GENETIC LAB REPORT

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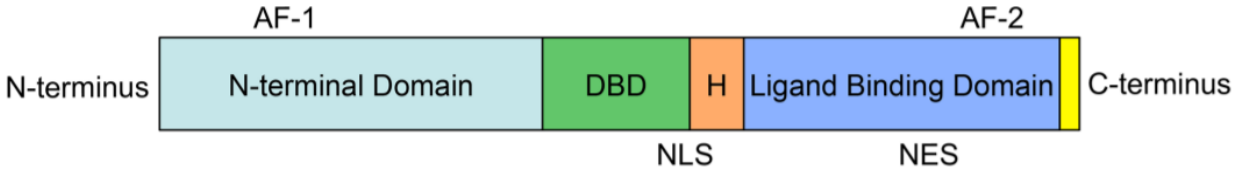
Background and Introduction

Androgens are chemical messengers or hormones that characterize important features of the male reproductive system, its development and the secondary sexual traits. Androgen receptors, expressed in a broad range of tissues, hails from the family of steroid hormones which contain varied members like glucocorticoid receptor, estrogen receptor and progesterone receptor families. Androgen receptors recognise their respective ligand of choice secreted from the adrenal glands, testes and ovaries. The receptors, after binding to the hormone, internalises it and takes it to the nucleus. These internalised androgen receptors act as DNA binding transcription factors and regulate the expression of specific gene targets, for eg. Prostate specific antigen. The two major androgens are testosterone and dihydrotestosterone, the latter of which is formed by enzymatic reduction of the first molecule and is more biologically active. The gene encoding for the Androgen receptor is located in the X chromosome and is vital for essential functions in the bone, prostate, muscle, reproductive and cardiovascular systems, neuronal system, in immune cells and in RBC generation. It is also prominently known as NR3C4 (nuclear receptor subfamily 3, group C, member 4). By acting as a ligand dependent DNA binding transcription factor, it up regulates or down regulates specific genes controlling important body functions other than the male sexual phenotype.

Below is the structural representation of the Androgen receptor:



The Androgen receptor (AR) is a 110kDa protein. Its structure is very similar to that of estrogen receptors α and β or to progesterone receptor. Starting with the exon 1, it encodes the N-terminal binding domain. It comprises of 60% of the AR protein but varies in length because of the difference in the amino acid codon for polyglycine (CGN)n repeats and polyglutamine (CAG)n repeats in the different polymorphs. Exon 2 consists of a zinc finger domain, the helix of which

recognizes the major groove of the DNA and hence forms the DNA –binding domain. Exon 3 also contains one zinc finger domain with the help of which it recognises another ligand bound AR binding to another DNA molecule and hence facilitates dimerization. Exons 4 to 8 contain 11 alpha helices and a hinge region to promote ligand binding. They form the AR carboxyl terminal domain. This region also contains the transcription activation function domain -2 which acts as a co-regulator interface site. It is normally present in the cytoplasm in association with chaperone proteins like Hsp 90. Under the influence of the ligand, it undergoes a conformational change that results in its nuclear localization. The ARs also contain the nuclear localization signal or NLS in between the DNA binding domain and the hinge region. The nuclear export signal to revert it back to the cytoplasm when the ligand wears off, is present in the ligand binding region.The activation of AR associated functions can happen in a ligand binding initiated DNA binding manner or in a ligand binding initiated non-DNA binding manner. The second one involves the activation of secondary signalling cascade by phosphorylation events.

Androgen Receptor is expressed in a wide variety of body tissues, therefore the pathology associated with it also spreads to multiple organ systems. The deficiency of this ligand can have an effect on the male and female behavioural pattern. The pathology is mostly linked with the hypothalamus-pituitary-gonadal axis. It can exhibit in the form of hypogonadism. Age related fall in the level of testosterone is a natural thing which does lead to helath problems like obesity but can very well be normalized with treatment and care, the symptoms as well as the cause. AR is associated with a very lethal form of cancer in men, i.e prostate cancer. Prostate specific antigen is activated in an AR based DNA binding transcriptional activity manner and hence AR deprivation helps with prostate cancer therapy. AR signalling is regulated in a very intricate manner by collective impacts of AR mutation or truncation, amplification, over expression of the AR protein and the gonadal release. Androgen insensitivity is a major issue associated with ARs when a mutation in AR acts as an inactivating one and hence causes resistance to the circulating testosterone. Some such mutations cause Complete Androgen Insensitivity Syndrome (CAIS) which results in a XY female with female phenotype and female features. Breasts develop normally though internal female genitalia are absent. Some other mutations which leave residual AR activity cause conditions called Partial AIS (PAIS). Any solution to this will involve the role of an endocrinologist, psychologist and gynaecologist. The structure of the AR also harbours

