Chapter 5: Investigation of the potential utility of bioluminescent resonance energy transfer to investigate the AR N/C interaction and bioactive androgens in male serum

5.1 Introduction

The previous chapters highlight the importance of the AF5 region in the N/C interaction as measured by a mammalian 2-hybrid interaction assay. Other methods also previously utilised to measure this interaction involve isolation of protein lysates and detection of functional interactions via techniques such as co-immunoprecipitation or GST-based pull downs involving column based methods. These techniques are often used to verify the results of a mammalian 2-hybrid due to the potential for activity in this assay to be independently mediated by each of the protein constructs utilised in the assay. However, all of these techniques have some common limitations in the context of interrogating the N/C interaction. Because the N/C interaction occurs between the AR NTD and the AF2 region, each of these assays require that partial proteins be utilised. It has also been shown that the AR dimerises between its DNA binding domain and LBD (Nemoto et al. 1994), and these interactions are indistinguishable from the N/C interaction in the context of a full length receptor in a mammalian 2-hybrid interaction assay or co-immunoprecipitation experiment. Additionally, the use of a full length AR in an N/C interaction assay leads to high intrinsic activity due to transcriptional activation by the AR itself therefore obscuring any activity as the result of an interaction. Finally, none of these methods of measuring interactions can be interpreted in real time in living cells, and therefore studying the kinetics of the N/C interaction utilising these techniques is not possible.

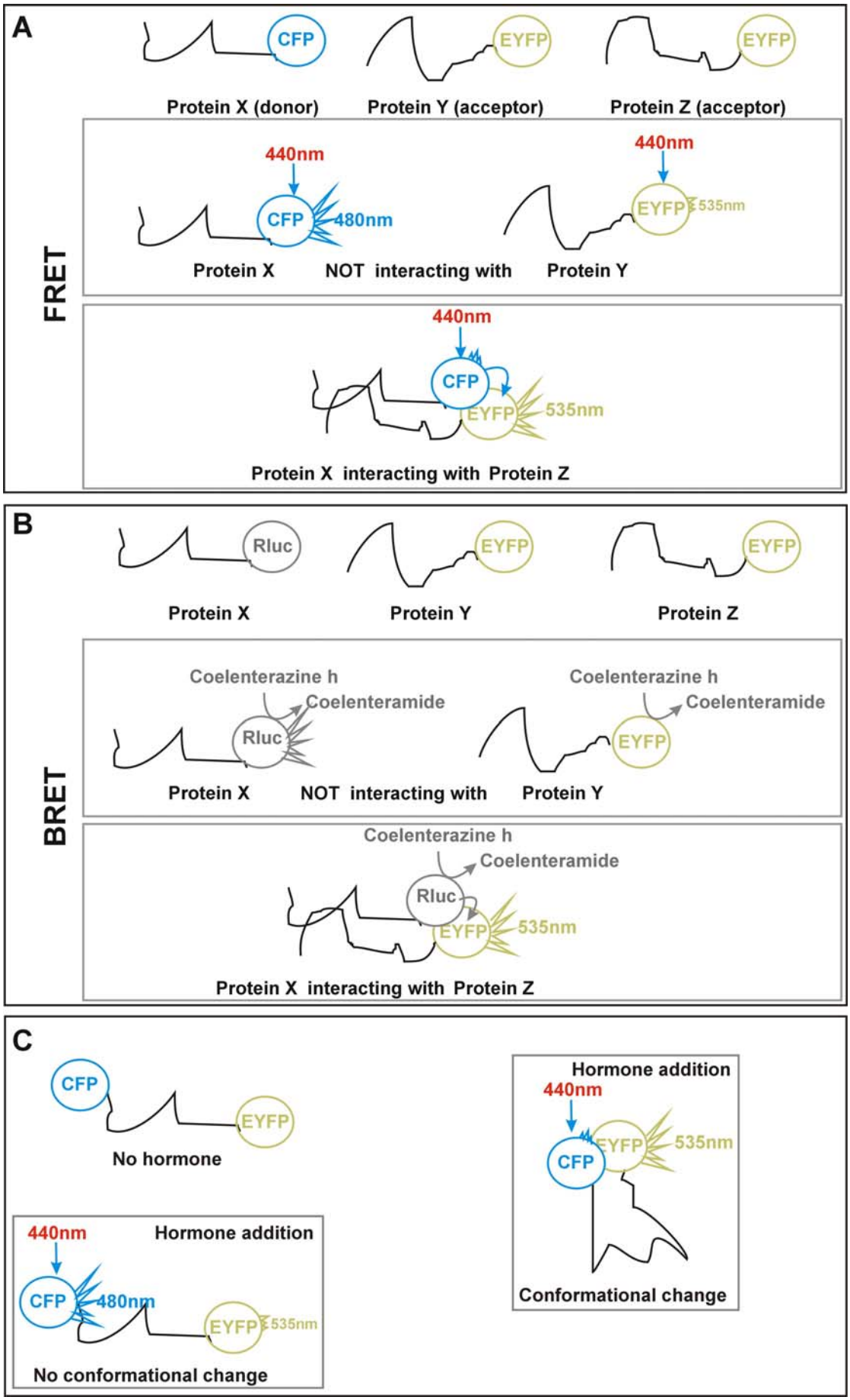
More recently, new techniques for the investigation of the AR N/C interaction have been developed which allow imaging full length AR interactions in real time in living cells. These novel imaging techniques, called fluorescence or bioluminescence resonance energy transfer (FRET or BRET), involve creating fluorescently or bioluminescently tagged proteins in mammalian expression vectors with non overlapping spectral distributions. These are then expressed in cells and upon excitation of one of the fluorophores (the donor) an electron is transferred to the other fluorophore (the acceptor), but only if the two proteins or domains of

interest are within close proximity (less than the width of one protein domain apart i.e. less than 100 angstroms). In the case of BRET, one of the protein tags is renilla luciferase and the other is a fluorophore. When the cells are excited at the donor wavelength of light or via substrate addition, the result of a functional interaction in FRET or BRET is a decrease in the emission of the donor fluorophore and an increase in the emission of the acceptor fluorophore (Fig 5.1) (Eidne et al. 2002). While FRET enables the location of interactions to be determined within a cell, the utilisation of Renilla luciferase in BRET allows for increased sensitivity of the assay due to lower background emission from the acceptor protein (Boute et al. 2002). This is because the method of excitation in BRET involves the addition of a substrate, coelenterazine h for excitation of Renilla luciferase rather than excitation with light, which results in non-specific excitation utilising the FRET method.

To date, two studies using FRET have demonstrated formation of an N/C interaction rapidly in the cytoplasm upon agonist treatment, and this preceeds an additional interaction in the nucleus which occurs following AR translocation (Schaufele et al. 2005). Furthermore, the ^{23}FQNLF^{27} motif is required for this interaction in both the cytoplasm and nucleus, indicating that the N/C interaction occurs as classically defined (that is, interaction of the amino-terminal ^{23}FQNLF^{27} domain with the AF2 surface) in both compartments.

Whereas the ablation of AR DNA binding capacity did not alter FRET activity, a decreased apparent N/C was observed when the AR was immobile inside nuclear foci (van Royen et al. 2007). These foci overlap sites of active transcription where the FXXLF-containing coregulator ARA54 binds preferentially to the AR. These findings indicate that the rapid induction of N/C in the cytoplasm upon agonist binding prevents the AR from untimely associations with coregulators (van Royen et al. 2007).

Figure 5.1: FRET and BRET techniques in the measurement of protein interactions and conformational change. A: FRET schematic. Proteins of interest are tagged with either Cyan fluorescent protein (CFP) the donor molecule which has an absorbance of 430-453nm and emission at 480 nm or enhanced yellow fluorescent protein (EYFP) the acceptor molecule which has an absorbance overlapping the emission spectrum of CFP but an emission of 535 nm. If the CFP tagged protein X donor molecule does not interact with the EYFP tagged protein Y acceptor molecule then excitation of CFP at 440 nM will result in emission at 480 nm. However, if CFP tagged protein X donor is interacting with EYFP tagged protein Z acceptor, this may result in the close proximity of CFP and EYFP (within 100 angstroms) and lead to the transfer of electrons to the EYFP acceptor molecule upon excitation of CFP at 440 nM resulting in emission at 535 nm. **B:** BRET schematic. Protein X is tagged with Renilla luciferase (Rluc) as the donor molecule and the acceptor molecule is tagged with EYFP as above. If the Rluc tagged protein X donor does not interact with the EYFP tagged protein Y acceptor upon excitation of Rluc by the addition of coelenterazine h substrate, energy will be released in the form of light at a wavelength of 464 nm. If the Rluc tagged protein X donor does interact with the EYFP tagged protein Z acceptor, the energy will be transferred to EYFP and emission at 535 nm will result. **C:** Measuring conformational changes within a protein using FRET involves double tagging at both the carboxyl and amino termini with the donor (CFP) and acceptor (EYFP) fluorophore. Addition of hormone may lead to structural changes in the protein sufficient to bring the donor and acceptor fluorophores together. If this is the case, excitation of the donor will result in a FRET signal at the acceptor's characteristic wavelength of light (535 nm) whereas lack of an appropriate conformational change will result in emission at the donor's wavelength of light (480 nm).


A
CFP
EYFP
EYFP
Protein X (donor)
Protein Y (acceptor)
Protein Z (acceptor)
FRET
440nm
CFP
480nm
Protein X
NOT interacting with
440nm
EYFP
535nm
Protein Y
440nm
CFP
EYFP
535nm
Protein X interacting with Protein Z
B
Rluc
EYFP
EYFP
Protein X
Protein Y
Protein Z
BRET
Coelenterazine h
Coelenteramide
Rluc
Protein X
NOT interacting with
Coelenterazine h
Coelenteramide
EYFP
Protein Y
Coelenterazine h
Coelenteramide
Rluc
EYFP
535nm
Protein X interacting with Protein Z
C
CFP
EYFP
No hormone
440nm
Hormone addition
CFP
480nm
EYFP
535nm
No conformational change
Hormone addition
440nm
EYFP
535nm
CFP
Conformational change

There are indications that the relationship between the N/C interaction and AR signalling is more complex. Firstly, the ^{23}FQNLF^{27} motif is required for initiation of transcription by AR from a chromatin integrated PSA promoter (Li et al. 2006). Secondly, the LXXLL-containing p160 coregulators modulate the N/C interaction and can rescue the interaction exhibited by an AR ^{23}AQNAA^{27} variant (Shen et al. 2005). Finally, as demonstrated in the previous chapter, the AF5 region is required for the N/C interaction. Consequently, it is possible that FRET may be measuring conformational changes associated with the N/C interaction, whereas other techniques such as the mammalian 2-hybrid N/C interaction assay measures the summation of N/C events that occur throughout the AR signalling cascade.

The N/C interaction occurs rapidly in the response to agonists but does not form when AR is bound to antagonists such as hydroxyflutamide (OHF), bicalutamide (BIC) or when bound to alternate hormones such as the synthetic progestin medroxy-progesterone acetate (MPA) (Kemppainen et al. 1999). This observation led Kemppainen et al to propose utilisation of the N/C interaction as a measure of agonist activity of androgenic ligands and the inhibition of the N/C as a measure of antagonism of the receptor. Thus, a sensitive BRET assay to measure the N/C interaction may have the potential to measure the androgenic activity of serum samples in real time. This would involve the addition of serum samples to the BRET interaction and quantification of the resultant BRET signal.

To date, the BRET technique has not been utilised to study the N/C interaction or AR interactions with other proteins. Before the intiation of this project, the FRET results mentioned above on the N/C interaction were also unpublished. Therefore, the objectives of the current study were to (i) create the required tagged AR constructs for utilisation of AR BRET, and (ii) assess the functional properties of these receptors in comparison to wtAR.

5.2 Methods

5.2.1 Plasmids

The pcDNA3.1-EYFP and pcDNA3.1-Rluc vectors were a gift of Associate Professor Karen Eidne (Western Australian Institute for Medical Research, Perth, Australia). pCDNA3.1-AR (wtAR), pcDNA3.1 (empty vector) ARR3-tk-Luc and pBS(sk-) have been previously described (Buchanan et al. 2004).

5.2.2 Cloning of amino- and carboxyl-tagged full length AR variants

Full length AR tagged at either terminus with Renilla luciferase in the pcDNA3.1-Rluc and pcDNA3.1-EYFP vectors were created utilising the PCR adapter method outlined in 2.3.1.13 from pCMV-AR3.1 AR coding sequence and the primers RlucS1, RLucAS1, EYFPS1 and EYFPAS1 (sequences and PCR conditions listed in appendix A1). The insertion sites and sequence of the product was verified on both strands utilising the sequencing primers pCMVS3 and pcDNA3.1AS1 up to the unique restriction enzyme sites *AflII* and *Bsu36I*. The verified sequence of pCMV-AR3.1 between these sites was inserted into the double digested AR BRET vectors as described in 2.3.1.13 and sequence verified over both insertion sites as described in section 2.3.1.10 with the primers N111, X4AS, pCMVS3 and pcDNA3.1AS1, sequences provided in appendix A2. The AR-EYFP tagged variants were created by sublconing the verified sequences from the Rluc-AR tagged variants and were sequence verified over the insertion sites, as outlined in Appendix A1.

5.2.3 Transactivation assays and immunoblots of tagged AR variants

Transactivation assays were performed as described in sections 2.3.3.1 and 2.3.3.2. The immunoblot presented in figure 5.4 was performed on luciferase assay lysate as described in section 2.3.5.1 and the immunoblot precented in figure 5.3 was performed utilising RIPA buffer lysis and Bradford quantification of protein as described in sections 2.3.5.3 and 2.3.5.4.

