Design Brief

Dihydrotestosterone (DHT) biosensor for accessible health monitoring*

Avraham Abbett, Caroline Kaplan, Charlotte Kaplan, Aaron Missaghi, Hayim Sims, and Shlomo Vedol.▫
Maimonides School, Brookline, MA

Reviewed on 4 May 2024; Accepted on 10 June 2024; Published on 26 October 2024

Dihydrotestosterone (DHT), is a hormone and an androgen that plays a crucial role in the development of male sexual organs. In excess, DHT is linked to various health concerns like Androgenic alopecia and prostate cancer. On the other hand, low DHT levels are associated with disorders such as 5-alpha-reductase deficiency (5ARD) leading to intersex conditions. Traditionally, DHT levels are measured using High-Performance Liquid Chromatography (HPLC), which is expensive, time-consuming, and inaccessible to many. Other techniques, such as enzyme-linked immunosorbent assay (ELISA) utilize antibodies that have the potential to cross-react with other hormones and androgens and produce inaccurate results. Our study introduces a more affordable, rapid, and accessible alternative using concepts from synthetic biology. We have developed a biosensor design employing a transcription factor (TF) from bacterial strain Pimelobacter simplex (P. simplex), known to bind well to hormones. The TF, SRTF1, previously identified to respond to progesterone (Grazon et. al, 2020), is hypothesized to also bind DHT. On that basis, this research utilizes a cell-free gene expression system (CFE) derived from Escherichia coli developed by Arbor Biosciences, which incorporates a DNA template with a T7 promoter and green fluorescent protein (GFP) as a visual output. The presence of DHT will promote the binding of SRTF1 to the Steroid Responsive Genomic Island (SRGI) site within the T7 promoter, consequently permitting GFP production. The fluorescent output varies according to the DHT concentration, offering an easily interpretable quantitative measurement of DHT levels. Our biosensor demonstrates great potential as an inexpensive, accessible means for DHT testing particularly beneficial for communities with limited access to medical facilities. This innovative approach not only aids in the early diagnosis and treatment of DHT-related health issues but also contributes to the broader field of diagnostics and hormone-sensing technologies.

Keywords: Dihydrotestosterone (DHT), biosensor, cell-free system, alopecia, 5-alpha-reductase disorder

Dihydrotestosterone (DHT) is an androgen, a hormone originating in the liver that plays an important role in various physiological processes. DHT particularly influences male sexual maturation and the development of secondary sex characteristics (Anawalt, 2017) by binding to androgen receptors in tissues, exerting a more potent effect than testosterone due to its high affinity for these receptors (Horton, 1992). The interaction between DHT and testosterone regulates the

* *The authors were mentored by Maria B. Lazebnik from Maimonides School and Scott Gaines from Boston University. Please direct correspondence to: simshayim@gmail.com.*

development of male genitalia during gestation and affects other male secondary characteristics during puberty. In adult males, DHT is essential in maintaining muscle mass, bone density, libido, hair growth, muscle development, and reproductive function (Marchetti & Barth, 2013). Therefore, it is necessary to ensure normalized levels of DHT of 50 ng/mL in both adolescence and adulthood, as it trends upward in later stages of life (Baetens et. al, 2014).

Due to its importance in growth, abnormal DHT levels can drastically affect health and lead to diseases (Azizi et. al, 2021). Higher levels of DHT (60-80 ng/mL) (Figure 1) have been shown to induce hair loss, such as in the case of Androgenic alopecia, inadult males due to the absence of testosterone (Urysiak-Czubatka et. al, 2014). Increased levels of DHT have been found to beprevalent in a large percentage of middle-aged males in the US (Swerdloff et. al, 2017) and globally (Gustaffson et. al, 1996). Increased levels have been correlated to an increased risk of prostate cancer (Kosaka et. al, 2014). Conversely, insufficient levels of DHT (15-40 ng/mL) (Figure 1), as seen in 5ARD, can lead to intersex conditions due to negative impacts on gestation (Nascimento et. al, 2018). Intersex conditions can affect self-perception and provide long-term psychological and physical challenges throughout an individual's lifetime (Esteban et. al, 2023). Underdeveloped nations have increased difficulty determining the incidence rate of DHT deficiency in their populations, where diagnosis techniques remain inaccessible. Certain regions of the Dominican Republic have that 1 in 29 children are afflicted with 5ARD, yet lack critical tools to perform the testing to determine if a child's sex at birth was misdiagnosed (Cai et. al, 1996). These children may only know their sex at birth more than a decade after conception, which can pose emotional and psychological burdens to these patients (Cheon, 2011).

The predominant methods for testing DHT levels involve either High Pressure Liquid Chromatography (HPLC) or an Enzyme-linked immunosorbent assay (ELISA). HPLC is a sophisticated, costly, and resource-intensive procedure, that requires close proximity to an urban environment to procure specialized laboratory tools necessary to function (Wang et. al, 2018). Even within developed nations, a substantial financial investment, often thousands of dollars, is required for HPLC services. Furthermore, the process is time-