some diseases. The transactivation domain consists of (CAG)n or polyglutamine repeats . Variation in the number of repeats causes problems. A marked increment of the CAG repeats above the normal 11-13 repeats results in Kennedy's disease or Bulbospinal Muscular Atrophy which is a neurodegenerative disorder.

The structure of Androgen receptor and the presence of so many different domains, each with a different feature allow it to have multiple spice variants with multiple functions in gene activation. Hence any mutation or truncation in any of these isoforms will manifest itself in a problem associated with any of the organs. There are some AR sequence alterations which result in change in the protein sequence and hence an impaired or non-functional AR which is not able to sense the hormone. This leads to loss of function of AR and in Androgen Insensitivity Syndrome. Normal or bona fide alternative spilcing is associated with normal isoforms like AR45 that does not express in brain. But any alteration in the normal splicing event would lead to disease conditions.

PCR Protocol and Analysis of Results

The genomic DNA sequence for the AR protein was first searched for in the databases or from links from the references. The sequence will consist of introns as well as exons with ends defined by transcription start codon AUG and termination codon UGA, UAA or UAG.This sequence gets transcribed into pre-mRNA. Then the introns are removed to form a mature mRNA with no introns (removed by splicing) and ends defined by 5'UTR and 3'UTR. This mRNA is first converted to cDNA using reverse transcription PCR. Complementary DNA or cDNA is synthesised from single-stranded mRNA in a reaction using a reverse transcriptase PCR. First of all, RNA is isolated from a tissue or organ. mRNA is isolated from this and then subjected to reverse transcription to give a single cDNA strand which is then made double-stranded by PCR.

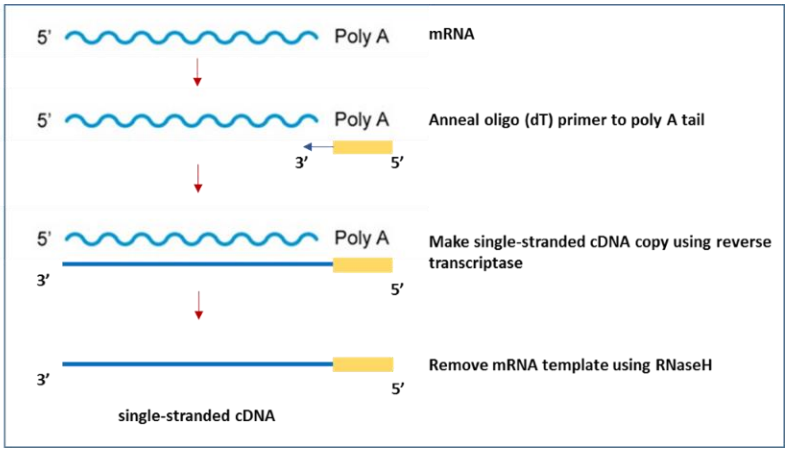


Fig: Reverse transcription

Now that the cDNA is available and the region to be amplified is known, specific set of oligonucleotides or primers complementary to the start and end region are used to amplify that sequence. The important parameters considered in the reaction are:

- Primer length: It is generally between 18-22 bps, long enough for primer to bind to the DNA and short enough so that the primer can bind at the annealing temperature used.

- Melting temperature of the pimers: It is the temperature at which half of the DNA duplex will become single-stranded. It should be nearly same for forward and reverse primers within ±5°C of each other. It should ideally range from 52-58°C. Tm above 65°C cab cause secondary annealing and hence can give non-specific bands also.