5.2.4 Nuclear localisation of tagged AR variants

Visualisation of the nuclear localisation of the tagged AR variants was performed according to section 2.3.6, utilising the AR-U407 rabbit polyclonal antibody with the DNA in nuclei stained with bisbenzimide H 33258 (Hoechst). All images were photographed at 60 X magnification and images were overlayed as described in section 2.3.6.

5.3 Results

5.3.1 The tagged AR variants have decreased transactivation activity

Four AR variants, presented schematically in figure 5.2, were created, cloned and sequence verified. The transactivation capacity and steady state levels of these variants were compared to wtAR. The renilla luciferase tagged AR's (Rluc-AR and AR-Rluc) demonstrated dramatically decreased DHT-induced transactivation activity (Fig 5.3A) and while immunoblotting of the these AR variants revealed the expected protein sizes of 135kD, Rluc-AR had dramatically reduced steady state levels in comparison to wtAR (Fig 5.3B). In contrast, the EYFP tagged AR variants displayed decreased but measurable DHT-induced transactivation activity (Fig 5.3A), and similar to wtAR steady state levels on immunoblot, with a predicted size of 127kD (Fig 5.3B).

5.3.2 Increasing steady state protein levels of tagged AR variants does not rescue AR transactivation activity

To test if differences in steady state levels were responsible for the decreased activity of the tagged AR variants, increasing plasmid DNA was transfected into COS-1 cells. While transfecting increased plasmid DNA of wtAR into the cells resulted in increasing steady state levels, transactivation activity did not follow the same trend. This suggests that treatment of the cells with 10 nM DHT and transfection of 0.5 ng of wtAR (pcDNA3.1-AR) leads to saturation of transactivation activity in this system (Fig 5.4). In contrast, the steady state level of both Rluc-AR and ARR-luc could be increased but both still failed to increase DHT-stimulated transactivation activity. The EYFP tagged AR variants exhibited a dose dependent increase in steady state levels and transactivation activity with increasing transfected plasmid albeit at a much lower maximal transactivation activity than wtAR (Fig 5.4).

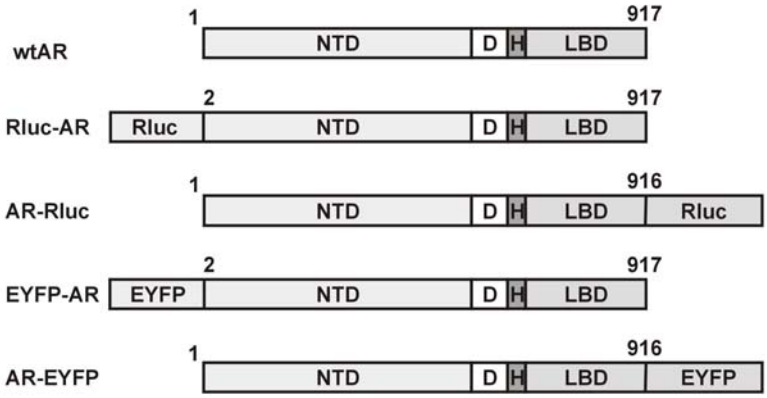


Figure 5.2: AR variant Constructs created to investigate the utility of BRET for AR protein interactions. Rluc-AR: AR tagged on the amino terminus with Rluc (with amino acid 1 of AR omitted); AR-Rluc: AR tagged on the carboxyl terminus with Rluc (and the stop codon, amino acid 917 of the AR omitted); EYFP-AR: AR tagged on the amino terminus with EYFP (with amino acid 1 of the AR omitted); AR-EYFP: AR tagged on the carboxyl terminus with EYFP (with amino acid 917 omitted). Wildtype AR is shown in the figure for comparison.

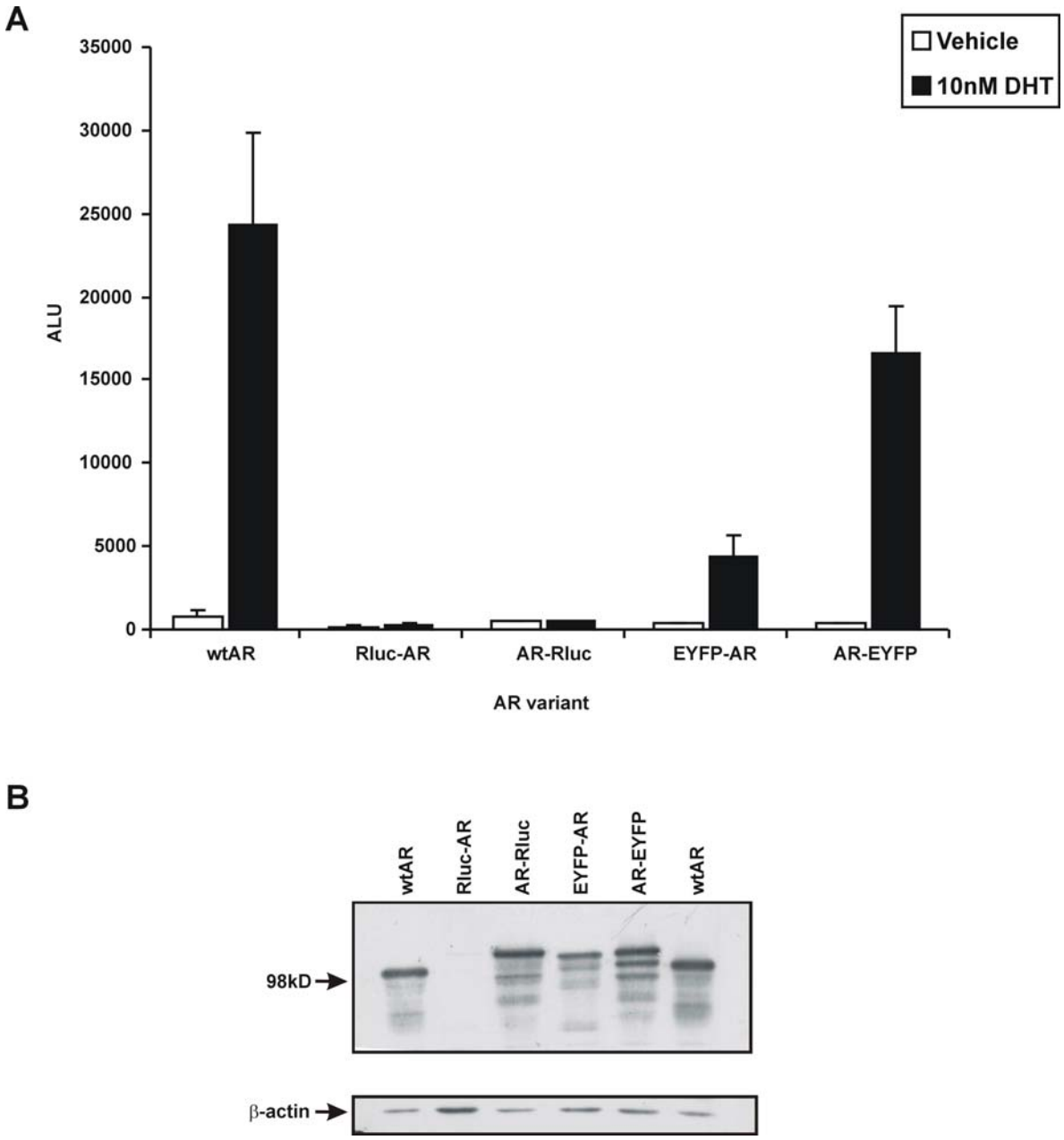


Figure 5.3: Tagging AR on either terminus interferes with transactivation activity and/or protein steady state levels. **A:** Transactivation analysis of AR variants involved transfection of COS-1 cells (10,000 per well in 96 well plates) with 2.5 ng of wtAR or the molar equivalent of the tagged AR variants and 100 ng of ARR3-tk-Luc. The prokaryotic plasmid pBS(sk-) was added to maintain total DNA in each transfection. Cells were treated with vehicle (EtOH) or 10 nM DHT for 24 h and assayed for luciferase activity. Data represents the mean $\pm$ SEM arbitrary light units (ALU) of 6 independently transfected wells. **B:** Immunoblot of AR variants demonstrated reduced Rluc-AR steady state levels. COS-1 cells (5 x 10^5 per well in 6 well plates) were transfected with 100 ng of wtAR or the molar equivalent of tagged AR variant and treated for 24 h with 10 nM DHT. Lysates were collected in RIPA buffer with protease inhibitors and blotted with AR N-20 antibody or anti-β actin. Data is representative of 2 independently performed experiments.

5.3.3 Nuclear localization of the tagged AR variants is significantly impaired

The ability to initiate transactivation is one of the final stages in the androgen receptor signalling cascade. Therefore, the ability of the tagged AR's to undergo one of the earlier events in activation; the ability to translocate to the nucleus upon hormone treatment was assessed. In COS-1 cells transfected with wtAR the receptor was almost exclusively cytoplasmic in vehicle treated cells and predominantly nuclear following 10 nM DHT treatment (Fig 5.5). Whereas Rluc-AR exhibited a similar pattern to wtAR, AR-Rluc was cytoplasmic even after 4 hours of 10 nM DHT treatment (Fig 5.5). In comparison, 10 nM DHT treatment of EYFP-AR and AR-EYFP resulted in more homogenous AR staining throughout the cell for both tagged AR variants (Fig 5.6). However, a lot of cytoplasmic staining for all of the variants except for Rluc-AR was noted, even in the presence of DHT. This may be a consequence of transfecting too much AR expression plasmid into the cells. Counting of several fields of cells for the distribution of staining with vehicle or DHT treatment highlighted that only Rluc-AR displays similar to wtAR cellular distribution in the presence and absence of DHT. All of the remaining tagged AR variants displayed altered cellular distribution compared to wtAR in the presence of DHT (Fig 5.7).

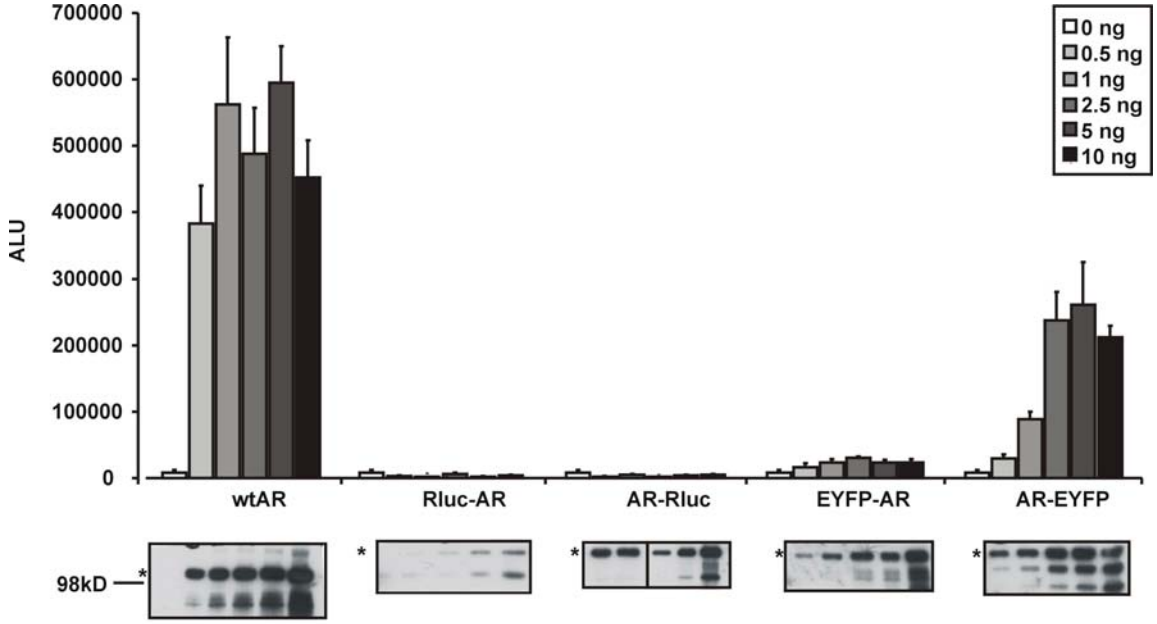


Figure 5.4: Increasing the steady state levels of tagged AR vectors increases the transactivation activity of EYFP but not Rluc-tagged AR vectors. Transactivation assays involving transfection of COS-1 cells (1 x 10^4 cells per well in 96 well plates) were transfected with increasing ng amounts of expression vector. Empty expression vector and prokaryotic plasmid were utilised to ensure that total reporter number and total DNA transfected was equal for each well. Wells were treated with 10 nM DHT and assayed for luciferase activity 24 hours later. Data represent the mean $\pm$ SEM of 6 independently transfected wells. Immunoblots were performed on an equal volume of transactivation assay lysate pooled from the replicate wells with the AR N-20 antibody. * represents the AR product of expected size. As not all of the lysates would fit on one gel, the samples for AR-Rluc were split over two gels, with the different gels separated by a line in the AR-Rluc immunoblot. Data is representative of 2 independently performed experiments.

Figure 5.5: AR tagged at the amino terminus with Rluc undergoes normal nuclear localisation following treatment with DHT whereas carboxyl tagging with Rluc abolishes nuclear translocation. COS-1 cells (5.6×10^4 per well in 8 well chamber slides) were transfected with 800 ng AR variant per well. Cells were incubated for 33 hours in stripped growth media and then treated with 10 nM DHT or vehicle (EtOH) for 4 hours. Wells were fixed with 4% paraformaldehyde and immunodetected utilising AR specific U402-s antibody, along with Alexa-fluor donkey anti sheep secondary antibody. Nuclei were stained with 200 µg/ml bisbenzimide H 33258 (Hoechst) before being mounted with fluorescent mounting medium. Cells were visualised and photographed on an Olympus reverse-phase fluorescent microscope at 60X magnification. Data is representative of 2 independently performed experiments.

