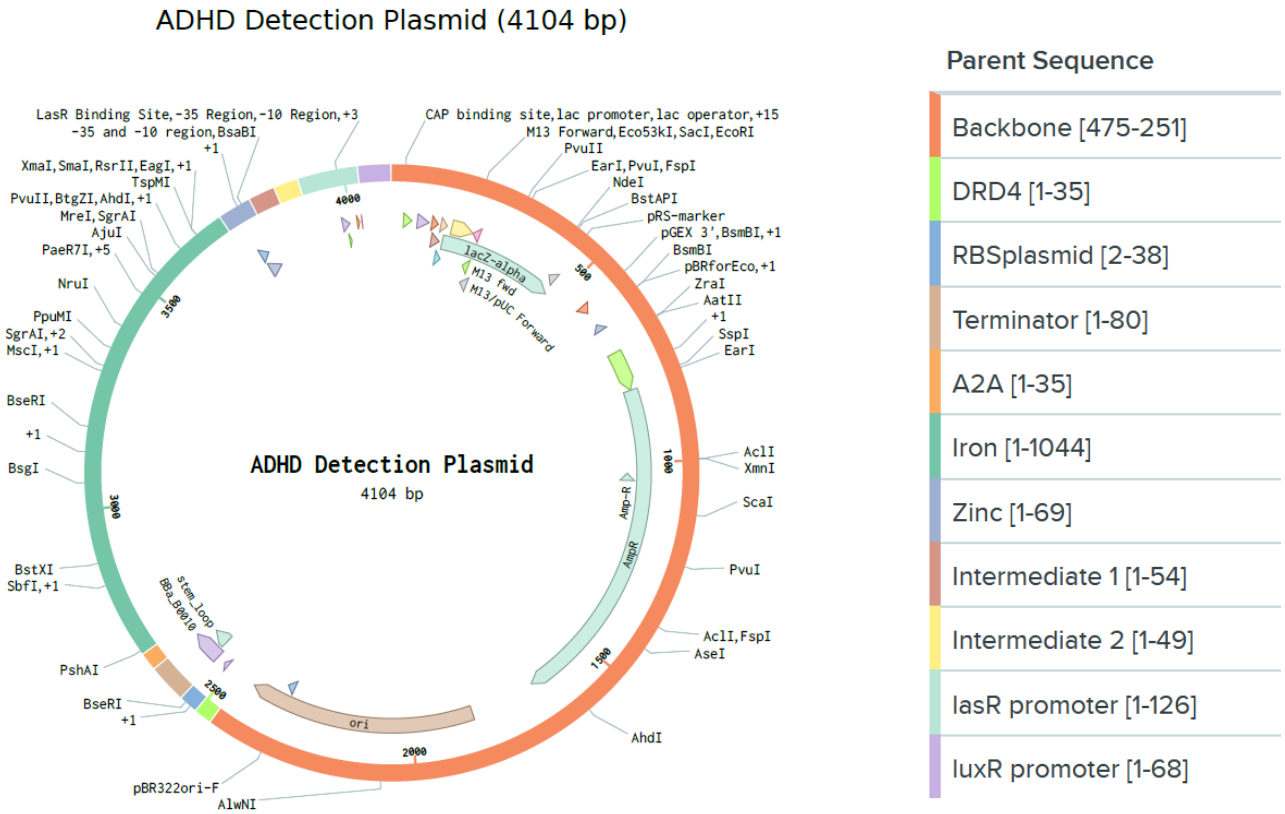

Figure 8: The final assembly of the different parts. Due to potential redundancy, only the first promoter had an RBS and a terminator added.