- Annealing temperature: Primer Tm is indicative of the stability of the DNA duplex and hence of the annealing temperature. Too high Ta can lead to less number of primer DNA template hybridizations causing low PCR yield and too low Ta will result in non-specific products.

- GC content of the primer should be from 50-60%.

- A GC clamp should be added to the 3' primer end to increase specific binding.

The PCR cycle should be designed based on the length of the amplification required.

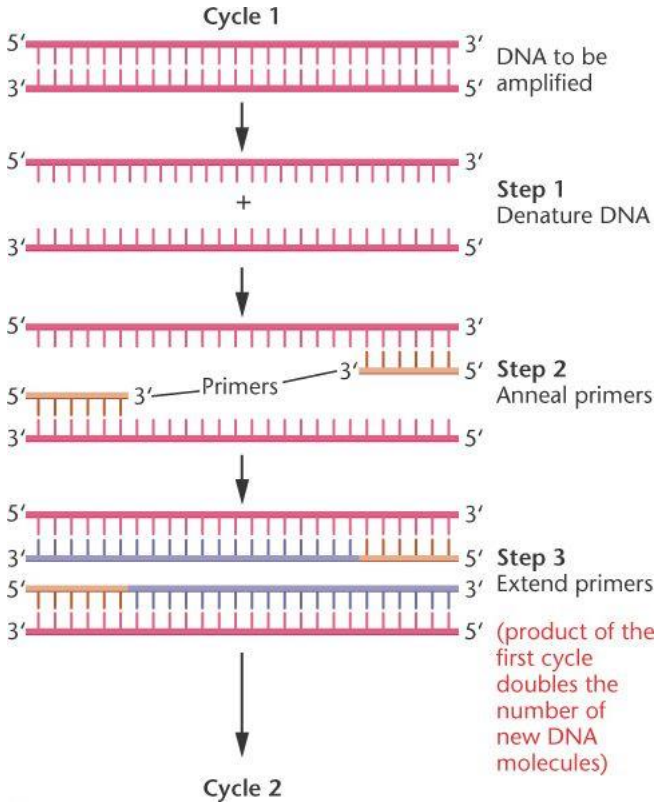


Figure . Polymerase Chain Reaction

It consists of the following steps:

- Denaturation: The dsDNA separates at a high temperature of 95°C into two separate strands allowing for base recognition. T
- Annealing: The forward and reverse primers anneal. FP anneals to the 3'-5' (bottom) strand and reverse primer to the 5'to 3' (top) strand .
- Extension: The bases are added at the 3' end of each primer by a specific DNA polymerase (Taq Polymerase) which functions at a higher temperature of 72°C.

- Repeat: This cycle repeats for normally 25-35 times.

Choosing the primer pairs and the expected amplicon length:

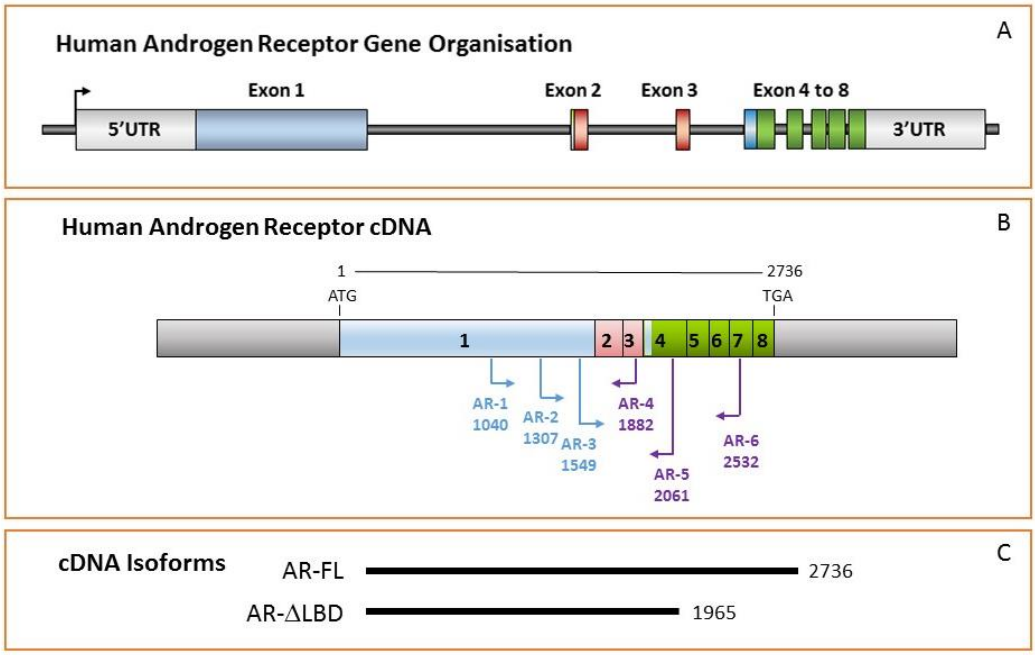


Figure 5. Organisation of the androgen receptor gene (**A**) and cDNA (**B**). Boxes represent exons; exons encoding protein domains are indicated: blue = N terminal domain, red = DNA binding domains and green = ligand binding domain. The 5' and 3' untranslated regions (UTR) are indicated by grey boxes. Arrows indicate the position and direction of the PCR primers AR1 – AR6. (**C**) Indicates regions covered by the full-length cDNA isoform (AR-FL) and the truncated cDNA isoform (AR-ΔLBD). Numbers refer to position relative to the ATG.

Fig: Human Androgen Receptor and its isoforms

Out of all the 6 primers provided, three forward and three reverse, following are chosen to distinguish between the two isoforms given.

- AR-FL: AR-1 as forward primer and AR-6 as reverse primer. Expected band length of amplicon in the agarose gel would be 1492 bps.
- AR-ΔLBD: AR-1 as forward primer and AR-4 as reverse primer. Expected band legth is 842 bps.

Following reagents were used to set up the reaction:

Reagent	Stock conc.	Final conc.	Dilution required	Volume in 50 µl
Sterile water				31µl
5 x GoTaq2 buffer	5x	1x	1:5	10 µl
dNTP mix	10mM	0.2 mM	1:50	1
cDNA template	10µM	0.2 µM	1:1	1
Primer 1	1 ng/µl	1	1:50	1
Primer 2	1 ng/µl	1	1:50	1
GoTaq2	5 U/µl	0.5	1:10	5

Fig: Table of reagents used

Following PCR cycle was used:

- Initial Denaturation: 95° C for 2 min
- Final Denaturation: 95° C for 30 sec
- Annealing: 58°C for 30 sec
- Extension: 72° C for 1 min per 1kb of DNA to be amplified

} 30 cycles

- Final Extension:72° C for 10 min
- Hold: 4°C

Interpretation of the gel results:

A1: The reaction has no DNA template and hence is a negative control. Hence we see no bands. This reaction is just run to check whether amplification is not due to any contamination in the other reaction components used.

A2: It is also a negative control with no template done with a different set of primers. A band that appears between 500 and 600 bp length is a contaminant band.