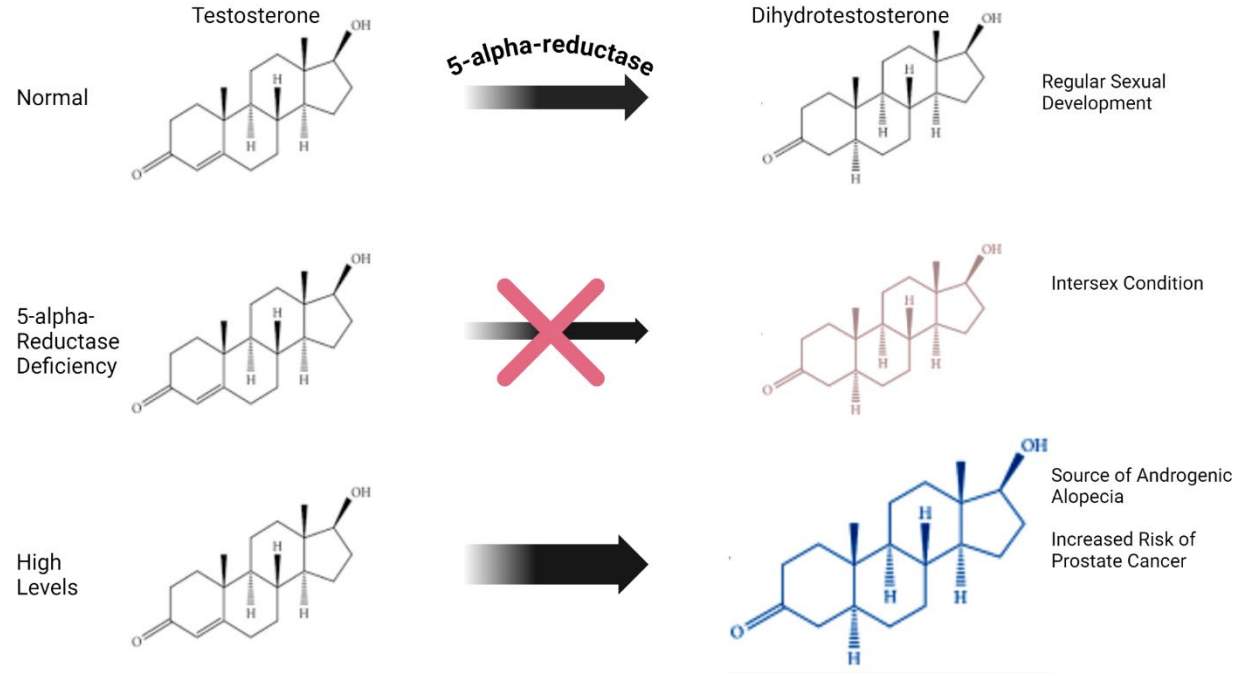

Figure 1. Phenotypic effects of DHT.

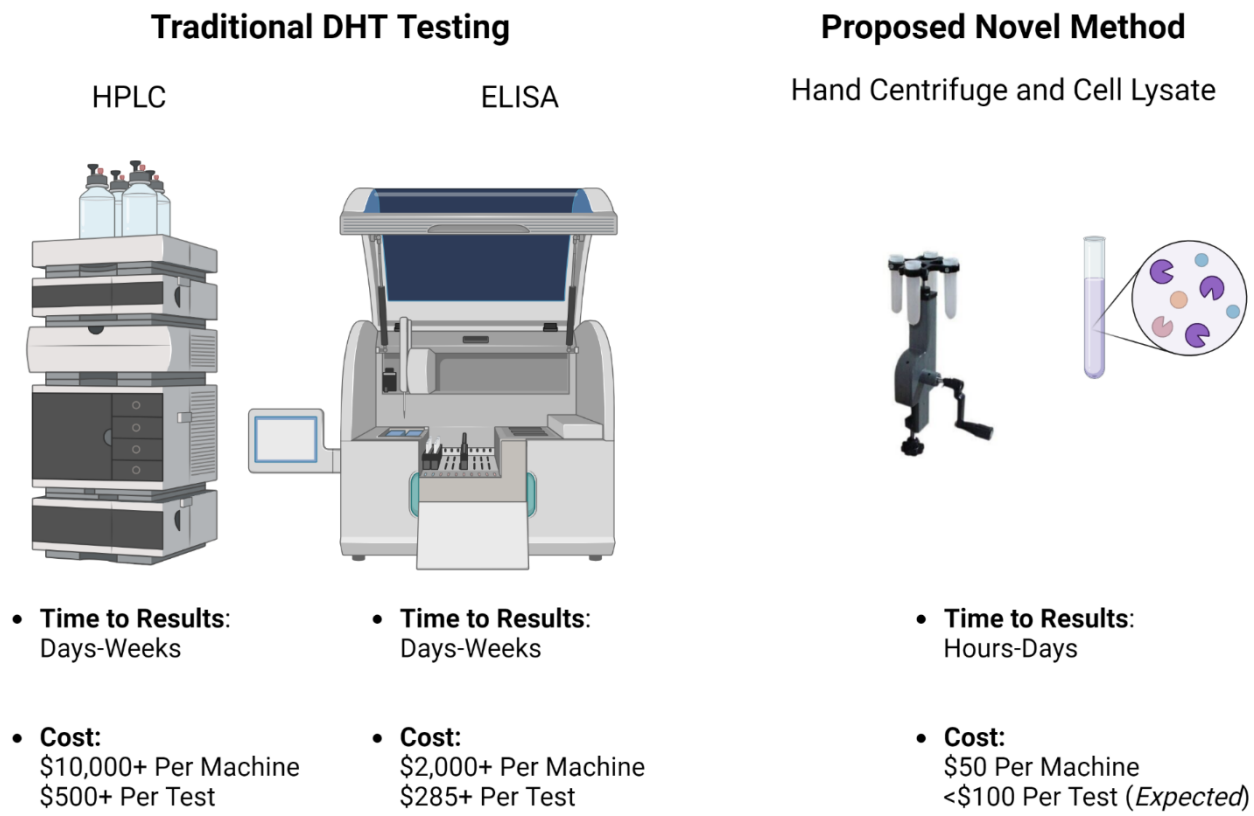

Figure 2. Comparison of DHT testing mechanisms.

consuming, potentially spanning days to yield results. HPLC is the standard for DHT testing, yet it is not easily accessible and costly.

ELISA-based DHT testing has several advantages over HPLC, but certain limitations prevent its pervasive use. Although cheaper than HPLC, ELISA kits typically cost several hundred dollars per package and necessitate adequate facilities and tools, such as a microplate reader, to perform measurements (ALPCO et. al, 2023). In addition to price, another limitation of ELISA testing lies in potential uncertainties in appraisal. The antibodies used in ELISA are liable to cross-react with other androgens, resulting in inaccurate results, contributing to errors in diagnosis (Tighe et. al, 2015).

Our novel DHT testing method (Figure 2) offers a more accessible and cost-effective approach, addressing the limitations of traditional techniques. Our approach utilizes a blood sample and the cranking of a hand-operated centrifuge to separate and test serum, circumventing the need for complex and costly equipment. This alternative employs an *in vitro* cell-free system that significantly reduces turnaround time from days to hours (Voloshin & Swartz, 2005). The swift response enables healthcare providers to diagnose DHT-related conditions and begin treatment earlier. By lowering the cost and complexity of testing, this method can reach a broader patient population and improve access to critical diagnostic services. The enhanced accessibility of this approach is particularly beneficial for healthcare systems in developing countries, remote locations, and underserved communities, where traditional HPLC and ELISA tests are impractical due to cost and logistical constraints.