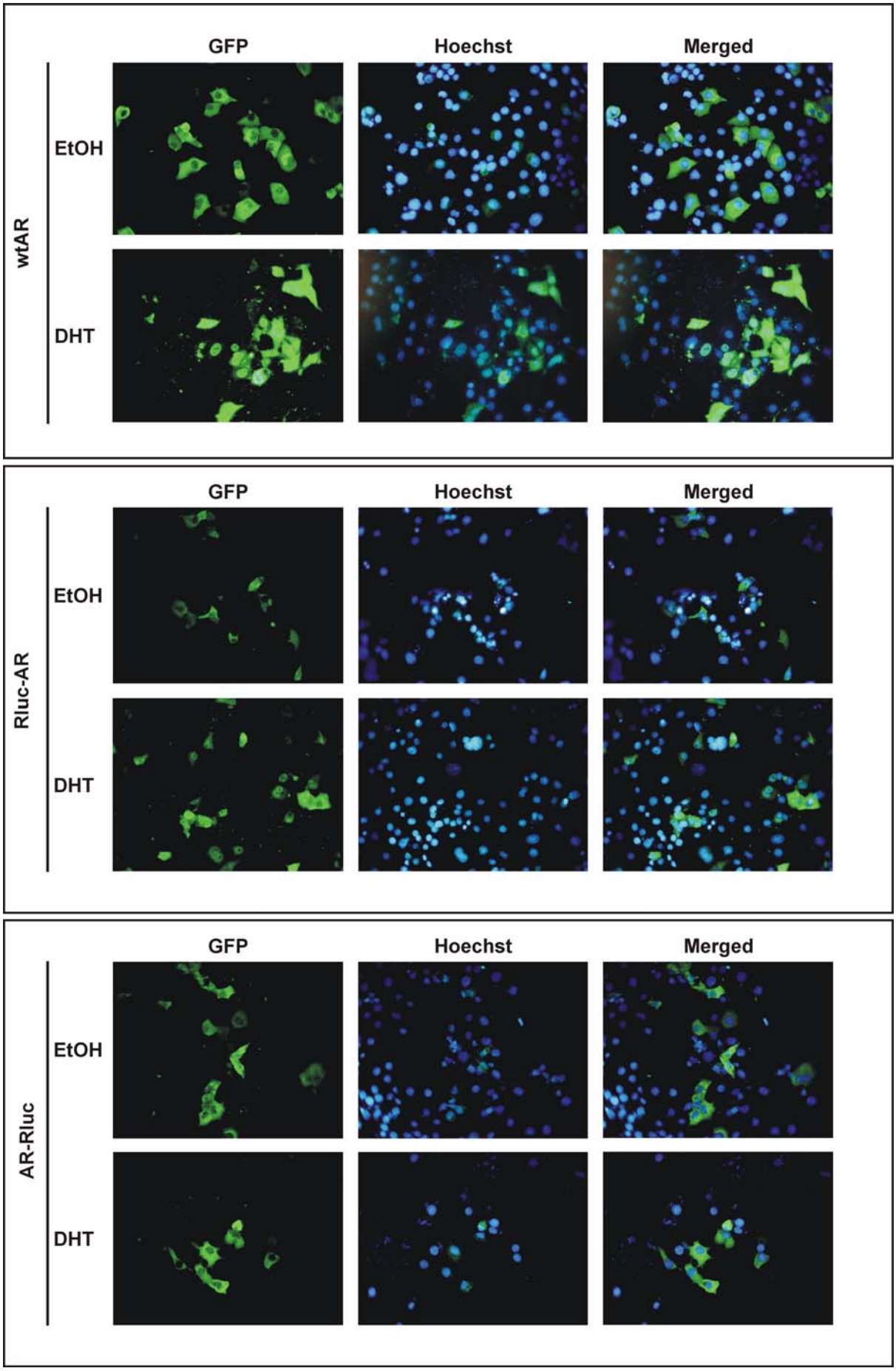
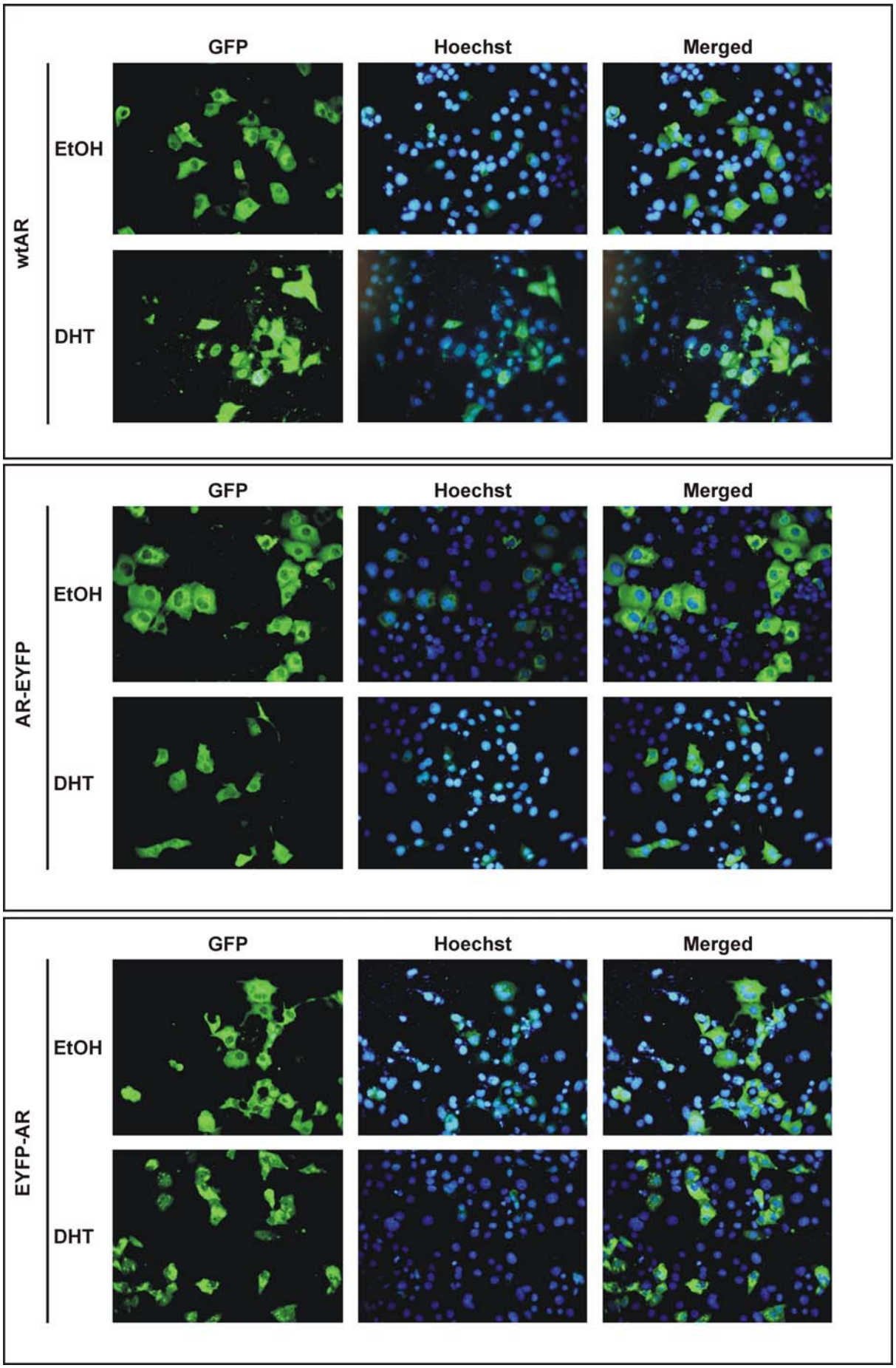

GFP
Hoechst
Merged
wtAR
EtOH
DHT
GFP
Hoechst
Merged
Rluc-AR
EtOH
DHT
GFP
Hoechst
Merged
AR-Rluc
EtOH
DHT

Figure 5.6: Amino and carboxyl terminal tagged AR with EYFP display homogenous AR localisation upon DHT treatment. Cells were transfected and treated as described in figure 5.5. Data is representative of 2 independently performed experiments.


GFP
Hoechst
Merged
wtAR
EtOH
DHT
GFP
Hoechst
Merged
AR-EYFP
EtOH
DHT
GFP
Hoechst
Merged
EYFP-AR
EtOH
DHT

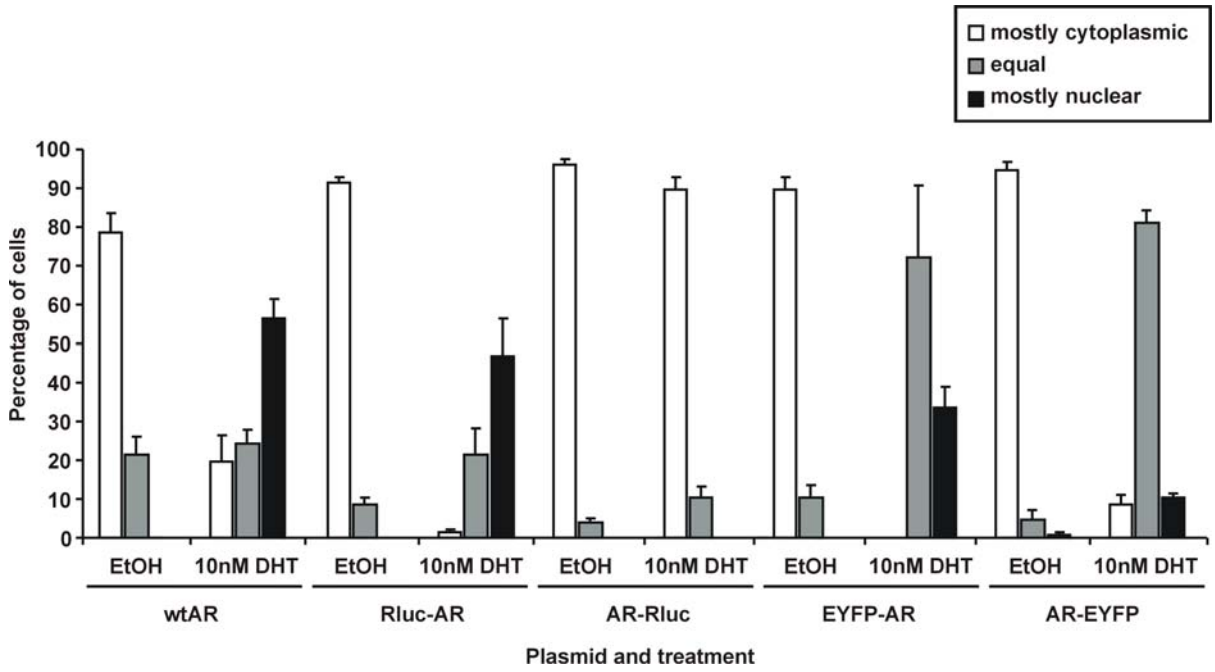


Figure 5.7: Summary of nuclear localisation of BRET vectors upon DHT treatment reveals that all of the tagged AR variants except Rluc-AR have aberrant localisation. Cells were treated and visualised as described in Figures 5.5 and 5.6. Greater than 100 cells were scored overall in 5 different fields for each different treatment group as displaying mostly cytoplasmic, mostly nuclear or equal nuclear and cytoplasmic GFP staining. Data is presented as the mean number of cells $\pm$ SEM of each staining type over 5 different fields.

5.4 Discussion

Over expression of proteins in mammalian cells is a common tool utilised to investigate protein function. For FRET and BRET procedures, over expression must be carefully controlled or cells become flooded with protein thereby increasing the background activity of the assay and dramatically reducing sensitivity (Eidne et al. 2002). In addition, it is important that tags introduced to either the amino or carboxyl terminus of proteins do not interfere with native protein function. The findings in this chapter demonstrate that the tags used for BRET compromise either receptor transactivation capacity or normal nuclear redistribution of the AR following DHT treatment, indicating that the function of the tagged AR variants is compromised.

Importantly, there have now been two studies using AR tagged on the amino and carboxyl termini, but both fail to present convincing data on correct translocation of the tagged AR variants. One study demonstrated only a modest increase compared to wtAR in the nuclear levels of the tagged AR's by immunoblotting of nuclear or cytoplasmic fractions (Schaufele et al. 2005). This slight increase may be akin to the re-distribution of the EYFP tagged AR in the current study. The second study utilised linker sequences between the tags and AR consisting of glycine and alanine residues, but did not present data on translocation (van Royen et al. 2007). Nuclear translocation is one of the crucial steps of AR signalling that is inseparable from the AR N/C interaction, as both occur rapidly in response to agonist. Indeed, if AR translocation is impaired with the addition of tags on the AR, then this could result in misleading results as to where and when the N/C interaction is formed. Given the inseparable nature of the N/C interaction and nuclear translocation, it is entirely possible that an as yet unmeasured N/C interaction occurs in the nucleus.

A future possibility that has been suggested is to place the tags in the middle of the protein of interest, for example in a flexible linker region. Placing one of the tags within the hinge or

amino terminal regions of the AR (or between domains) may prevent some of the problems currently associated with the end-tagging of the AR. However, this is probably conducive only for investigation of the interaction of the AR with other protein partners and not for the investigation of the N/C interaction if indeed it is a rapid conformational change of the AR that occurs upon ligand binding. In addition, it is now clear that the hinge region is not solely a flexible spacer region but is a protein interaction domain (Buchanan et al. 2001; Buchanan et al. 2007) and contains sites of acetylation and phosphorylation necessary for normal receptor function (Haelens et al. 2007). While also considered a flexible region, structural redistribution and changes in the AR-NTD are essential components of receptor activity, and this domain also contains many phosphorylation and protein interaction sites vital for transcriptional function (Alen et al. 1999; Ueda et al. 2002; Kumar et al. 2003; Buchanan et al. 2004; Lavery et al. 2005). Placement of internal tags would therefore require a more detailed understanding of the structure of the AR NTD and hinge regions to avoid interference with AR function.