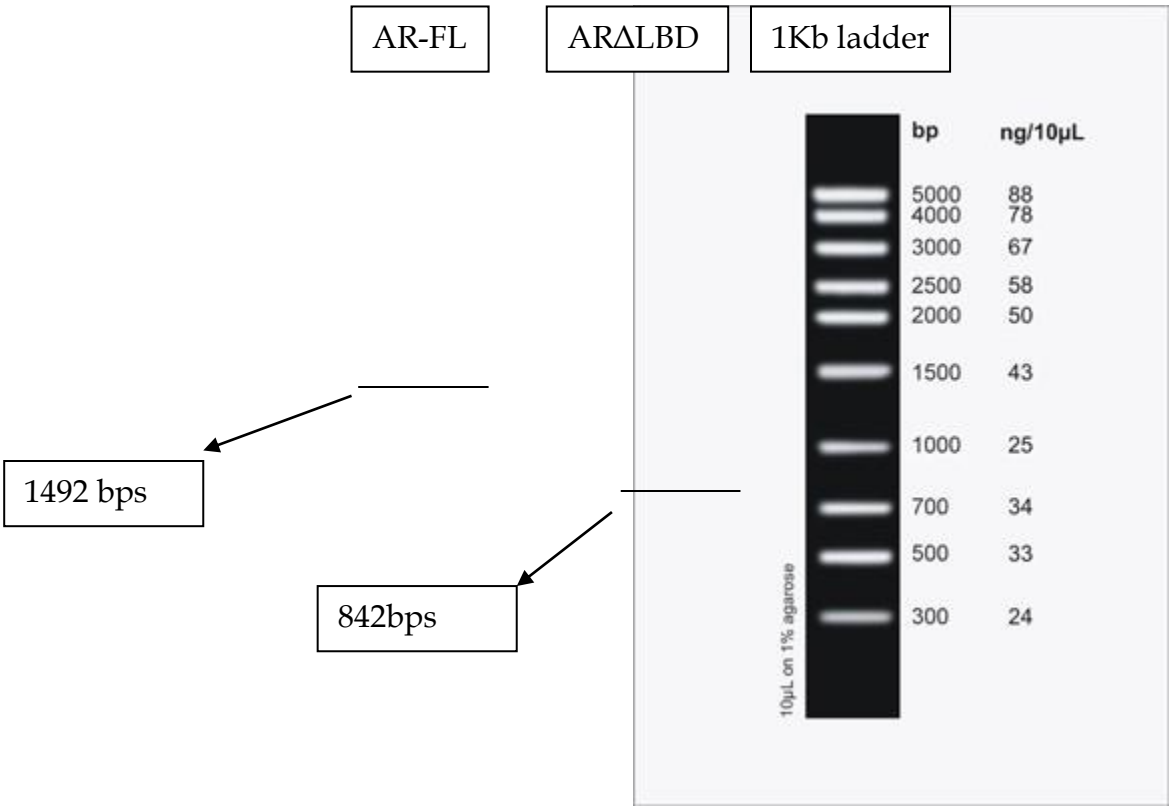
A3: It uses primers AR-1 and AR-6 and should give a band of 1492 base pair. But the lane shows nothing.

A4: It uses primers AR-2 and AR-6 and expected band length is 1225 bps. The result shows a good band corresponding to 1200 bp in the 100 bp ladder. This can be considered as a positive control too for comparing other reactions.

A5: It uses primers AR-2 and AR-4 and expected band length is 575 bps. The result shows a good band corresponding to 500 bp in the 100 bp ladder.

A6: It uses the same set of primers as A5 and hence gives the exact same band. Only a different cDNA template has been used.

Figure of gel to distinguish the two isoforms given:



Experimental Design: Multiplex PCR to distinguish between several different alternative splice variants of the androgen receptor gene

Multiplex PCR is a molecular biology technique. It is a modification of the basic PCR amplification technique. It enables to amplify different or multiple target sequences in a template at the same time in a single reaction using multiple primer pairs suited to amplify the specific targets. This saves time, effort as well as reagents for setting multiple PCR reactions.