Systems level

Our novel means of sensing DHT levels uses a biosensor. Biosensors are biological devices that detect biological substances such as proteins or chemicals through targeted binding. These sensors have various

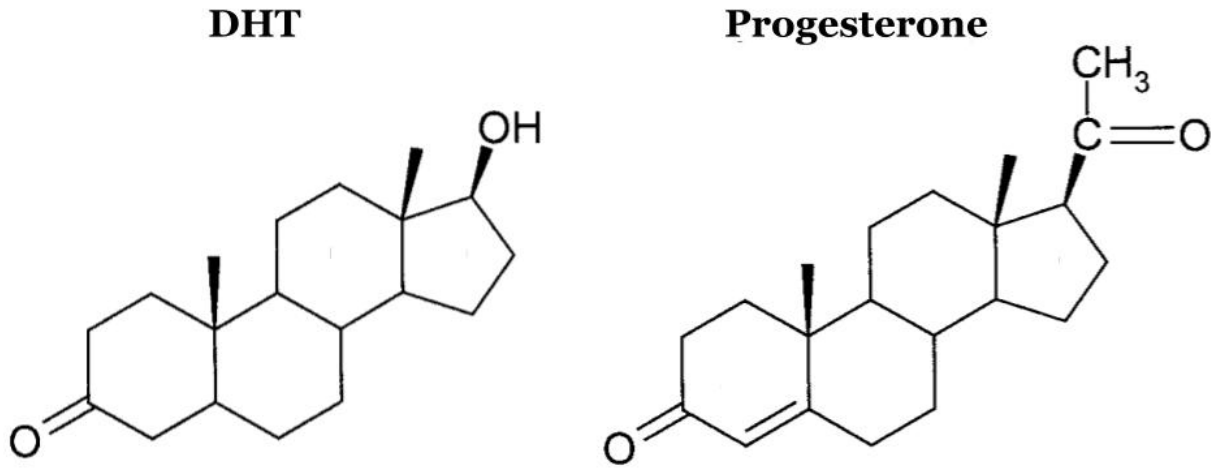

Figure 3. Chemical similarities between DHT and progesterone.

applications, such as medical diagnostics, environmental monitoring, and food safety (Mehrotra, 2016). The typical biosensor uses a biorecognition element and a transduction mechanism. The biorecognition element identifies the substance being measured, and the transduction mechanism turns the identification into a measurable signal that can be altered depending on the levels of said substances.

Transcription Factors (TF) are molecules commonly used in organisms to sense and respond to their environment. TFs allow for quick responses to situational changes in a multitude of circumstances. TFs have a special structure composed of a DNA binding domain and a protein interaction domain. We will use a TF as a part of the biosensor system in our experimentation. While the TF we are utilizing, SRTF1, is known to act as a biosensor for the progesterone hormone (Grazon et. al, 2020), our study investigates its ability to effectively detect DHT. Although DHT is not completely analogous to progesterone, current investigation has shown that the protein interaction domain is receptive to similar hormones; consequently, DHT is hypothesized to bind similarly to SRTF1 (Charney & Herzog, 2014). In this novel study, we propose to test the ability of SRTF1 to measure levels of DHT by determining its binding potential. This willopen up new possibilities for creating effective yet cost-effective biosensors to help improve DHT testing (Sacks et. al, 2001).

Cell-free gene expression (CFE) systems allow biochemical reactions to occur outside of living cells (Brookwell et. al, 2021) (Figure 3). CFEs let us create proteins outside of living cells by using the key components needed for transcription and translation. These systems use cell lysates, components of broken cells, that contain essential biological machinery necessary for *in vitro* protein synthesis. Unlike living cells, cell lysates can be freeze-dried, allowing for bulk, long-term storage without refrigeration (Garenne et. al, 2021). This makes CFE systems convenient for building a simple and portable biosensor, which could be accessible to those who lack resources and laboratory spaces.

For our project, we develop a systemic biosensor to detect DHT levels. We will use the myTXTL Linear DNA Expression Kit, which includes an *E. coli*-based Master Mix and a linear DNA vector encoding green fluorescent protein (GFP). GFP gives a clear observable signal when protein synthesis occurs; green illumination will be emitted as a visual product on the basis of when the system is activated to produce the GFP protein. The production of GFP protein can be detected by direct observation in the presence of ultraviolet light. High levels of GFP will indicate high levels of system activation, while low levels of GFP will indicate low levels of system activation.