While it is demonstrated here that tagging the AR on the amino or carboxyl termini with either EYFP or Rluc compromises AR nuclear translocation or transactivation function, the FRET studies utilising double tagged full length AR variants to investigate the N/C interaction, have nevertheless provided some useful and novel information on the N/C interaction but must be interpreted with caution. While the tags themselves may cause a conformational change within the AR thereby inhibiting the binding of AR by coregulators, these results from FRET fit not only with the working model of N/C interaction and coregulator binding proposed in the previous chapter, but also with data defining the antagonistic nature of AR coregulator interactions and the N/C interaction (He et al. 2001; He et al. 2006; Toumazou et al. 2007).

Development of new classes of fluorophores or technologies to label specific proteins within living cells such as the use of nanoparticles, or smaller genetically encoded proteins may allow tagging of AR without the undesirable biological consequences (Sapsford et al. 2006). A recent report comparing the use of CFP/YFP to the use of CFP and a membrane permeable fluorescin derivative targeted to a short sequence of cysteines encoded into the acceptor protein demonstrated improved fold response to a stimulus in the FRET signal and normal downstream signalling of a membrane bound G protein coupled receptor (Hoffmann et al. 2005). Future work should be aimed at developing tagged AR molecules that do not compromise biological function. Once such molecules have been established then the application of FRET could provide a means of further examination of the role of AF5 in the N/C and coregulator interactions and allow verification of the current FRET results with respect to the N/C interaction.

Overall, these findings indicate that AR tagged on either the carboxyl or amino terminus with Rluc or EYFP interferes with AR transactivation activity and/or nuclear translocation. Given that N/C interaction and AR translocation may be mutually inseparable, the placement of the reporter tags to investigate the AR N/C interaction needs to be considered and carefully optimised so that results are not distorted towards a particular cellular compartment. It may be possible in the future to create tagged AR variants that are minimally compromised by fluorescent-derivative tags and thereby gain a more concrete evaluation of the N/C interaction with full length AR molecules in real time in living cells.

Chapter 6: Development and optimisation of a bioassay to measure bioactive testosterone in male human serum

6.1 Introduction

It has been estimated that as many as 1 in 200 men in the Australian population may have hypogonadism which manifests as low levels of the circulating androgen, testosterone (T) (Handelsman et al. 2004). While the previous chapters define a model for the involvement of each of the transactivation domains of the AR in transcriptional activation, the ability of androgens to enter a cell will ultimately lead to transcriptional activation and therefore also warrants detailed investigation. As discussed in section 1.3, most of the T in circulation is bound to the serum binding proteins albumin or sex hormone binding globulin (SHBG). A small proportion of unbound T (2%) is called free testosterone (FT) and is thought to be readily available to cells while albumin bound T (45-50%) by virtue of binding T less tightly than SHBG is thought to be more available to cells than SHBG bound T (45-50%) (Sodergard et al. 1982; Mendel 1992). Commonly, total T is measured in the serum (total T) to gain a measure of the androgenic status of an individual but in large research studies other measures are utilised which attempt to account for effects on cellular availability of the serum binding of T. Estimation of the serum levels of calculated FT (cFT) involves the measurement of total T, serum SHBG and serum albumin and the calculation of FT utilising the binding coefficients of T to each of these serum binding proteins (Vermeulen et al. 1999). Levels of calculated bioavailable T (cBT) in serum are similarly evaluated via the measurement of serum SHBT and total T and the subsequent calculation of the non SHBG-bound portion of T(van den Beld et al. 2000). Serum levels of immunoassayed bioavailable T (iBT) by contrast are evaluated by the direct measurement of non SHBG bound T involving precipitation of SHBG-bound T from the serum sample and the subsequent measurement of the remaining T (O'Connor et al. 1973). The interpretation of each of these parameters is based on controversial assumptions regarding the physiologically relevant fraction of serum T (Pardridge 1987; Mendel 1992). The fraction of serum T capable of entering cells and mediating transcriptional activation via the actions of the AR is currently unknown.

The measurement of androgenic substances in biological samples other than serum to identify endocrine disrupting chemicals has been investigated in numerous assay systems including yeast, mammalian cells and whole organisms (Gray et al. 2002). Many of these assays have provided a useful tool to assess the androgenicity of either specific chemical compounds or the androgenicity of complex biological samples (Hennessey et al. 1986; Raivio et al. 2001; Paris et al. 2002; Lee et al. 2003; Roy et al. 2004; Sonneveld et al. 2004; Chen et al. 2006; Roy et al. 2008). However, to date only five have used a mammalian cell system to describe the ability of androgens in human serum samples to mediate transactivation of an androgen regulated reporter gene through the AR. The earliest of these investigated the androgenic potential of human serum by utilising the N/C interaction assay (Chapters 3 and 4) demonstrating a strong correlation with total T measured by immunoassay ($r = 0.93$, $p>0.0001$, $n = 22$) along with low intra and interassay coefficients of variation (8.3% and 21% respectively) (Table 1)(Raivio et al. 2001). Bioactive androgen in serum as measured by this method was lower in males with prostate cancer compared to those without (Raivio et al. 2001; Raivio et al. 2003). This assay has also been subsequently utilised to measure bioactive androgens in testicular fluid, correlating positively with pubertal changes in males with constitutional delay of puberty as well as showing increased levels of serum bioactivity in elderly males treated with DHT replacement therapy (Raivio et al. 2002; Raivio et al. 2004; Jarow et al. 2005). Currently unclear, however, is whether this measure simply reflects total T in the serum or is an independent measure of androgen action.

Subsequent assays have been developed using a more conventional approach; they utilise stable integration into either the liver cell line HEK 293 or Chinese hamster ovary cells (CHO) of a constitutive AR expression plasmid and a luciferase reporter gene utilising an MMTV response element. Despite this response element being a general hormone response element (Ham et al. 1988), all of these assays demonstrate androgenic selectivity of response

either due to the absence of the other steroid receptors or as a consequence of the over expression of AR in these cells or both. These assays require overnight or 24 hour incubation of the cells with human serum varying from 1 to 10% of the medium incubation volume per well. All of these assays demonstrated intra and inter assay correlation coefficients below 10 and 20% respectively (Table 1)(Paris et al. 2002; Chen et al. 2006; Roy et al. 2006). The largest study involved 68 individuals, mostly females, and showed no correlation with total T as measured by radioimmunoassay but selected samples from this cohort demonstrated a good correlation with total serum T measured with gas chromatography tandem mass spectroscopy (GC MS/MS)($r = 0.99$, $p<0.05$, $n=15$). Furthermore, this assay also showed that female samples with higher levels of SHBG had lower values on the assay when spiked with 1 nM T in comparison to samples with lower levels of SHBG, indicating that T-SHBG binding was influencing the assay measurement (Table 1) (Chen et al. 2006).

An assay comprising the stable integration of a synthetic androgen selective promoter containing 3 androgen response elements and a minimal promoter element and an AR expression plasmid into human bone U2-OS cells has been described. Twenty-four hours of treatment with human serum at a maximum of 10% of the total treatment volume mediated significant luciferase activity in a dose dependent fashion. No inter or intra assay coefficients were reported and it appears that the assay was only tested on one serum sample (Table 1) (Sonneveld et al. 2004).

Reference	Transfected Or stable	Treatment time	Midrange of assay (nmol/L T)	Inter-assay CV	Intra-assay CV	Least detectable concentration nmol/L T	Cohort size
Raivio et al (2001)	Transfected	Overnight	0.675	21%	8.3%	0.1	23
Chen et al (2006)	Stable	16 h	0.535	7.4%	7.5%	0.015	49
Paris et al (2002)	Stable	24 h	0.04	<20%	<10%	0.010	17
Sonneveld et al (2004)	Stable	24 h	0.66	22%	NR	NR	1
Roy et al (2006)	Stable	24 h	0.3	19.4%	8.5%	0.1	30

Table 1: Comparison of currently reported androgen mammalian cell-based bioassay characteristics

Thus there is currently no extensively optimised bioassay that utilises a male human cell line and a strictly androgen responsive promoter element. Consequently, the aims addressed in this chapter are to (i) to establish an assay in a male human cell line that can specifically measure T in a human serum sample and (ii) to optimise this assay in order to reliably and reproducibly measure bioavailable androgens in male human serum samples.

6.2 Materials and methods

6.2.1. Materials

Cyanoketone was a gift from Professor David Armstrong (Department of Gynaecology and Obstetrics, Adelaide University). Other reagents and chemicals along with their suppliers are listed in table 2.1.

6.2.2 Plasmids

Plasmids for transactivation assays are listed in appendix A.2.1.1. The pGL4.14-ARR3-tk-LUC plasmid was subcloned from pGL3-tk-ARR3-Luc as described in 2.3.4.1.

6.2.3 Human serum sample collection and dextran charcoal stripping

Male human blood was kindly donated by a healthy male 25 year old volunteer via venous collection into vacuette serum collection tubes. Blood was allowed to clot for 30 minutes, and tubes were spun at 1000 g for 15 minutes. Serum was isolated from the blood cells and placed into sterile 50 mL centrifuge tubes. One half of the serum volume was dextran charcoal stripped according to the method outlined in section 2.3.2.5. Stripped and non stripped serum was sent to the IMVS clinical pathology laboratory for measurement of the levels of cortisol, T and E2 utilising the Immulite 2000 immunoassay platform (DPC, Los Angeles, CA, USA).

6.2.4 In vitro transactivation assay

Transient transactivation assays were performed with the amount of reporter and AR expression plasmid indicated according to the general method outlined in section 2.3.3.2.

6.2.4.1 Treatment of cells with hormone for 24 hours

Following transfection, cells were treated with 100µl of phenol red free RPMI 1640 with 5% destran charcoal stripped FCS (treatment medium) containing the indicated concentrations of test or control human serum or for calibrations, containing the indicated concentration of T

spiked into charcoal stripped human serum and diluted in treatment medium. The concentrations of both human serum and vehicle (EtOH) was constant across all wells of each experiment. Each test, control and calibration sample was performed in 6 replicate wells for 24 hours. Cells were lysed and assayed for luciferase activity as described in section 2.3.3.1.

6.2.4.2 Treatment of cells with hormone for 5 minutes

Following the transfection procedure, the transfection medium was removed from the plates by inversion and 25 µL of the respective treatment was placed into each well. Calibrations were prepared by spiking the indicated concentration of T into dextran charcoal stripped human serum and serial dilution. Test wells contained 25 µL 100% human serum per well. For FCS standard curves, 100% dextran charcoal stripped FCS was substituted for the human serum. Vehicle (EtOH) was kept constant across all wells of the experiment. Plates were incubated at 37^{o}C for 5 minutes, after which the treatment was removed by inversion of the plate and wells were washed once with PBS. Phenol red free RPMI 1640 with 5% dextran charcoal stripped FCS was then placed onto the wells and plates incubated at 37^{o}C with 5% CO_2 for 24 hours before lysis and luciferase measurement as described in section 2.3.3.1.

6.2.5 Electroporation delivery of DNA for transactivation assay

PC3^{AR+} cells were harvested at log phase by trypsinisation, resuspended in growth medium and then pelleted by centrifugation at 2000 g for 5 minutes. Cells were washed once with double the volume of room temperature PBS and pelleted as above. Following determination of cell number by trypan blue exclusion, cells were resuspended at approximately 1 x 10^7 cells/mL in PBS. An aliquot of 0.5 mL of cells was mixed with 15 µg of pGL4.14-ARR3-LUC and 5 µg pCMV-AR with 400 µL of these samples transferred to a 2 mm gap cuvette (BTX International, Massachusetts, USA) and electroporated on a square wave electroporator ECM 630 (BTX International) at 280 V for 2 msec. The cell mixtures were immediately resuspended in 40 mL of dextran charcoal stripped growth medium, plated at 100 µL per well (approximately 10,000 cells per well in 96 well plates) and left to adhere for 24 hours before

hormone or serum treatments were added to the cells for a further 24 hours as described above.

6.2.6 *In vivo* transactivation assay

Stable cell lines were created by the method outlined in 2.3.4.4. Cells were then transfected with either 100 ng pCMV-AR or 100 ng pGL4.14-ARR3-LUC according to the method outlined in section 2.3.3.3. Cells were treated with the appropriate hormones or equivalent concentration of vehicle (EtOH) in phenol red free RPMI 1640 with 5% dextran charcoal stripped FCS for 24 hours and assayed as described in section 2.3.3.1. Immunoblots were performed according to the method outlined in section 2.3.5.1.

6.2.7 Cyanoketone inhibition transactivation assay and radioimmunoassay

Cells were plated and transfected as described above in 6.2.4 and 6.2.4.1 and treated for 24 hours with 20 nM T, 10 µM DHEA(S) or the equivalent vehicle control (EtOH) in the presence or absence of 0.01, 0.1 or 1 µM cyanoketone diluted in phenol red free RPMI 1640 with 5% dextran charcoal stripped FCS. All treatments were duplicated in wells containing no cells to provide a baseline estimate of the added hormone. After 24 hours of incubation, media was collected from all wells, transferred to a fresh 96 well plate and frozen until radioimmunoassay at -20^{o}C. Cells were lysed and assayed according to the method in section 2.3.3.1. Radioimmunoassays for androstenedione (ASD) and testosterone were performed by Professor David Armstrong (Discipline of Obstetrics and Gynaecology, The University of Adelaide) utilising either the androstenedione or testosterone DPC coat-a-count kit according to manufacturer's instructions. All treatments were performed in triplicate wells, with non phenol red free RPMI with 5% dextran charcoal stripped FCS spiked with increasing concentrations of ASD or T to form standard curves.