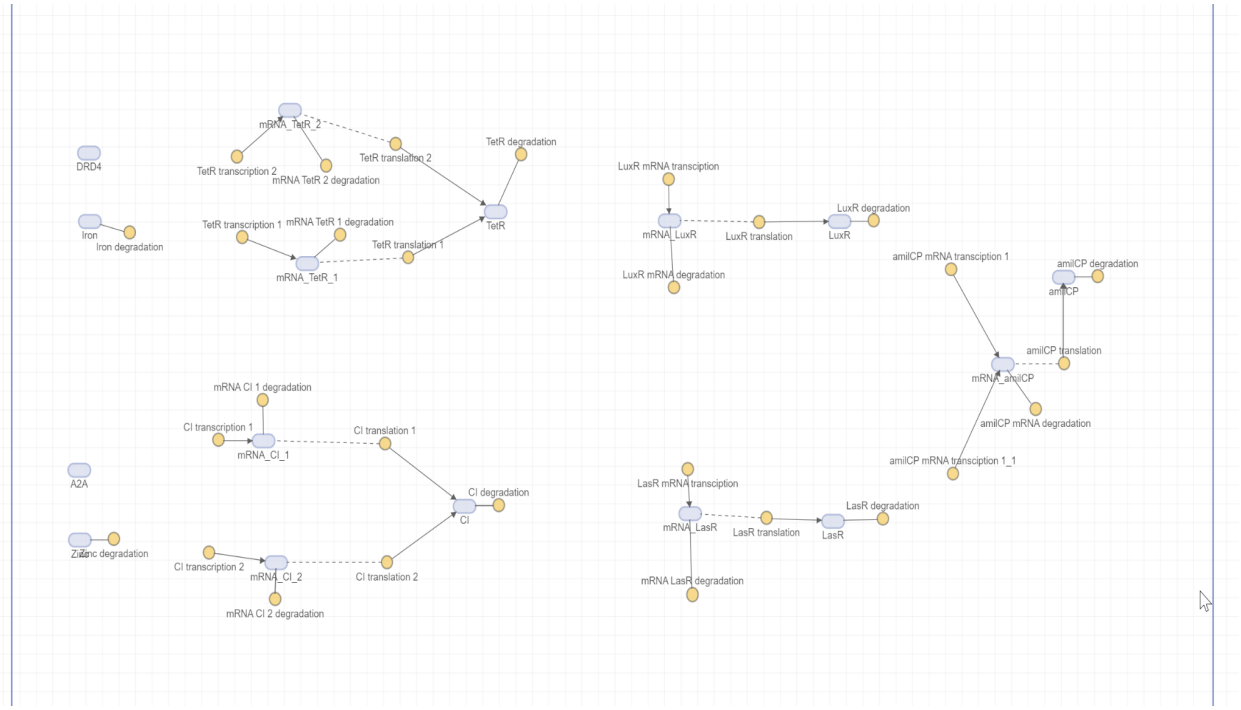

Figure 9: As a summary of the circuit, biomarkers like DRD4, iron, A2A, and zinc are used as the first layers of input. DRD4 forms a NOR gate with iron, and A2A forms another NOR gate with zinc. Under normal conditions, the outputs would be TetR and Cl repressor, they serve to inhibit the production of LuxR and LasR respectively. This is because only when LuxR and LasR are both present would amilCP be produced, and. Therefore, having normal levels of the biomarkers would actually prevent the output of amilCP. On the other hand However, under conditions where DRD4 and A2A are mutated, as well as iron and zinc deficiency, amilCP would be produced due to no TetR and Cl repression.

Appendix B

Table 1: the forward and reverse primer of each part. BbsI restriction enzyme recognition sites in bold, sticky ends underlined, bases that bind to parents in italics.

Part	Forward primer	Reverse primer
Backbone	**GAAGAC**GCAACG*ctggaaagcgggcagtgagc*	**GAAGAC**GCGTAT*ccaggaaccgtaaaaaggcc*
DRD4	**GAAGAC**GCATAC*ttgacggctagctcagtcct*	**GAAGAC**GCCGAA*gctagcactgtacctaggac*
RBS	**GAAGAC**GCTTCG*actagagaaagaggagaaa*	**GAAGAC**GCGCCC*taggtctctcatttagtatt*
Terminator	**GAAGAC**GCGGGC*ccaggcatcaaataaaacga*	**GAAGAC**GCGCAC*gagagcgttcaccgacaaac*
A2A	**GAAGAC**GCGTGC*ttgacggctagctcagtcct*	**GAAGAC**GCGACC*gctagcactgtacctaggac*
Iron	**GAAGAC**CCGGTC*atgacgcttgtcgacagatt*	**GAAGAC**GGGCCT*ttaggtgccctggccgttcc*
Zinc	**GAAGAC**GGAGGC*cgtccgctcgctgtatctct*	**GAAGAC**GGTGGG*attaaccgaaggatacactc*
Intermediate1	**GAAGAC**GGCCCA*tccctatcagtgatagagat*	**GAAGAC**GGAGAG*gtgctcagtatctctatcac*
Intermediate2	**GAAGAC**GGCTCT*taacaccgtgcgtgttgact*	**GAAGAC**GGTCAG*gcaaccattatcaccgccag*
lasR promoter	**GAAGAC**GGCTGA*tgttctcgtgtgaagccatt*	**GAAGAC**GGCGGC*ctgaagaatttatacaaatt*
luxR promoter	**GAAGAC**GGGCCG*acctgtaggatcgtacaggt*	**GAAGAC**GGCGTT*tatcaccgccagaggtattc*