In this CFE system, protein synthesis begins almost immediately after the Master

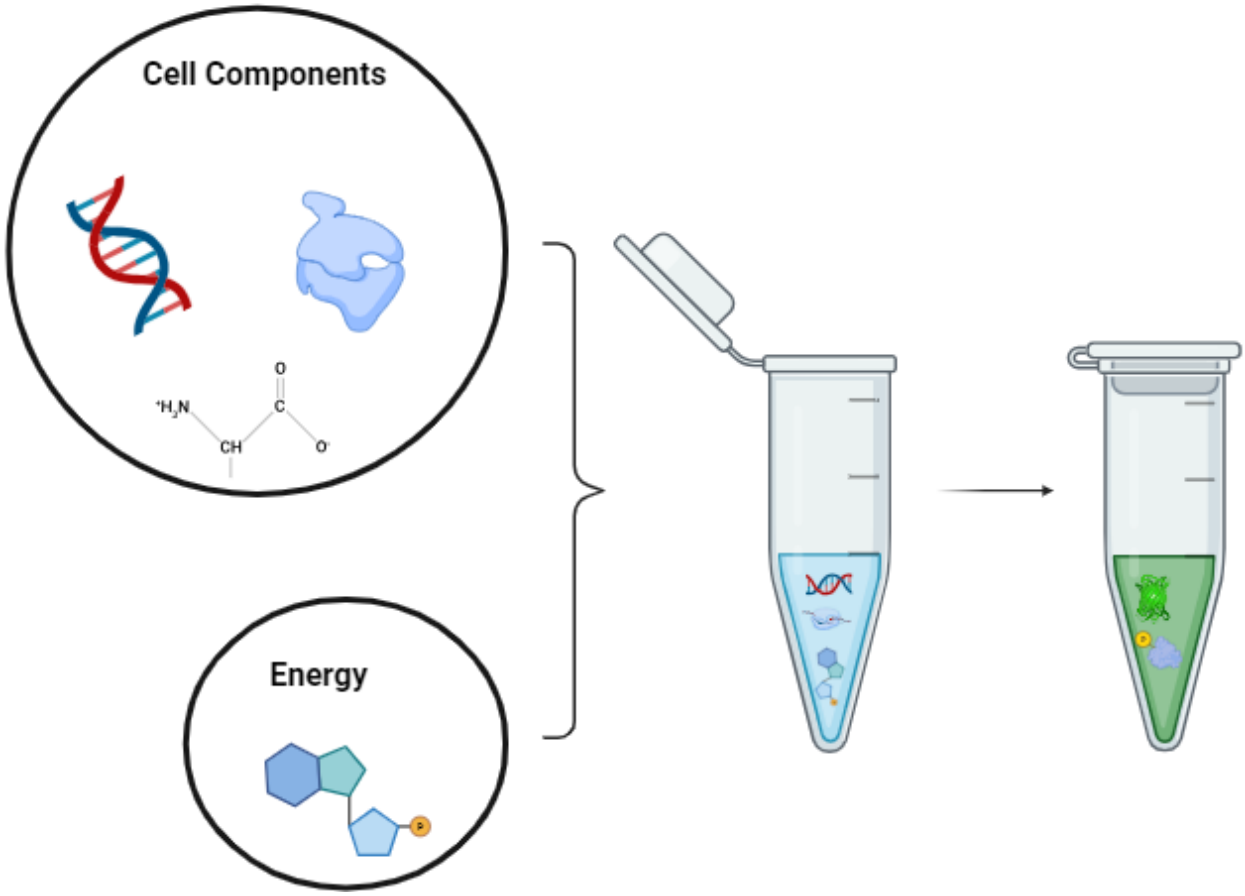

Figure 3. Cell-free genetic expression components model.

Mix has been added with the linear DNA, providing rapid results. This kit works with both linear and circular DNA templates; however, we chose linear DNA because it is simpler and cheaper, especially since greater quantities of DNA can be produced by PCR amplification (Daicel Arbor et. al, 2023). The system allows for gene expression using various common promoters, including Sigma70 and T7. The T7 system, which uses T7 RNA polymerase and the T7p14 promoter, allows for high-yield GFP protein production. Because of its efficiency, versatility, affordability and low cost, this CFE system is perfect for designing an accessible health-monitoring biosensor such as ours.

Device level

Our experiment utilizes a CFE derived from *E. coli*, that involves the use of a carefully structured DNA template containing all necessary components for mRNA transcription and protein translation (Brookwell et al., 2021). The central element in our design is the T7 promoter, which is recognized by RNA polymerase (RNAP), ensuring efficient transcription of the DNA template into mRNA, which can then be translated into protein.

Sigma70, a transcription factor in *E. coli* that is used in our system, plays a pivotal role in guiding RNAP to the appropriate transcription initiation sites. It assists in binding RNAP to the T7 promoter, beginning transcription (Stephens-Shields et al., 2022).