6.2.8 Megalin immunofluorescence

Both COS-1 and $PC3^{AR+}$ cells were plated and fixed according to sections 2.3.6.2 and 2.3.6.3 except for the omission of the methanol and acetone washes to prevent membrane permeabilisation. Cells were then incubated overnight at $4^{o}C$ in a humid chamber with 5% donkey serum in PBS containing 1:50 or 1:100 anti megalin C-19 antibody. To demonstrate specificity of staining, cells in control wells were incubated with 1:100 anti megalin pre-incubated for 2 hours with 50 fold excess of blocking peptide. The slides were then washed twice for five minutes in PBS twice and then incubated for 30 min in the dark in 1:400 donkey anti-sheep Alexa fluor 488 in 5% donkey serum in PBS. The remainder of the staining and imaging procedure was performed according to section 2.3.6.4.

6.2.9 Statistical analyses

All statistics in this chapter were performed utilising Graphpad prism 5 for windows. Where there were more than three comparisons, differences between groups were analysed with the Kruskal-Wallis test with a Dunn's multiple comparison post hoc test which does not assume normality of the data. Where there were only two comparisons, a Mann Whitney U assessment of medians was performed. All results that are presented with an asterix (*) are significant to the level of $p<0.05$.

6.3 Results

6.3.1 Determination of most responsive and sensitive androgen response element

In order to determine the most sensitive transient transactivation assay system, the reporters utilised in chapters 3 and 4 were tested for their responsiveness to T in PC3 cells and compared to the previously utilised bioassay cell lines CHO-K1 and COS-1. The MMTV reporter investigated in previous chapters was not assessed because it is not selectively androgen responsive. Compared to the ARR3-tk-luc reporter, both the PSA540 and 5.8PSA reporters demonstrated reduced fold activation in response to T treatment at all concentrations of reporter transfected in all of the cell lines, despite an equimolar concentration of all 3 reporters being transfected into the cells (data not shown). Furthermore, while wells transfected with 50 ng and 100 ng of reporter responded equally to T treatment for each of the promoters, the luciferase response in wells transfected with 200 ng of reporter was lower. Transfecting 100 ng of reporter and 2.5 ng of the pCMV-AR expression plasmid per well into the cell lines COS1, CHO-K1 and PC3 resulted in optimal activity over the vehicle treated wells from all reporters when treated with 10 nM T except in CHO-K1 cells which demonstrated significant activity above vehicle treated control on the ARR3 reporter only (Figure 6.1A). In contrast, the prostate cancer cell line $PC3^{AR+}$ or the breast cancer cell line T47D resulted in increased sensitivity in comparison to the PC3 parental cell line, demonstrating a response significantly above vehicle treated wells when treated with 0.1 nM T. Further, the $PC3^{AR+}$ cells treated with 0.1 nM T had a smaller standard error in comparison to similarly treated T47D cells (Fig 6.1B). Increasing the transfected amount of AR expression plasmid to 5 or 10 ng per well did not further increase the sensitivity of the assay (Fig 6.2A). At 1 nM T the response in wells transfected with greater than or equal to 2.5 ng AR expression plasmid was therefore considred optimal (Fig 6.2A).

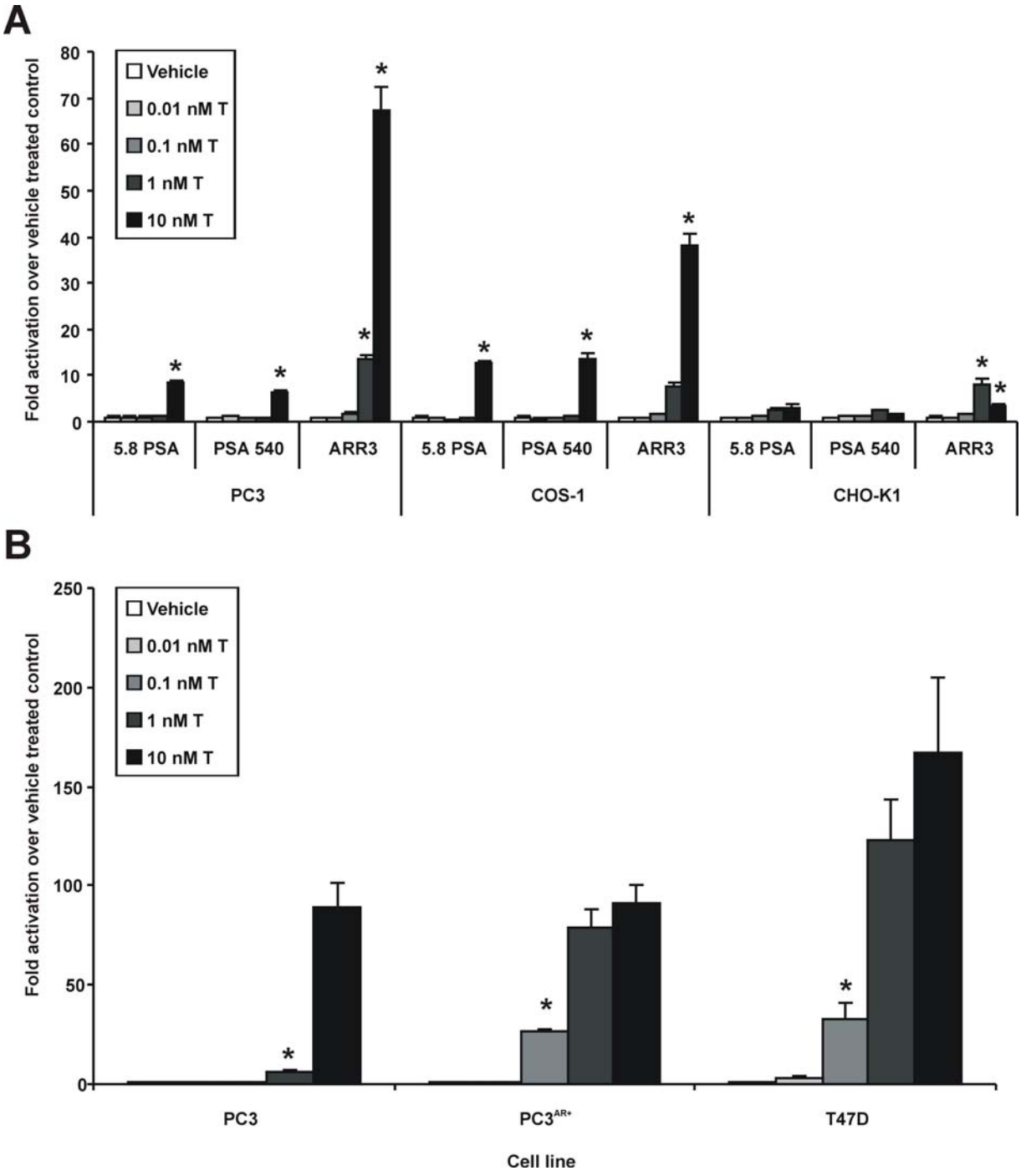
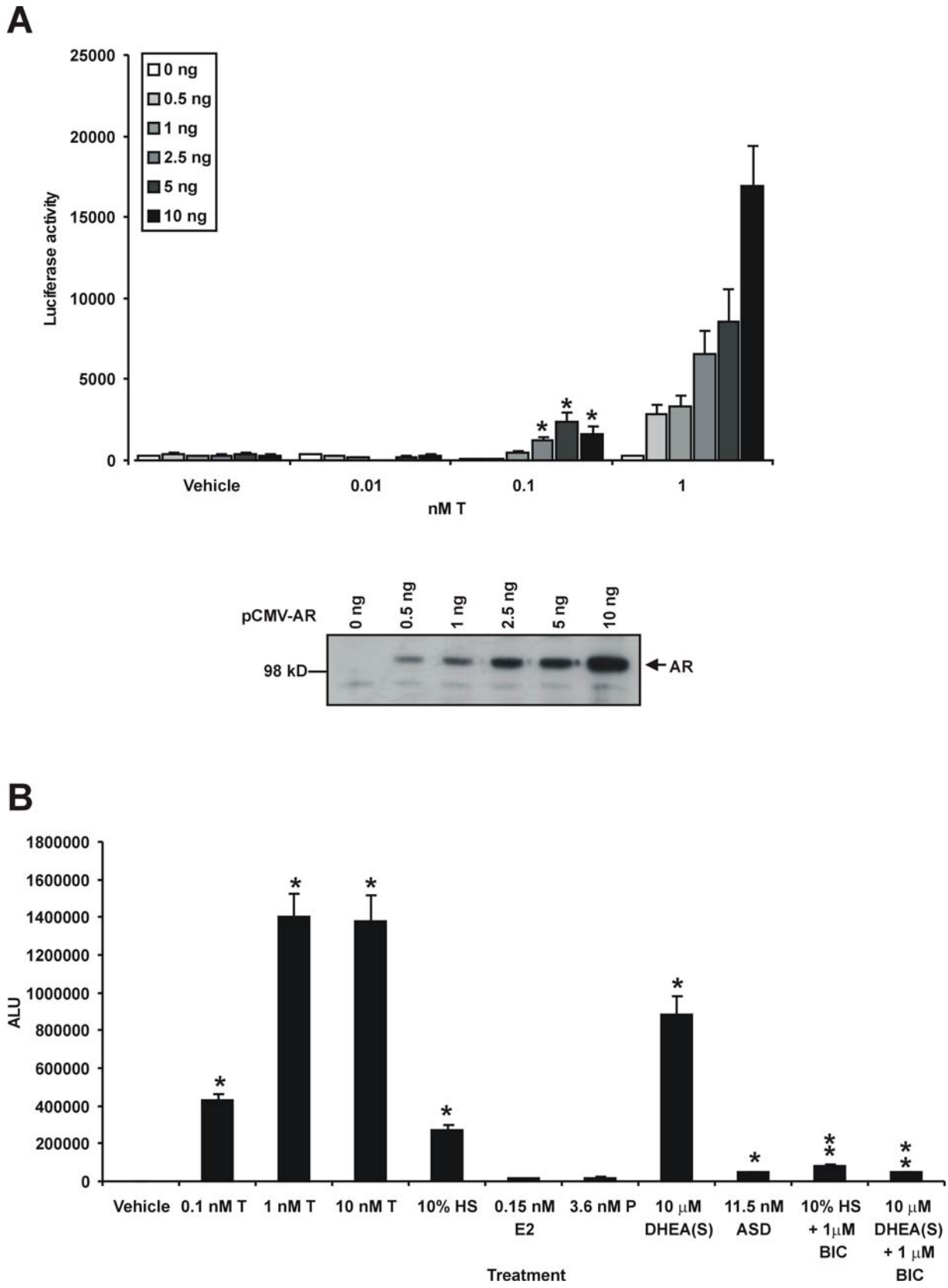


Figure 6.1: The ARR3 reporter in PC3^{AR+} cells demonstrates sensitive reporter gene transcription in response to T. **A:** PC3, COS-1 or CHO-K1 cells (10,000 per well in 96 well plates) were transfected with 2.5 ng pCMV-AR and 100 ng of the respective reporter plasmid ARR3, 5.8PSA or PSA 540. Wells were treated for 24 hours with increasing amounts of T from 0.01 nM to 10 nM, or vehicle (EtOH), after which they were lysed and assayed for luciferase activity. Data represents the mean fold activation over ethanol treated wells ± SEM of 6 independently transfected wells. **B:** PC3, PC3^{AR+} or T47D cells were transfected, assayed and presented as described in A. *p<0.05 with two tailed Mann Whitney U assessment of means in comparison to the vehicle only treated data for the specific cell line. All data is representative of at least 2 independently performed experiments.

Figure 6.2: Response of the transactivation assay to increasing steady state AR levels and alternate hormones found in male serum. A: Increasing the amount of AR per well increases AR steady state levels but not sensitivity of transactivation response to T. $PC3^{AR+}$ cells (10,000 per well in 96 well plates) were transfected with 100 ng ARR3-tk-Luc and increasing ng amounts of pCMV-AR from 0.5 ng to 10 ng per well, or no pCMV-AR per well. The molar concentration of expression vector and total DNA per well was equivalent between the wells via the utilisation of the empty expression vector pCMV and the prokaryotic plasmid pBS(sk-). Wells were treated with increasing concentrations of T from 0.01 to 1 nM T or vehicle control only (EtOH) for 24 hours before lysis. Immunoblot was preformed on the pooled lysates of the 6 independently transfected wells with an equal volume of lysate (25 µL) resolved for AR with the AR N-20 antibody. Data on graph represent the mean ± SEM luciferase activity in arbitrary light units (ALU) of 6 independently transfected wells. *$p<0.05$ for Kruskal-Wallis analysis of variance with Dunns post hoc analysis in comparison to the 0.1 nM T treated well with no AR transfected. **B:** Treatment of the assay with various hormones for 24 hours demonstrates significant responses in comparison to vehicle treated wells from physiological levels of the hormones T, dehydroepiandrosterone sulphate (DHEA(S)) and androstenedione (ASD) and human serum but not 17β-estradiol (E2) or progesterone (P). $PC3^{AR+}$ cells were transfected with 2.5 ng pCMV-AR and 100 ng ARR3-tk-Luc per well and incubated for 24 hours. Levels of hormone correspond to average hormone levels found in the serum of a healthy adult male. Data represents the mean ± SEM ALU of six independently transfected wells. * $p<0.05$ for Kruskal-Wallis analysis of variance with Dunns post hoc analysis in comparison to the vehicle treated data. **$p<0.001$ for Mann Whitney U analysis of medians in comparison to the wells with the same treatment without bicalutamide (BIC). P = progesterone, HS = control human serum. All data is representative of at least 2 independently performed experiments.