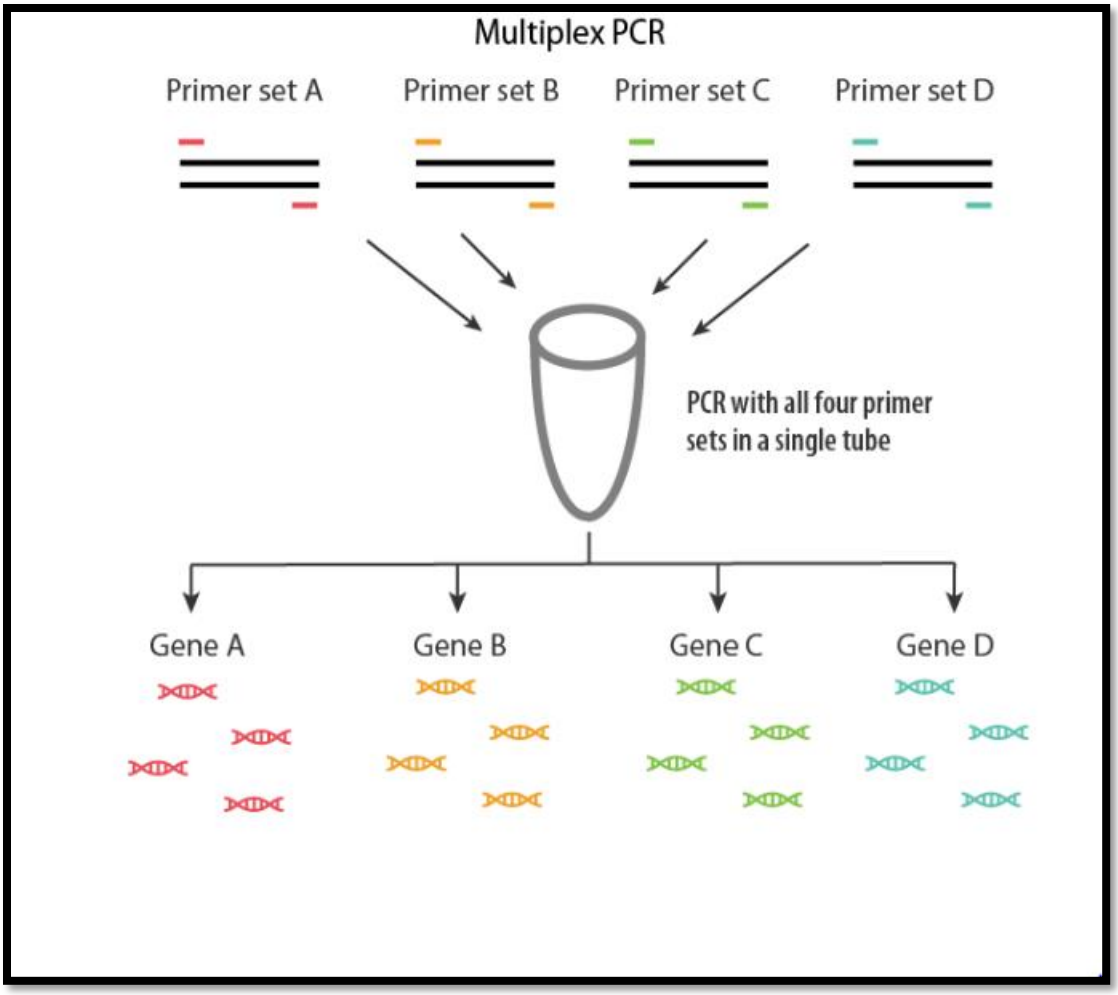


Fig: Multiplex PCR

For setting up the multiplex PCR reaction to distinguish between the different spice variants of androgen receptor gene, different set of primer pairs should be used. Like the given set of primers, AR-1 to AR-6 can distinguish between variants lacking exon 2: DNA binding domain and an isoform lacking the exons 4-8: ligand binding domain. A single cDNA template can be taken in a vial with different primer sets, each of equal concentration. Annealing temperature of the different primer pairs should be normalised so that they can work in a single reaction vial. But before setting the multiplex PCR, it should be ensured that the the different target sequences are of distinct length. Only then they can be seen in the agarose electrophoresis gel with marked distinction and interpreted to be a specific isoform.

Multiplex PCR does save time and effort but has several disadvantages like less amplification efficiency and no two target sequences are amplified with the same efficiency.

References:

Antonarakis ES et al., AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer, N Engl J Med 2014.

Cao B et al., Androgen receptor splice variants activating the full-length receptor in mediating resistance to androgen-directed therapy, Oncotarget 2014.

Davey et al., Androgen Receptor Structure, Function and Biology: From Bench to Bedside, Clin Biochem Rev 37 (1) 2016.

Dehm et al., Alternatively Spliced Androgen Receptor Variants, Endocr Relat Cancer. 2011.

Hornberg E et al., Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival, PLoS One 2011.

Kumar R et al., Allosteric modulators of steroid hormone receptors: structural dynamics and gene regulation, Endocr Rev 2012.

McEwan IJ et al., Intrinsic disorder in the androgen receptor: identification, characterisation and drugability, Mol Biosyst 2012.

Monaghan et al., A sting in the tail: the N-terminal domain of the androgen receptor as a drug target, Asian Journal of Andrology 2016.

Sun S et al., Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant, J Clin Invest 2010.