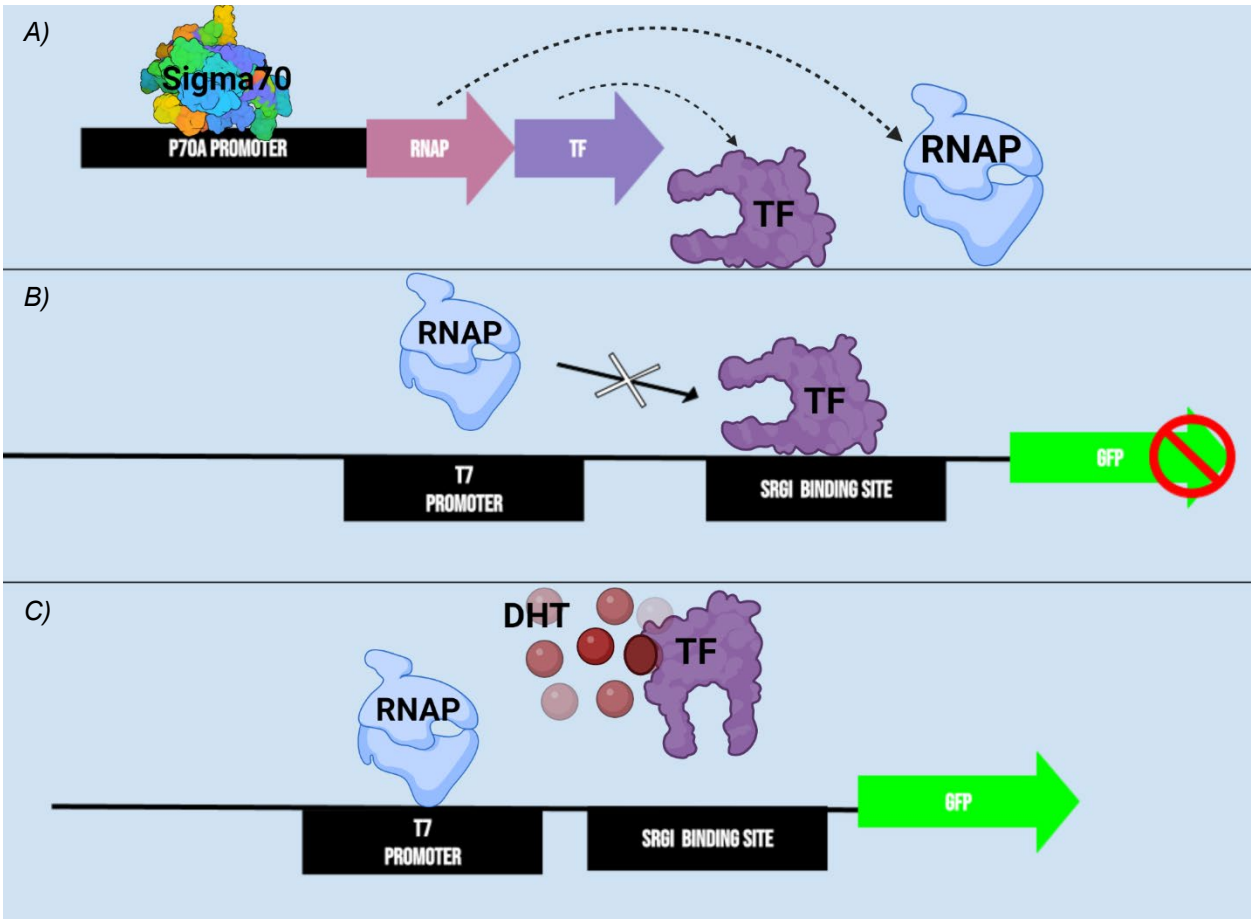

Figure 4. DHT measurement expression circuit.

In this design (Figure 4A), Sigma70 enables RNAP to target and transcribe specific downstream segments, including *SRTF1* and *GFP* genes. The *SRTF1* gene encodes for the production of SRTF1 TF, a key regulatory element in this system. The GFP protein encoded by the *GFP* gene serves as the output, providing a fluorescent signal to indicate gene expression.

SRTF1 acts as a regulator of *GFP* transcription. SRTF1 blocks RNAP from transcribing the *GFP* gene, preventing effective production of GFP when bound to the SRGI site within the T7 promoter (Figure 4B). If DHT is present in significant concentrations, SRTF1 undergoes a conformational change in response that disrupts its binding to SRGI (Figure 4C). Due to the shift, RNAP is no longer blocked by SRTF1 and transcribes *GFP*, resulting in green fluorescence when the system is exposed to blue or ultraviolet light. Conversely, when DHT concentrations are low, some SRTF1 can partially bind to the SRGI site, obstructing RNAP to a certain extent, and reducing GFP production (Figure 4B). This leads to lower GFP fluorescence. The proposed behavior creates a clear operational framework for our biosensor's functionality, with GFP production indicating the presence of higher DHT levels.

The fluorescence level of GFP directly correlates with the presence of DHT. When DHT levels are high, GFP is strongly expressed, indicating the ‘on’ state of the system. Conversely, when DHT levels are low, SRTF1retains its binding to the SRGI site, resulting in minimal transcription and low GFP fluorescence, indicating the ‘off’ state.

Parts Level

The description below delineates the working of the cell-free genetic expression (CFE) system. Parts are listed in order of their appearance in the sequence leading to the production of the GFP signal as an output. The first DNA construct (Figure 4A) contains the p70a promoter. As Sigma70 binds to this promoter, it initiates the eventual transcription of two important proteins: RNAP and SRTF1, used as parts to measure and correspond to levels of DHT, respectively. While the myTXTL system already has the RNAP sequence inserted in the first linear construct, we will redesign it to incorporate the specific SRTF1 coding region.

The second DNA construct (Figure 4B) is where the process of DHT detection begins. This template has the genetic information for making GFP protein. It contains important regions such as the T7 promoter, where transcription of the *GFP* gene starts, and the SRTF1 binding site, where *GFP* transcription can then be regulated. While the manufacturer does include the T7 promoter region in the sourced strand of linear DNA, the SRTF1 binding site will be inserted by us.

The T7 promoter found in the second DNA construct (Figure 4B) is the specific sequence on the DNA template where transcription is initiated, functionally serving as a 'start' signal that attracts RNAP to begin transcribing genes. Sigma70 assists the RNAP in finding the T7 promoter, acting as a guide that ensures it binds to the right place on the DNA template. RNAP attaches to the T7 promoter and begins transcribing the DNA into mRNA, the first step toward protein production.

The SRGI binding site, a repressor binding site, plays a key role in our system's transcription regulation. When SRTF1 binds to this site, it physically blocks RNAP from continuing to move and transcribe the *GFP* gene located downstream of the DNA construct (Figure 4B). Thus, the binding or release of SRTF1 determines whether transcription continues and if GFP is synthesized. When SRTF1 is bound, transcription stops; when SRTF1 is not bound, transcription resumes (Figure 4C). DHT is the hormone able to mediate SRTF1 binding. (Grazon et. al, 2020). The higher the levels of DHT present in the samples, the more the hormone interacts and binds to the SRTF1, causing it to undergo a conformational change, which releases it from the SRGI site. Without the presence of a physical blockade, RNAP can then move forward and transcribe the downstream genes, including *GFP*.

GFP is the final product and serves as a visual output. When SRTF1 no longer blocks RNAP, GFP is produced, emitting green fluorescence when exposed to blue or ultraviolet light. This fluorescence indicates that transcription has occurred and that ahigh level of DHT is present. Together, these parts form the operational framework for this CFE system. By controlling the interactions between these different components, we can determine whether GFP is produced and to what extent it is produced, providing a clear visual indication of DHT hormone levels. This is a useful method for developing a biosensor to detect and measure concentrations.

The additional sequences included in this construct are the ribosome binding sites (RBS) and terminator sequence, which allow for a fast and efficient cessation of the encoding process. RBSare located upstream of each gene to guide ribosomes to the correct start point for translation. Additionally, start (AUG) and stop codons flank the coding sequence and are essential for mRNA stability, translation efficiency, and proper regulation by terminating the system when the process has concluded.

Safety

This study utilizes a cell-free system derived from *E. coli*, which will be obtained as part of a kit from Arbor Biosciences. Specifically, the myTXTL® Sigma 70 Master Mix Kit and myTXTL T7 eGFP Expression Kit will be used as described by the manufacturer, according to the safety protocols (Daicel Arbor et. al, 2023).

Both kits contain linear DNA constructs, which will be modified by cutting with appropriate restriction enzymes and introducing the following sequencing:

SRTF1 coding sequence (as described by Galagan lab, private communication) in the myTXTL Sigma70 construct, and SRGI binding site into the myTXTL T7 expression vector. Ligation reactions will be set-up to introduce the specified sequences into these vectors. Once the vectors are modified, they will be combined with Master Mix as described by Arbor Biosciences to induce *in vitro* transcription and translation to produce the desirable mRNA and protein, and determine GFP production. All safety molecular biology and recombinant DNA protocols will be followed as described by Cold Spring Harbor Laboratory Manual (Sambrook et. al, 1989).