A
25000
20000
15000
10000
5000
0
Luciferase activity
0 ng
0.5 ng
1 ng
2.5 ng
5 ng
10 ng
Vehicle
0.01
0.1
1
nM T
pCMV-AR
0 ng
0.5 ng
1 ng
2.5 ng
5 ng
10 ng
98 kD
AR
B
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
ALU
Vehicle
0.1 nM T
1 nM T
10 nM T
10% HS
0.15 nM E2
3.6 nM P
10 μM DHEA(S)
11.5 nM ASD
10% HS + 1μM BIC
10 μM DHEA(S) + 1 μM BIC
Treatment

6.3.2 Treatment of the cells with alternate ligands results in activity from T, human serum and the androgenic precursors DHEA(S) and androstenedione only

Human serum obtained from a healthy male volunteer was dextran charcoal stripped and hormone levels were subsequently measured to validate the effectiveness of the stripping process. The results for T, cortisol and E2 are presented in table 6.1. Dextran-charcoal stripping reduced the hormone levels of the serum to below detectable levels for many of the assays, except for cortisol where it reduced the level to approximately one third of that found in the non stripped sample. In comparison, the hormone levels of dextran charcoal stripped FCS were below the detectable range (Table 6.1). Transfection of $PC3^{AR+}$ cells with 100 ng ARR3, 2.5 ng AR and treatment for 24 h with a panel of steroids at concentrations found in human male serum resulted in increased luciferase activity in comparison to vehicle only treated wells in the wells treated with charcoal stripped growth medium spiked with T, 10% human serum, 10 μM DHEA(S) or 11.5 nM ASD only (Fig 6.2B). Although it may appear surprising that DHEA(S) is capable of inducing activity in the assay as there has been one report of the polar sulphated form of DHEA, DHEA(S) being biologically unavailable to liver cells (Hammer et al. 2005), two other reports have shown that synovial cells and granulosa cells can convert DHEA(S) into more potent steroids (Bonser et al. 2000; Weidler et al. 2005) so it remains possible that this cell line is capable of active conversion of this conjugated hormone. The AR specific antagonist bicalutamide (BIC) was capable of significantly decreasing transactivation activity mediated by 10 μM DHEA(S) and 10% human serum in comparison to the wells treated with these hormones but without BIC, indicating that this activity is mediated via the AR (Fig 6.2B). The hormones estradiol and progesterone were not capable of generating significant activity above the vehicle treated wells.

Hormone	Method of measurement	Limit of detection	Non-stripped human serum	Stripped human serum	Stripped FCS
T (nmol/L)	Immulite 2000	2	21.9	ND	ND
E2 (pmol/L)	Roche E 170	5	96	ND	ND
Cortisol (nmol/L)	Roche E 170	2	290	102	ND

Table 6.1: Hormone levels in the control dextran charcoal stripped and non stripped human serum samples and dextran charcoal stripped FCS as measured by the immulite 2000 platform.

6.3.3 The activity generated by DHEA(S) in the assay is dependent on the metabolism of the androgenic precursor within $PC3^{AR+}$ cells

To further investigate if DHEA(S) was mediating effects by binding directly to the AR or via intracellular metabolic conversion to more active androgenic metabolites, the specific 3β-hydroxysteroid dehydrogenase inhibitor, cyanoketone, was utilised to inhibit the conversion from DHEA to ASD in the $PC3^{AR+}$ cells (Cooke 1996). Treatment of the cells with 0.01 μM to 1 μM cyanoketone resulted in a stepwise decrease in transactivation activity generated from DHEA(S) while having no effect on T generated or basal transactivation activity (Fig 6.3A). These results were reflected in a radioimmunoassay of the media from the cells collected at 24 hours; while the vehicle treated cells had similar levels of ASD formed from treatment of the cells with 20 nM T or 10 μM DHEA(S), 1 μM cyanoketone reduced ASD production in DHEA(S) treated wells but did not affect ASD levels in 20 nM T treated wells (Figure 6.3B). Together, these results demonstrate that the activity generated from DHEA(S) in the assay is most likely the result of conversion to more active androgen metabolites within the $PC3^{AR+}$ cells.

6.3.4 Treatment of the cells for 5 minutes only with human serum or hormone abolishes DHEA(S) and ASD-mediated transactivation activity

As it was most likely that the activity of 10 μM DHEA(S) in the assay was generated via conversion to more active androgenic metabolites, it was postulated that a shorter treatment time may decrease the transactivation activity mediated via this conversion. Therefore, cells

were treated with a pulse of hormone or 100% human serum for periods of time from 5 minutes to 24 hours. While 24 hours of treatment with 100% human serum resulted in cell death and no activity generated from the assay, 5 minutes of treatment followed by 24 hours of culture in medium stripped of hormones resulted in significant accumulated luciferase activity for both 100% human serum and 10 nM T treated samples (Fig 6.4A). Furthermore, 5 minutes of treatment with 10 μM DHEA(S) and 11.5 nM ASD resulted in no significant activity above vehicle treated control samples (Fig 6.4B). As 100% serum treatment of the cells may lead to non specific effects on the assay due to the serum matrix, the effect of a 5 minute treatment of the cells with increasing nM amounts of T spiked into charcoal stripped human serum or charcoal stripped FCS was investigated. At all concentrations of T, the wells treated with 100% HS or 100% FCS were similar, and there was no significant difference between the two treatments at any concentration of T (Fig 6.4C). The linearity of the response was assessed by spiking hormone stripped male serum with known nanomolar quantities of T and then using a calibration curve to calculate concentration of T in the assay. This assessment yielded a line of best fit with an R2 value of 0.9991, indicating a strong positive association between the spiked levels of T and the assay output (Fig 6.4D). Finally, the sensitivity to T of the 5 minute treatment was assessed with a side by side analysis of the dose response curves in response to T. This analysis demonstrated that the 5 minute treatment resulted in a 10 fold reduction in sensitivity to T (Figs 6.4E and F).

6.3.5 Megalin, a receptor involved in the active transport of SHBG-bound T into cells, is not expressed in PC3^{AR+} cells

Megalin is a member of the low density lipoprotein related receptor family which are cell surface endocytotic receptors that have been demonstrated to recognise specific plasma binding proteins. These receptors bind their specific proteins and internalise them by endocytosis. Recently, megalin has been found to actively transport SHBG-bound T into cells (Hammes et al. 2005). Therefore, the expression of this protein was investigated in PC3^{AR+} cells. As seen in Figure 6.5, although megalin protein could be observed in the positive

control cells (COS-1) with GFP immunofluorescent detection and could be blocked with a 50 fold excess of blocking peptide, there was no visible expression in the $PC3^{AR+}$ subline.

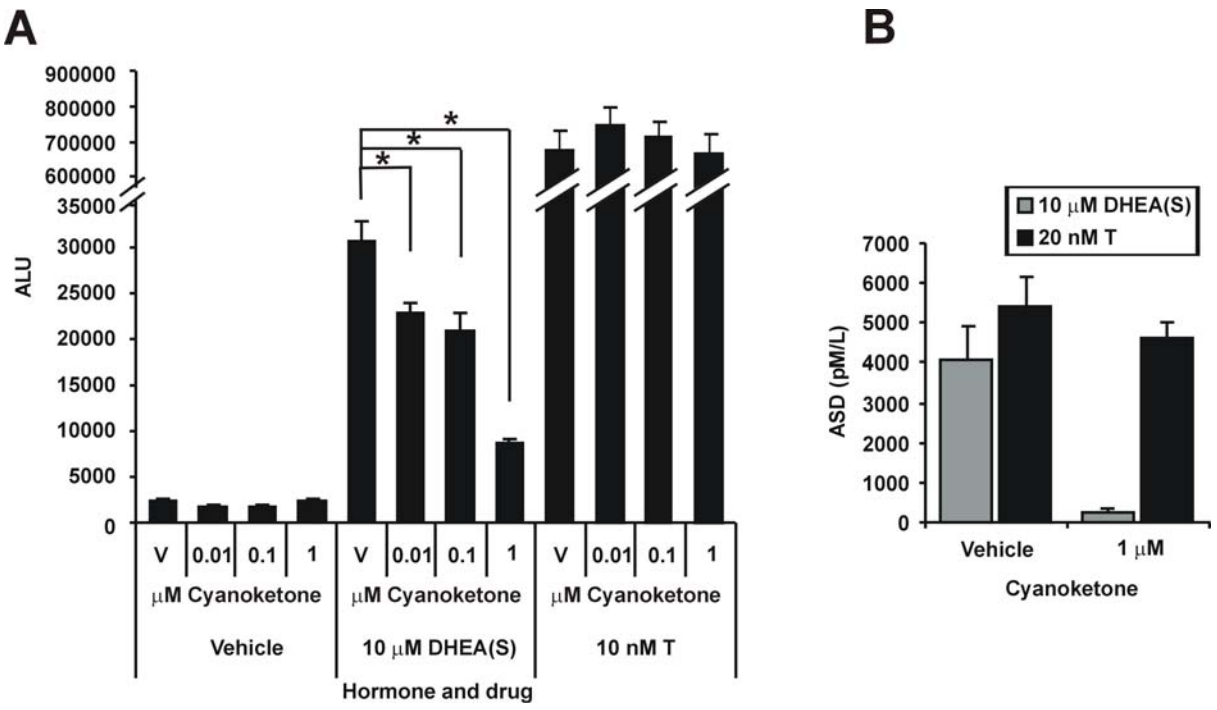


Figure 6.3: Inhibition of 3β-hydroxysteroid dehydrogenase results in decreased DHEA(S)-induced luciferase activity and reduced levels of ASD formation by DHEA(S) treated cells. A: Cells were transfected as described in Figure 6.2B and treated with Vehicle (EtOH), 10 nM T or 10 μM DHEA(S) and with increasing concentrations of the 3β-hydroxysteroid inhibitor cyanoketone to inhibit intracellular conversion of the androgenic precursor DHEA to ASD. Vehicle (EtOH) was constant per well with 24 hours treatment. Data represents the mean ± SEM ALU of six independently transfected wells. **B:** Specific inhibition of formation of ASD from DHEA(S) in the presence of 1 μM cyanoketone. Medium was collected from the wells after 24 hours of hormone treatment as described in A, along with medium placed into wells containing no cells. Data represents mean ± SD of three independently transfected wells in pM/L of gross ASD minus the mean ASD level in wells treated with 10 μM DHEA(S) but no cells. All data is representative of at least 2 independently performed experiments. * $p<0.05$ on two tailed Mann Whitney U analysis of medians in comparison to non-cyanoketone treated wells.