References

[1] “What is ADHD?,” *Centers for Disease Control and Prevention*, 23-Sep-2021. [Online]. Available:https://www.cdc.gov/ncbddd/adhd/facts.html#:~:text=ADHD%20is%20one%20of%20the,)%2C%20or%20be%20overly%20active. [Accessed: 06-Feb-2022].

[2] “Team:Groningen/pigmentproduction - 2012.Igem.Org,” Igem.org. [Online]. Available: http://2012.igem.org/Team:Groningen/pigmentproduction. [Accessed: 12-Apr-2022]

[3] S. B. Guze, “Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (DSM-IV),” Am. J. Psychiatry, vol. 152, no. 8, pp. 1228–1228, 1995.

[4] T. S. Emser, B. A. Johnston, J. D. Steele, S. Kooij, L. Thorell, and H. Christiansen, “Assessing ADHD symptoms in children and adults: evaluating the role of objective measures”, *Behavioral and Brain Functions*, vol. 14, no. 1, 2018.

[5] P. O. Quinn and M. Madhoo, “A review of attention-deficit/hyperactivity disorder in women and girls,” *The Primary Care Companion For CNS Disorders*, 2014.

[6] A. Thapar and E. Stergiakouli, “An overview on the genetics of ADHD,” *Xin li xue bao. Acta psychologica Sinica*, Aug-2008. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2854824/. [Accessed: 06-Feb-2022].

[7] S. V. Faraone, C. Bonvicini, and C. Scassellati, “Biomarkers in the diagnosis of ADHD – promising directions,” *Current Psychiatry Reports*, vol. 16, no. 11, 2014.

[8] J. Gelernter, H. Kranzler, E. Coccaro, L. Siever, A. New, and C. L. Mulgrew, “D4 dopamine-receptor (DRD4) alleles and novelty seeking in substance-dependent, personality-disorder, and control subjects,” *The American Journal of Human Genetics*, vol. 61, no. 5, pp. 1144–1152, 1997.

[9] T. Roman, G. V. Polanczyk, C. Zeni, J. P. Genro, L. A. Rohde, and M. H. Hutz, “Further evidence of the involvement of alpha-2a-adrenergic receptor gene (ADRA2A) in inattentive dimensional scores of attention-deficit/hyperactivity disorder,” *Molecular Psychiatry*, vol. 11, no. 1, pp. 8–10, 2005.

[10] “BBa_J23100,” *Part:BBa_J23100*. [Online]. Available: http://parts.igem.org/Part:BBa_J23100. [Accessed: 11-Apr-2022].

[11] “BBa_I1765000,” *Part:BBa_I1765000*. [Online]. Available: http://parts.igem.org/Part:BBa_I1765000. [Accessed: 11-Apr-2022].

[12] “BBa_K190016,” *Part:BBa_K190016*. [Online]. Available: http://parts.igem.org/Part:BBa_K190016. [Accessed: 11-Apr-2022].

[13] “BBa_R0040,” *Part:BBa_R0040*. [Online]. Available: http://parts.igem.org/Part:BBa_R0040. [Accessed: 11-Apr-2022].

[14] “BBa_R0051,” *Part:BBa_R0051*. [Online]. Available: http://parts.igem.org/Part:BBa_R0051. [Accessed: 11-Apr-2022].