To generate the standard curve of DHT levels to GFP production, DHT (>99% pure Sigma Aldrich, St. Louis, MO) will be obtained and diluted according to manufacturer's specifications and safety protocol and used as calibration standards. Then DHT will be added to the CFE system to determine the outcomes of the experiment.

Discussions

The benefits of using a CFE system with GFP as a visual indicator give our biosensor a rapid method to detect DHT levels through fluorescence. The ease of CFE and correlation between DHT levels and GFP allows us to synthesize proteins and analyze results within a single day, allow quick and efficient evaluation of DHT levels. The Master Mix in the CFE system is user-friendly and encourages consistent results with minimal pipetting, enhancing its reliability and efficiency for our diagnostic project.

Communities that do not have access to the expensive or laboratory-grade traditional diagnostics used to examine DHT levels, such as HPLC or ELISA, would benefit greatly from a fast, efficient, and easily understood solution. The high prevalence of 5ARD among adolescents in many nations can now be adequately addressed through our diagnostic product, successfully confirming low DHT levels in these individuals. Due to improvements in turnaround time, even affluent countries could benefit from this facile means of testing. Despite these benefits, several challenges must be addressed. Constructing the DNA sequences in the correct order and ensuring proper interaction with both the SRTF1 and RNAP can be complex. Identifying suitable restriction enzymes requires obtaining the complete DNA sequencemaps from the manufacturer, and finding optimal conditions for restriction, digestion, and ligation may demand further optimization. Additionally, the production of a functional TF from the SRTF1 coding sequence must be validated experimentally.

Other uncertainties relate to the performance of SRTF1 in the cell-free system from myTXTL, as it has only been tested in *E. coli*. It is unclear whether SRTF1 can recognize and bind to the SRGI binding site effectively in the cell-free system The interaction between DHT and SRTF1 must also be determined since the Galagan lab has only conclusively demonstrated SRTF1 to bind progesterone. Nevertheless, since DHT is a steroid hormone with structural similarities to progesterone, it is reasonable to hypothesize that DHT can also interact and bind to SRTF1 (Figure 3). Furthermore, the role of SRTF1as a repressor that prevents RNAP from transcribing *GFP* remains undetermined. Future investigations will focus on refining the system's reliability, exploring new approaches to enhance its effectiveness, and addressing concerns through bench experimentation.

Our system utilizes the interaction between DHT and the SRTF1, to provide a reliable means to interpret the level of DHT in samples on the basis of fluorescence produced. As depicted (Figure 5), the relationship between hormone concentrations and resulting GFP fluorescence has been shown by studies to be sigmoidal, a common biological response (Hebisch et. al, 2013). DHT, also a hormone, would produce similar results when measured. This pattern demonstrates that as DHT levels increase, the inhibition exerted by SRTF1 rapidly decreases, allowing RNAP to transcribe the *GFP* gene, leading to heightened GFP production and fluorescence.

These findings have implications for understanding the role of DHT role in various biological contexts. The sigmoidal response

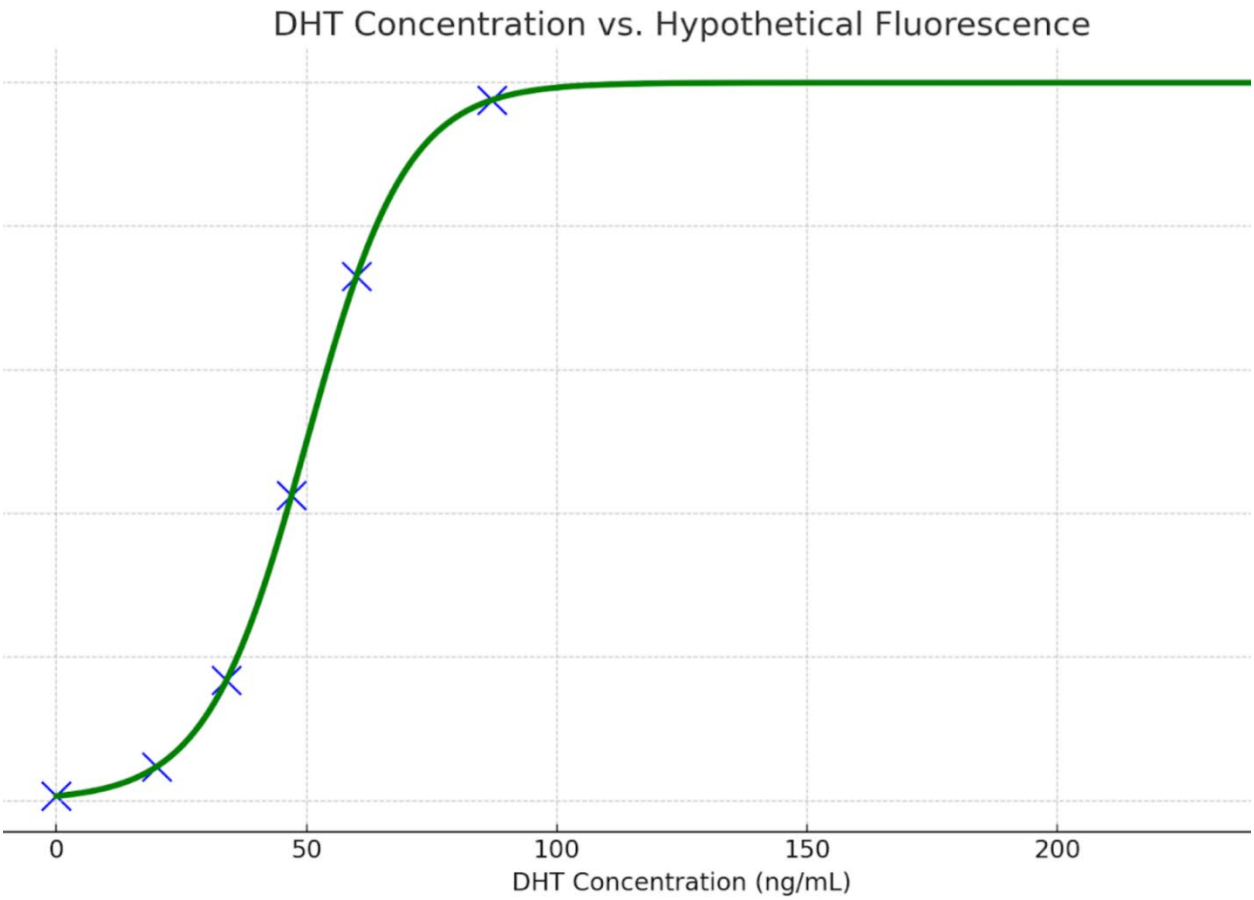

Figure 5. DHT-Fluorescence standard correlation.