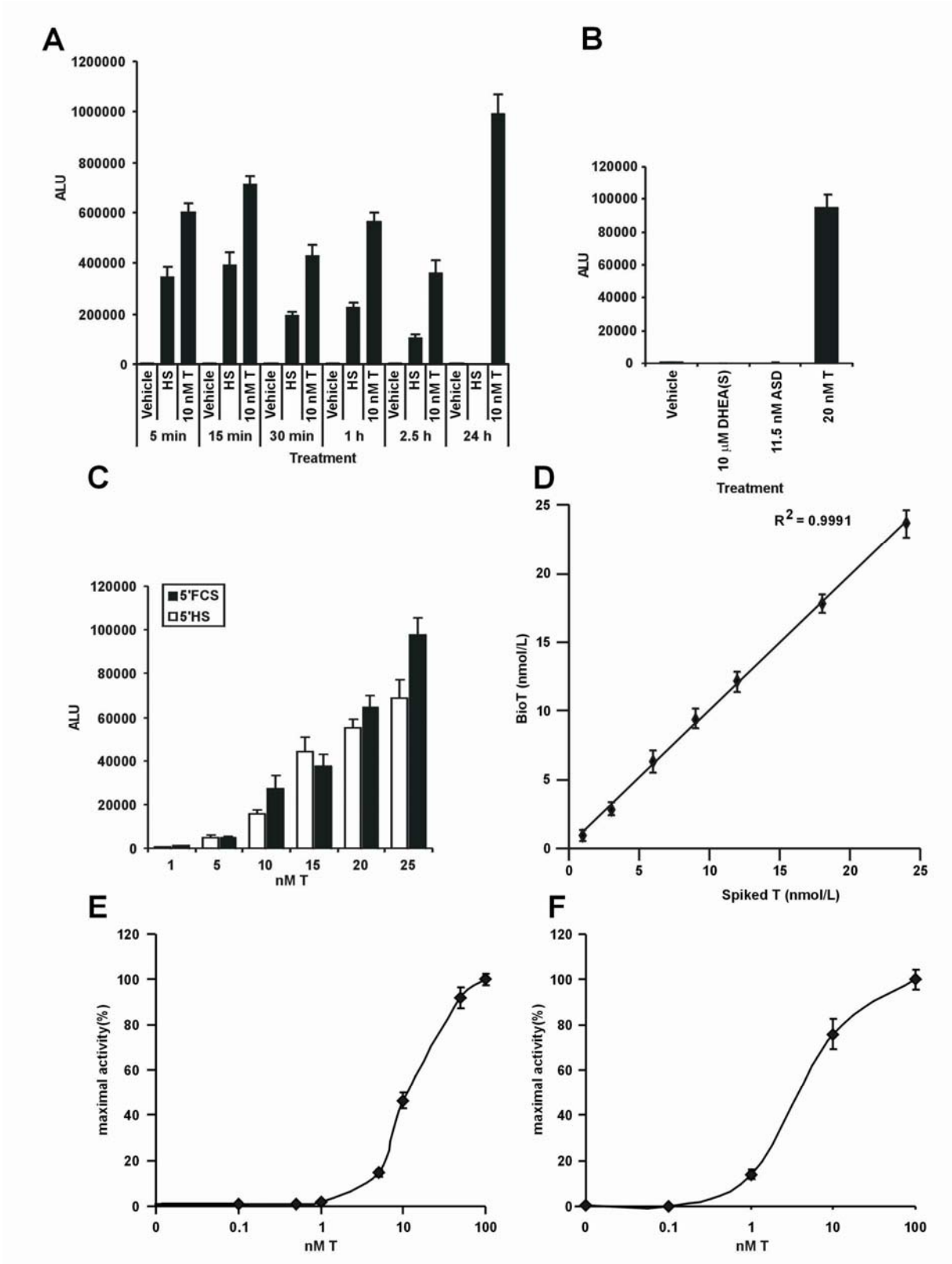
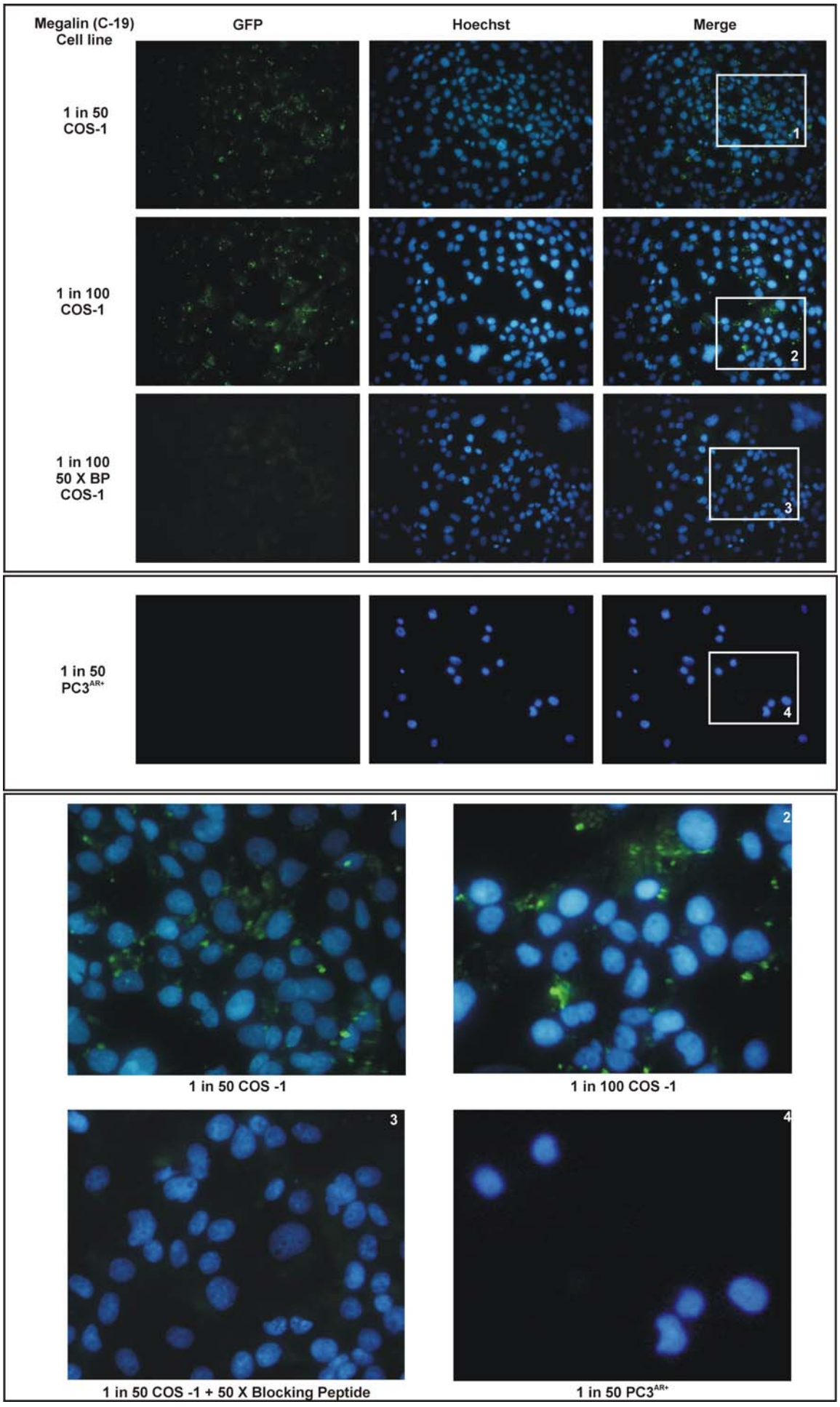

A
ALU
Vehicle
HS
10 nM T
5 min
15 min
30 min
1 h
2.5 h
24 h
Treatment
B
ALU
Vehicle
10 μM DHEA(S)
11.5 nM ASD
20 nM T
Treatment
C
5'FCS
5'HS
ALU
nM T
D
R2 = 0.9991
BioT (nmol/L)
Spiked T (nmol/L)
E
maximal activity(%)
nM T
F
maximal activity(%)
nM T

Figure 6.4: Treatment of cells for 5 minutes abrogates DHEA(S) induced activity and decreases the sensitivity of response to T in cmparison to a 24 hour treatment along with potential non specific effects of human serum on the bioassay. A: Cells were plated and transfected as described in Figure 6.2B and treated for increasing time periods between 5 minutes to 24 hours with 25 µL per well of vehicle (EtOH), 100% human serum (HS) or 10 nM T. In the remaining time to 24 hours for each period the treatment was removed and replaced with 100 µL per well of hormone stripped growth medium. Data represents the mean ± SEM ALU of six independently transfected wells. **B:** Cells were transfected as described in figure 6.2B and treated for 5 minutes at 37^{o}C with vehicle, DHEA(S), ASD or T, or equivalent amount of vehicle (EtOH). Wells were washed once with PBS and then incubated for 24 hours at 37^{o}C in 100 µl of hormone stripped growth medium to allow luciferase expression. Data represents the mean ± SEM ALU of six independently transfected wells. **C:** Cells were transfected as described in figure 6.2B and treated for 5 minutes with dextran charcoal stripped human serum (5'HS) or FCS (5'FCS) spiked with increasing concentrations of T. Data represents the mean ± SEM ALU of six independently transfected wells. All treatments were assessed by Mann Whitney U analysis of medians and were non significant. **D:** Cells were transfected and treated for 5 minutes with stripped human serum spiked with known concentrations of T. BioT was calculated based on the calibration curve and linear regression was performed on the resulting points. **E:** Cells were transfected as described in Figure 6.2B and treated for 5 minutes with 100% hormone stripped human serum spiked with increasing concentrations of T. Data represents the mean ± SEM of percent maximal activity (at 100nM T) of six independently transfected wells. **F:** Cells were transfected as described in Figure 6.2B and treated for 24 hours with cell growth medium spiked with increasing concentrations of T. Data represents the mean ± SEM of percent maximal activity (at 100nM T) of six independently transfected wells. All data is representative of at least 2 independently performed experiments.

6.3.6 Stably transfected cells are not capable of providing the viability or discrimination required for a bioassay

In addition to the transient system presented above, stable cell lines were also developed as an alternative assay system as several publications have now suggested without solid evidence that stable transfection decreases the variability of the assay in comparison to transient transfection. Two different lines were established, one constitutively over expressing AR protein and one with the ARR3 reporter cassette stably integrated into $PC3^{AR+}$ cells. Over 20 cell lines were tested for each variant, with a representative sample from the cell lines depicted in Fig 6.6A and B respectively. While the stable over expression of AR protein resulted in significant fold activation when transfected with ARR3-tk-Luc, these cells grew very slowly and were only viable for approximately 10 passages, even when selected and grown in hormone stripped medium. Therefore, these cell lines were unusable in an assay that requires a large cell number for successful completion. In contrast, all of the ARR3-tk-Luc-stably integrated cell lines grew robustly but when transfected with pCMV-AR3.1 expression vector failed to give the fold induction required for the discrimination and accuracy of measurement of bioactive androgen within serum samples. In addition to stable cell lines, electroporation was also attempted to deliver the DNA required to a large batch of cells at one time, which were then plated into individual wells and then treated as described. Once optimised, this method did result in dose responses to increasing nM amounts of T, but also resulted in significant cell death and variability within the dose response (data not shown). Additionally, upon examination of the variability exhibited by previously published stable cell bioassays and the assay reported here, there was no evidence that stably transfected systems reduced variability as assessed by inter and intra assay coefficients of variation of these published assays (Table 2).

Figure 6.5: COS-1 cells express megalin while PC3^{AR+} cells do not. Cells (54,000 per chamber) were plated onto glass chamber slides and allowed to attach for 48 hours. Cells were fixed in 4.5% paraformaldehyde and treated overnight with no primary antibody, 1:50, 1:100 or 1:100 with 50 fold blocking peptide of anti-megalin (C-19) goat polyclonal antibody in PBS with 5% donkey serum (blocking serum). Slides were treated with 1:400 Alexa 488-conjugated donkey anti-sheep secondary antibody and DNA in nuclei were stained with bisbenzimide H 33258 (Hoechst). Slides were viewed at 60X magnification at a wavelength of 495 nm and visualised with an FITC filter (megalin staining – green) and then followed by viewing at a wavelength of 356 nm visualised with a DAPI filter (nuclei staining – blue). No megalin staining was visible in any of the PC3^{AR+} frames viewed. Images on far right and in the bottom section are overlayed. The larger images in the bottom section correspond to the numbered boxed white segment from the top section. Images shown are representative of the whole chamber in 1 of a total of 2 experiments.


Megalin (C-19)
Cell line
GFP
Hoechst
Merge
1 in 50
COS-1
1 in 100
COS-1
1 in 100
50 X BP
COS-1
1 in 50
PC3AR+
1
2
3
4
1 in 50 COS -1
1 in 100 COS -1
1 in 50 COS -1 + 50 X Blocking Peptide
1 in 50 PC3AR+

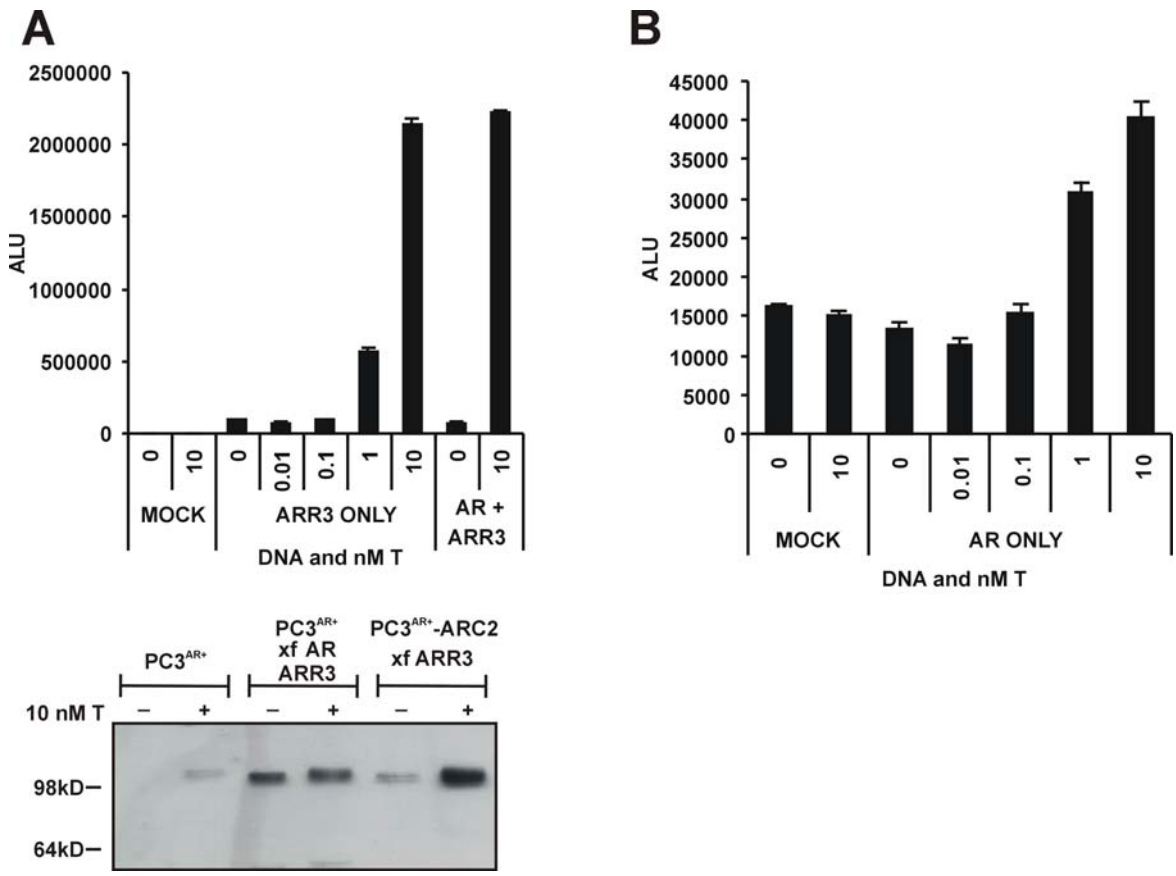


Figure 6.6: Stable cell lines either overexpressing AR or with an integrated reporter fail to provide the viability or discrimination required for the assay. A: A representative $PC3^{AR+}$ stable cell line overexpressing AR was transfected with 100 ng ARR3 reporter only. In comparison, $PC3^{AR+}$ cells were transfected with 2.5 ng pCMV-AR and 100 ng ARR3 reporter. $PC3^{AR+}$-ARC1 and parental $PC3^{AR+}$ cells were plated, transfected and treated with the indicated hormone concentration for 24 hours as described in figure 6.2B. Data represent the mean ± SEM of six independently transfected wells, with the immunoblot demonstrating the level of endogenous AR in the $PC3^{AR+}$-ARC1 cells performed on the transactivation assay lysates as described in Figure 6.2A. xf: transfected with. **B:** A representative cell line with the ARR3-tk-Luc reporter cassette integrated into the genome ($PC3^{AR+}$-ARR3LUC clone 1) was transfected with 100 ng pCMV-AR per well and treated and assayed as described in figure 6.2A. Data represents the mean ± SEM ALU of six independently transfected wells and is representative of at least 2 independently performed experiments for each cell line.