[15] “BBa_B0034,” *Part:BBa_B0034*. [Online]. Available: http://parts.igem.org/Part:BBa_B0034. [Accessed: 11-Apr-2022].

[16] “BBa_K3024003,” *Part:BBa_K3024003*. [Online]. Available: http://parts.igem.org/Part:BBa_K3024003. [Accessed: 11-Apr-2022].

[17] “BBa_C0081,” *Part:BBa_C0081*. [Online]. Available: http://parts.igem.org/Part:BBa_C0081. [Accessed: 11-Apr-2022].

[18] “BBa_C0062,” *Part:BBa_C0062*. [Online]. Available: http://parts.igem.org/Part:BBa_C0062. [Accessed: 11-Apr-2022].

[19] “BBa_C0079,” *Part:BBa_C0079*. [Online]. Available: http://parts.igem.org/Part:BBa_C0079. [Accessed: 11-Apr-2022].

[20] “BBa_K091117,” *Part:BBa_K091117*. [Online]. Available: http://parts.igem.org/Part:BBa_K091117. [Accessed: 11-Apr-2022].

[21] “BBa_I1051,” *Part:BBa_I1051*. [Online]. Available: http://parts.igem.org/Part:BBa_I1051. [Accessed: 11-Apr-2022].

[22] “Part:BBa K592009 - parts.Igem.Org,” *Igem.org*. [Online]. Available: http://parts.igem.org/Part:BBa_K592009. [Accessed: 12-Apr-2022].

[23] “BBa_B0010,” *Part:BBa_B0010*. [Online]. Available: http://parts.igem.org/Part:BBa_B0010. [Accessed: 11-Apr-2022].

[24] “Puc19 (plasmid #50005),” *Addgene*. [Online]. Available: https://www.addgene.org/50005/. [Accessed: 11-Apr-2022].

[25] “DH5α competent cells,” *Thermo Fisher Scientific - CA*. [Online]. Available: https://www.thermofisher.com/ca/en/home/life-science/cloning/competent-cells-for-transformation/competent-cells-strains/dh5a-competent-cells.html. [Accessed: 11-Apr-2022].

[26] N. E. Biolabs, “BbsI,” *NEB*. [Online]. Available: https://international.neb.com/products/r0539-bbsi#Product%20Information. [Accessed: 11-Apr-2022].

[27] “PureLink™ PCR Purification Kit,” *Thermo Fisher Scientific - US*. [Online]. Available: https://www.thermofisher.com/order/catalog/product/K310001. [Accessed: 11-Apr-2022].

[28] “Phusion High-Fidelity PCR kit,” *Thermo Fisher Scientific - US*. [Online]. Available: https://www.thermofisher.com/order/catalog/product/F553S?SID=srch-srp-F553S. [Accessed: 11-Apr-2022].

[29] N. E. Biolabs, “Restriction endonucleases,” *NEB*. [Online]. Available: https://international.neb.com/products/restriction-endonucleases/restriction-endonucleases. [Accessed: 11-Apr-2022].

[30] “Rapid DNA ligation kit,” *Thermo Fisher Scientific - US*. [Online]. Available: https://www.thermofisher.com/order/catalog/product/K1422?SID=srch-srp-K1422. [Accessed: 11-Apr-2022].

[31] “Blood agar base (dehydrated),” *Thermo Fisher Scientific - US*. [Online]. Available: https://www.thermofisher.com/order/catalog/product/CM0055B. [Accessed: 11-Apr-2022].

[32] “Plasmid cloning by PCR,” *Addgene*. [Online]. Available: https://www.addgene.org/protocols/pcr-cloning/. [Accessed: 01-Mar-2022].

[33] “Restriction digest of plasmid DNA,” *Addgene*. [Online]. Available: https://www.addgene.org/protocols/restriction-digest/. [Accessed: 01-Mar-2022].

[34] “PureLink PCR purification kit - thermo fisher scientific.” [Online]. Available: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/purelink_pcr_man.pdf. [Accessed: 01-Mar-2022].