curve suggests a threshold effect, where a specific concentration of DHT is required to overcome the inhibitory action of SRTF1 on GFP expression. This threshold behavior is critical for regulatory mechanisms in biological systems, where precise control of gene expression is essential. Moreover, such widespread variance between low, normal, and high levels of DHT greatly increases its utilization as an identification factor. Visual observation alone will be more reliable in determining the DHT levels among individuals - high levels (strong fluorescence) or low levels (low fluorescence).

Altered DHT levels in individuals often manifest physiologically. However, by the period of such an occurrence disease has already progressed. Therefore, there is a need for a more quantifiable method of measuring DHT to conclusively determine if the disease is due to alterations in hormonal concentrations or external factors, allow better diagnosis and treatment As an example, if low levels of DHT were detected in a patient, such as a person suffering from 5ARD, low GFP fluorescence will be detected using our biosensor system, indicating that SRTF1 is effectively bound to SRGI binding site, inhibiting RNAP activity, thus preventing GFP production (Jia et. al, 2018). Specific concentrations of DHT could even be obtained using a spectrophotometer which detects the absorbance values of the GFP produced. However, even without a spectrophotometer, DHT can still be determined visually based on intensity of GFP signal in the presence of ultraviolet light.

Next steps

In the future steps, we will obtain DHT from sample patient blood, and then use our

biosensor to measure the levels of DHT. The blood will be collected using sterile techniques to minimize contamination risk. The collected blood will be distributed into various blood collection tubes: plain glass (red top), clot activator and gel (gold top), heparin (green top), EDTA (lavender top), and sodium citrate (blue top). After allowing blood to clot at room temperature (23°C) for 30 minutes, the samples will be spun using a hand-centrifuge to separate serum from blood cells, and carefully isolated to avoid contamination with blood cells. Additional tubes with fluoride, in light and dark gray coloration, will be used to inhibit non-specific esterases when studying testosterone esters (Wang et. al, 2018). The isolated serum will be stored at room temperature for up to 7 days, refrigerated for up to 14 days, or frozen for up to 6 months to maintain stability. A minimum of 1 mL of serum from red-top tubes will be used for analysis. Safety procedures include proper disposal of biohazardous waste and adherence to laboratory protocols.

Author contributions

H.S. conceived the system, design, device, parts, working on all aspects of the project. H. S. wrote the abstract, discussion, safety, and future steps, wrote the paper at all stages, wrote and edited all sections and sourcing citations, and created the video. A.M. and S.V. helped brainstorm the initial ideas for the project, and S.V., C.K., and C.K. contributed ideas to a draft in the preliminary Background section. A.A. made initial models for graphics of Figures 1 and 4 in Google Draw using designs provided by H.S., which H.S. subsequently finalized using BioRender. While H.S. provided guiding direction, leading the group and motivated the group, all attended meetings and worked collaboratively throughout.

Acknowledgements

We would like to thank Scott Gaines for being our mentor, giving invaluable advice and insights, providing us with training and inspiration, and always being willing to lend a hand even in late-night Zoom meetings. We would like to thank our club advisor, Maria Lazebnik, for her continuous support of this project - volunteering her time, sponsoring our club, and teaching us topics in Synthetic Biology.

References

Anawalt, B. D. (2017). Is Dihydrotestosterone a Classic Hormone?, *Endocrine Reviews*, 38(1-3), 170–172, https://doi.org/10.1210/er.2017-00091

Azizi, A., Mumin, N. H., & Shafqat, N. (2021). Phytochemicals With Anti 5-alpha-reductase Activity: A Prospective For Prostate Cancer Treatment. *F1000Research*, 10, 221. https://doi.org/10.12688/f1000research.51066.3

Baetens, D., Mladenov, W., Delle Chiaie, B., Menten, B., Desloovere, A., Iotova, V., Callewaert, B., Van Laecke, E., Hoebeke, P., De Baere, E., & Cools, M. (2014). Extensive clinical, hormonal and genetic screening in a large consecutive series of 46,XY neonates and infants with atypical sexual development. *Orphanet Journal of Rare Diseases*, 9, 209. https://doi.org/10.1186/s13023-014-0209-2

Brookwell, A., Oza, J. P., & Caschera, F. (2021). Biotechnology Applications of Cell-Free Expression Systems. *Life.* Basel, Switzerland, 11(12), 1367. https://doi.org/10.3390/life11121367

Cai, L. Q., Zhu, Y. S., Katz, M. D., Herrera, C., Baéz, J., DeFillo-Ricart, M., Shackleton, C. H., & Imperato-McGinley, J. (1996). 5 alpha-reductase-2 gene mutations in the Dominican Republic. *The Journal of Clinical Endocrinology and Metabolism*, 81(5), 1730–1735. https://doi.org/10.1210/jcem.81.5.8626825

Charney, W. & Herzog, H. L. (2014). Microbial Transformations of Steroids: A Handbook. *Academic Press*.

Cheon C. K. (2011). Practical approach to steroid 5alpha-reductase type 2

deficiency. *European Journal of Pediatrics*, 170(1), 1–8. https://doi.org/10.1007/s00431-010-1189-4

"Dihydrotestosterone (DHT) ELISA." *American Laboratory Products (ALPCO) Diagnostics,* 11 October, 2023, https://www.alpco.com/dihydrotestosterone-dht-elisa.html

Esteban, C., Ortiz-Rodz, D. I., Muñiz-Pérez, Y. I., Ramírez-Vega, L., Jiménez-Ricaurte, C., Mattei-Torres, E., & Finkel-Aguilar, V. (2023). Quality of Life and Psychosocial Well-Being among Intersex-Identifying Individuals in Puerto Rico: An Exploratory Study. *International Journal of Environmental Research and Public Health*, 20(4), 2899. https://doi.org/10.3390/ijerph20042899

Garenne, D., Haines, M.C., Romantseva, E.F., Fremont, P., Strychalski., E. A., & Noireaux, V. (2021). Cell-free gene expression. *Nature Review Methods Primers* 1, 49. https://doi.org/10.1038/s43586-021-00046-x

Grazon, C., Baer, R.C., Kuzmanović, U. et al. (2020). A progesterone biosensor derived from microbial screening. *Nature Communications*, 11, 1276. https://doi.org/10.1038/s41467-020-14942-5

Gustafsson, O., Norming, U., Gustafsson, S., Eneroth, P., Aström, G., & Nyman, C. R. (1996). Dihydrotestosterone and testosterone levels in men screened for prostate cancer: a study of a randomized population. *British Journal of Urology*, 77(3), 433–440. https://doi.org/10.1046/j.1464-410x.1996.89120.x

Hebisch, E., Knebel, J., Landsberg, J., Frey, E., & Leisner, M. (2013). High variation of fluorescence protein maturation times in closely related Escherichia coli strains. *PloS One*, 8(10), e75991. https://doi.org/10.1371/journal.pone.0075991

Horton R. (1992). Dihydrotestosterone is a peripheral paracrine hormone. *Journal of Andrology*, 13(1), 23–27.

Jia, W., Zheng, D., Zhang, L., Li, C., Zhang, X., Wang, F., Guan, Q., Fang, L., Zhao, J., & Xu, C. (2018). Clinical and molecular characterization of 5α-reductase type 2 deficiency due to mutations (p.Q6X, p.R246Q) in SRD5A2 gene. *Endocrine Journal*, 65(6), 645–655. https://doi.org/10.1507/endocrj.EJ17-0542

Kosaka, T., Miyajima, A., & Oya, M. (2014). Is DHT Production by 5α-Reductase Friend or Foe in Prostate Cancer?. *Frontiers in Oncology*, 4, 247. https://doi.org/10.3389/fonc.2014.00247

Marchetti, P. M., & Barth, J. H. (2013). Clinical biochemistry of dihydrotestosterone. *Annals of Clinical Biochemistry*, 50(Pt 2), 95–107. https://doi.org/10.1258/acb.2012.012159

Mehrotra P. (2016). Biosensors and their applications - A review. *Journal of Oral Biology and Craniofacial Research*, 6(2), 153–159. https://doi.org/10.1016/j.jobcr.2015.12.002

"MyTXTL Linear DNA Expression Kit." *Daicel Arbor Biosciences*, 6 July 2023, arborbiosci.com/products/cell-free-protein-synthesis/mytxtl-cell-free-expression-kits/mytxtl-linear-dna-expression-kit/

Nascimento, R. L. P., de Andrade Mesquita, I. M., Gondim, R., Dos Apóstolos, R. A. A. C., Toralles, M. B., de Oliveira, L. B., Canguçu-Campinho, A. K., & Barroso, U., Jr (2018). Gender identity in patients with 5-alpha reductase deficiency raised as females. *Journal of Pediatric Urology*, 14(5), 419.e1–419.e6. https://doi.org/10.1016/j.jpurol.2018.08.021

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Plainview, N.Y.: Cold Spring Harbor Laboratory Press.

Stephens-Shields, A. J., Snyder, P. J., Ellenberg, S. S., Taylor, L., & Bhasin, S. (2022). Relation of Testosterone, Dihydrotestosterone, and Estradiol With Changes in Outcomes Measures in

the Testosterone Trials. *The Journal of Clinical Endocrinology and Metabolism*, 107(5), 1257–1269. https://doi.org/10.1210/clinem/dgac028

Swerdloff, R. S., Dudley, R. E., Page, S. T., Wang, C., & Salameh, W. A. (2017). Dihydrotestosterone: Biochemistry, Physiology, and Clinical Implications of Elevated Blood Levels. *Endocrine Reviews*, 38(3), 220–254. https://doi.org/10.1210/er.2016-1067

Thirumalai, A., Cooper, L. A., Rubinow, K. B., Amory, J. K., Lin, D. W., Wright, J. L., Marck, B. T., Matsumoto, A. M., & Page, S. T. (2016). Stable Intraprostatic Dihydrotestosterone in Healthy Medically Castrate Men Treated With Exogenous Testosterone. *The Journal of Clinical Endocrinology and Metabolism*, 101(7), 2937–2944. https://doi.org/10.1210/jc.2016-1483

Tighe, P. J., Ryder, R. R., Todd, I., & Fairclough, L. C. (2015). ELISA in the multiplex era: potentials and pitfalls. *Proteomics Clinical Applications*, 9(3-4), 406–422. https://doi.org/10.1002/prca.201400130

Urysiak-Czubatka, I., Kmieć, M. L., & Broniarczyk-Dyła, G. (2014). Assessment of the usefulness of dihydrotestosterone in the diagnostics of patients with androgenetic alopecia. *Postepy Dermatologii i Alergologii*, 31(4), 207–215. https://doi.org/10.5114/pdia.2014.40925

Voloshin, A. M. & Swartz, J. R. (2005). Efficient and scalable method for scaling up cell free protein synthesis in batch mode. *Biotechnology Bioengineering,* 91, 516–521.

Wang, C., Shiraishi, S., Leung, A., Baravarian, S., Hull, L., Goh, V., Lee, P. W. N., & Swerdloff, R. S. (2018). Corrigendum to "Validation of a testosterone and dihydrotestosterone liquid chromatography tandem mass spectrometry assay: interference and comparison with established methods" [Steroids Volume 73, Issue 13, 12 December 2008, Pages 1345-1352]. *Steroids*, 135, 108. https://doi.org/10.1016/j.steroids.2018.05.006