[35] New England Biolabs, “Golden Gate assembly,” *Neb.com*. [Online]. Available: https://international.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/golden-gate-assembly. [Accessed: 11-Apr-2022].

[36] “DNA ligation,” *Addgene*. [Online]. Available: https://www.addgene.org/protocols/dna-ligation/. [Accessed: 01-Mar-2022].

[37] M. Drancourt and D. Raoult, “Cost-effectiveness of blood agar for isolation of mycobacteria,” *PLoS Negl. Trop. Dis.*, vol. 1, no. 2, p. e83, 2007. Available: http://dx.doi.org/10.1371/journal.pntd.0000083. [Accessed: 11-Apr-2022].

[38] “Serum Iron Test,” *Mount Sinai Health System*. [Online]. Available: https://www.mountsinai.org/health-library/tests/serum-iron-test#:~:text=Normal%20value%20range%20is%3A,per%20liter%20(micromol%2FL). [Accessed: 01-Mar-2022].

[39] “Test ID : ZNS,” *ZNS - Overview: Zinc, Serum*. [Online]. Available: https://www.mayocliniclabs.com/test-catalog/overview/8620. [Accessed: 01-Mar-2022].

[40] I. I. Geneva, B. Cuzzo, T. Fazili, and W. Javaid, “Normal body temperature: A systematic review,” *Open Forum Infect. Dis.*, vol. 6, no. 4, p. ofz032, 2019.

[41] T. Roman, G. V. Polanczyk, C. Zeni, J. P. Genro, L. A. Rohde, and M. H. Hutz, “Further evidence of the involvement of alpha-2a-adrenergic receptor gene (ADRA2A) in inattentive dimensional scores of attention-deficit/hyperactivity disorder,” *Molecular Psychiatry*, vol. 11, no. 1, pp. 8–10, 2005.

[42] P. K. Mishra and V. Thawani, “Effect of temperature in growth of E. Coli,” Effect of Temperature in growth of E. coli | International Journal of Current Research, 30-Jul-2018. [Online]. Available: https://www.journalcra.com/article/effect-temperature-growth-e-coli. [Accessed: 01-Mar-2022].

[43] H. Robberecht, A. A. J. Verlaet, A. Breynaert, T. De Bruyne, and N. Hermans, “Magnesium, iron, zinc, copper and selenium status in attention-deficit/hyperactivity disorder (ADHD),” *Molecules (Basel, Switzerland)*, 27-Sep-2020. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7583976/. [Accessed: 11-Apr-2022].

[44] N. M. Myer, J. R. Boland, and S. V. Faraone, “Pharmacogenetics predictors of methylphenidate efficacy in childhood ADHD,” *Molecular psychiatry*, Sep-2018. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7039663/. [Accessed: 11-Apr-2022].

[45] A. P. A.-B. J; “Annual research review: Does late-onset attention-deficit/hyperactivity disorder exist?,” *Journal of child psychology and psychiatry, and allied disciplines*. [Online]. Available: https://pubmed.ncbi.nlm.nih.gov/30843223/. [Accessed: 11-Apr-2022].

[46] A. Tamatam, F. Khanum, and A. S. Bawa, “Genetic biomarkers of depression,” *Indian journal of human genetics*, Jan-2012. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3385174/. [Accessed: 11-Apr-2022].

[47] L. Tovo-Rodrigues, L. A. Rohde, A. M. B. Menezes, G. V. Polanczyk, C. Kieling, J. P. Genro, L. Anselmi, and M. H. Hutz, “DRD4 rare variants in attention-deficit/hyperactivity disorder (ADHD): Further evidence from a birth cohort study,” *PloS one*, 31-Dec-2013. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3877354/. [Accessed: 11-Apr-2022].

[48] “Get to know a gene: ADRA2A,” *GeneSight*, 13-Mar-2022. [Online]. Available: https://genesight.com/white-papers/get-to-know-a-gene-adra2a/. [Accessed: 11-Apr-2022].

[49] J. Cafasso, “Iron deficiency anemia secondary to inadequate dietary iron intake,” Healthline, 24-Dec-2017. [Online]. Available: https://www.healthline.com/health/iron-deficiency-inadequate-dietary-iron. [Accessed: 03-April 2022].