Reference	Transient Or stable	Treatment time	Midrange of assay (nmol/L T)	Inter-assay CV	Intra-assay CV	Least detectable concentration nmol/L T
This report	Transient	5 min	8.5	16.2%	11.67	0.91
Raivio et al (2001)	Transient	Overnight	0.675	21%	8.3%	0.1
Chen et al (2006)	Stable	16 h	0.535	7.4%	7.5%	0.015
Paris et al (2004)	Stable	24 h	0.04	<20%	<10%	0.010
Sonneveld et al (2006)	Stable	24 h	0.66	22%	NR	NR
Roy et al (2006)	Stable	24 h	0.3	19.4%	8.5%	0.1

Table 2: Comparison of the bioassay characteristics with other currently reported androgen mammalian cell-based bioassays

6.3.7 Increasing treatment time does not decrease assay variability

The time of treatment was also investigated to determine if variability could be decreased with a longer incubation of the cells with serum. Treatment of cells for 15 minutes with serum did not increase luciferase activity in comparison to 5 minutes of treatment and in fact resulted in greater variability between plates than the 5 minute treatment (data not shown). Finally, the cells were starved of hormone by growth in hormone depleted medium for 48 hours before plating for the assay to determine if residual hormones in the growth media were having an effect on the assay. This starvation resulted in variable attachment of the cells to the plate and lower cell viability upon transfection, thereby resulting in increased variation between plates in comparison to cells that were not grown in stripped medium. It was decided overall that a 5 minute treatment involving a transiently transfected system with a lipid based method would give the most robust result to measure bioactive T in male serum samples and that a standard curve would be required on each plate due to the variability in absolute luciferase activity over plates.

6.3.8 Adapted assay procedure

Consequently, the assay involves the following process. Cells ($PC3^{AR+}$) are plated at 10,000 cells per well in 96 well plates and allowed to attach for 24 hours. The wells are washed once in phenol red free unsupplemented RPMI 1640 and then transfected utilising lipofectamine 2000® with 2.5 ng pCMV-AR and 100 ng ARR3-tk-LUC per well. The transfection is allowed to proceed for 4 hours after which the transfection medium is removed and 25µl of T calibrator (1, 3, 6, 12, 18 or 24 nM T in dextran charcoal stripped human serum) or patient sample is added to 6 replicate wells and incubated for 5 minutes at 37^{o}C. A calibration curve is included on each plate in the assay.The serum samples are removed from the wells by inversion of the plate and the wells are washed once with PBS. Then 100 µL of hormone stripped growth medium is placed into the wells for 24 hours. All the wells are lysed, frozen at -80^{o}C overnight, thawed and one third of the volume is measured in a standard luciferase assay. Standard curves are fitted to the data and only points that form a straight line are utilised. The curve then allows the extrapolation of the BioT concentration for each serum sample. The intra and inter assay coefficient of variation of the assay was 11.6 and 16.7% respectively. The variation within a day for the measurement of bioactive T on a single human serum sample is presented in Figure 6.7A while the variation between days is presented graphically in Figure 6.7B.

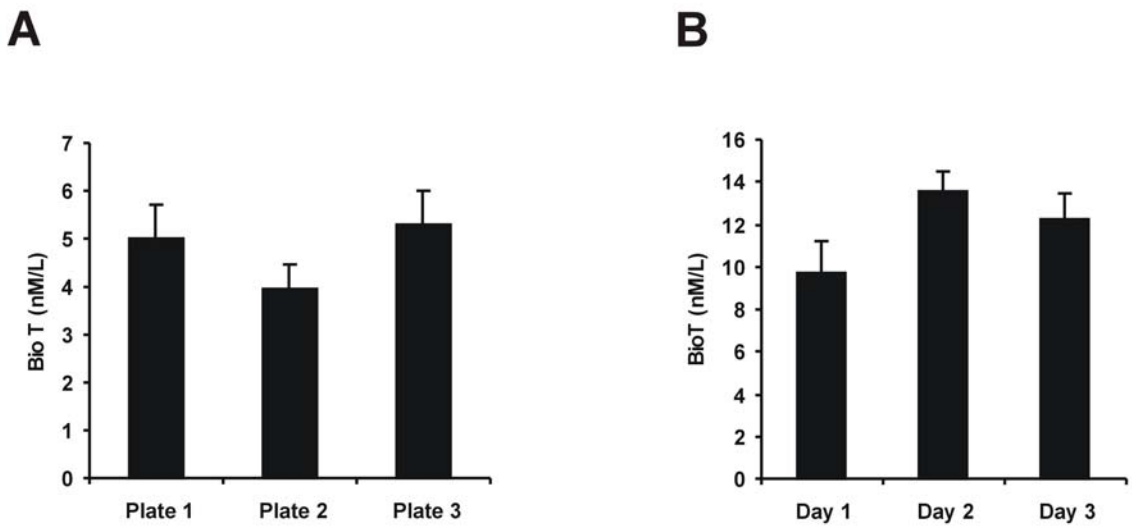


Figure 6.7: Measurement of a human serum sample between plates on a single day and over plates on three separate days demonstrates an intra assay and inter assay coefficient of variance of 11.6% and 16.7% respectively. **A:** Cells were transfected and treated as described in figure 6.4A with the addition of the treatment of 6 wells with the same human serum sample from a FAMAS participant over three separate plates. Each plate was individually treated with the same standards in 100% dextran charcoal stripped human serum) spiked with T from 1 to 24 nM with six independently transfected wells devoted to each point on the standard curve. Unknowns were extrapolated from the standard curve run on each plate **B:** Cells were transfected and treated as described in A, but each of the transfections were independently repeated over 3 days with the same standards and control human serum sample utilised over days. Data represents the mean ± SEM of six independently transfected wells, and the bioavailable T measure was extrapolated from the standard curve for the corresponding day according to the method described in A.

6.4 Discussion

The finding that a reporter has a different ability and sensitivity to be transactivated by the AR in different cell lines has been reported previously and attributed to different levels of coregulators, and the relative levels of coactivators to corepressors within different cell lines (Buchanan et al. 2004; Bebermeier et al. 2006). The results from chapters 3 and 4 demonstrate the importance of the level of coregulators within cells on the determination of the AR transactivation function. Preliminary data from our laboratory indicates that the levels of the p160 coregulators do vary between the COS-1, PC3, CHO-K1 and $PC3^{AR+}$ cell lines (Dr Tina Bianco-Miotto, unpublished observation) and a previous report demonstrated that the steady state levels of NCOA2 were higher in PC3 cells in comparison to COS-1 cells (Gregory et al. 2004). However, there are a number of other AR coregulators that have been described that may be the determinants of the cellular responses demonstrated in this chapter, such as those described in chapter 1. In addition to this, the ability of the cells to internalise T in serum may be mediated by megalin which is present in COS-1 cells but not in $PC3^{AR+}$. Therefore, it is unlikely that an active transport mechanism for androgen uptake is the cause of the increased sensitivity of the $PC3^{AR+}$ cell line to androgens. Subsequent experiments will focus on the use of a protein that specifically inhibits all of the low density lipoprotein related receptor family members, receptor accessory protein (RAP) (Croy et al. 2003; Hammes et al. 2005) and determine if this protein modifies the results of the bioassay. Preliminary data indicate that the use of RAP protein in the cells does not influence the activity induced by human serum in the $PC3^{AR+}$ cells. It will be interesting in the future to contrast results from a COS-1 cell based bioassay with the $PC3^{AR+}$ cell based bioassay described in this chapter.

The response to DHEA(S) of the $PC3^{AR+}$ cells also demonstrates the importance of cell choice for a bioassay. Although one report has suggested that DHEA(S) is not available in vivo or in vitro, the findings here and in two other reports would suggest otherwise (Bonser et al. 2000;

Weidler et al. 2005). Indeed, this chapter demonstrates that these cells have a functional DHEA(S) to T pathway that when blocked results in decreased ASD formation and decreased activity in the bioassay. Given that the affinity of the AR for DHEA binding is approximately 2200 lower than that of T in prostate cancer cells (Chen et al. 2005), it is most likely that the effects of DHEA(S) on the assay are mediated via intracellular conversion of this androgenic precursor within the cells. While other assays do assess activity in response to DHEA(S), none use a physiological concentration of DHEA(S) in male serum which can be as high as 10 µM (Zumoff et al. 1980; Vermeulen 1995). The finding that prostate cells are capable of intracellular conversion of the sulphated form of DHEA when treated for 24 hours while 5 minutes of treatment is ineffective also highlights the importance of treatment time for bioassay results. Previous bioassays all utilise an overnight or 24 hour treatment time, and dilute the serum in medium containing 5% FCS, thereby producing assays that may be capable of intracellular metabolism of various hormones, depending upon the expression of the metabolic enzymes in the cell line chosen. These assays may thereby be measuring levels of androgens other than T.

The possible effects of cortisol in the bioassay have not yet been assessed. A previous bioassay has shown a response to cortisol but the correlation observed between cortisol levels and the bioactive T measure is not as strong as the correlation of bioactive T with total T in this assay (Chen et al. 2006). Our observation that 5 min of treatment of cells with 100% FCS (with undetectable levels of cortisol) was comparable to stripped human serum (containing cortisol of 100 nmol/L) suggests that in this assay design, cortisol may not affect the induction of the ARR3 reporter. The possible effects of cortisol in our bioassay will be further investigated in both 5 miunte and 24 hour treatment options in future experiments.

Several reports have asserted that utilisation of stable cell lines reduces variability of response. The extensive optimisation undertaken in this chapter indicates that stable cell lines

have similar variability in bioassay systems in comparison to transiently transfected assay systems. Theroughout the development of this bioassay it was incidentally noted that one of the biggest factors governing variability of response in both the stable cell line assays and the transient assays was cell density, a factor that is common to both assay methods (data not shown). Adding to this source of variability that applies equally to stable and transiently transfected cells is the observation that not all cells in a stable cell line have equal levels of expression, indicating variability may also be inherent in stable cell lines. Collectively, the data presented here would argue that there is no clear benefit of the utilisation of a stable cell line besides ease of use. As this assay was designed to measure male serum samples in the context of the large male cohort presented in Chapter 7 and not to promote as a clinically or commercially viable assay, the transient bioassay system is both appropriate and suitable for this purpose.

Others have advocated the utilisation of extraction of the hormones from serum samples in their bioassays to avoid the effects of serum matrix in general and SHBG in particular. Roy and coworkers (2006) demonstrated that extracted serum samples exhibit increased activity in comparison to unextracted serum samples. This difference has been attributed to the presence of SHBG in the unextracted samples. However, the demonstration of similar dose response curves for extracted serum in comparison to serum that is unextracted (Roy et al. 2006) would seem to argue that the extraction process is unnecessary. Furthermore, given the assumption that SHBG and albumin act to limit androgen bioavailability to cells, the removal of these proteins from the serum samples could therefore conceivably result in an assay that measures total T like currently utilised radioimmunoassays or immunoassays.

The inter and intra assay coefficients of variation of the assay were 16.7% and 11.6% respectively. This variation and the variation observed for another bioassay that utilises transient transfection (Raivio et al. 2001) is no larger than that observed for bioassays

consisting of stably transfected cells thereby refuting claims that transient transfection is a source of variation in bioassay systems (Paris et al. 2002; Sonneveld et al. 2004; Chen et al. 2006). In addition, this assay is not designed with the intention of becoming a routine clinical assay, rather it was designed to assess the levels of androgen in serum that are capable of activating the AR in a large cohort of men to thereby assess what proportion of T in serum bioassays actually measure. To this end, the design of the bioassay is sufficient and appropriate to achieve this aim.

In conclusion, this chapter demonstrates the detailed development and optimisation of a bioassay for the first time in a male target tissue with an androgen receptor specific response element. Furthermore, the novel treatment protocol of 5 minutes overcomes the ability of DHEA(S) and ASD to activate the assay and the lack of megalin expression in these cells renders the data from the assay more relevant to cells that do not express megalin. Together, these results demonstrate that this bioassay measures BioT in male serum samples rather than the androgenic potential of the serum samples. The results of the utilisation and validation of this assay in the serum samples from a large cohort of men is analysed in chapter 7.

Chapter 7: Bioactive T measurement of the Florey Adelaide Male Aging serum samples reveals a measure which reflects total T.

7.1 Introduction

The relationship between the decline in serum T and the health status of males as they age is complex and the concept of androgen replacement in aging males is a topic of considerable controversy. Current Endocrine Society of Australia guidelines for androgen replacement in adult-onset hypogonadism (males over 40 years of age) state that androgen replacement is only required for individuals exhibiting symptoms of androgen deficieny (discussed below) and with total serum T below 8 nmol/L or below 15 nmol/L if the male has high LH levels (ESA 1999). While studies of T replacement have been performed on elderly men, benefits such as improvement of erectile function and increases in energy levels and motivation are generally confined to individuals that had frankly low baseline levels of testosterone in comparison to men that were borderline androgen deficient (Kaufman et al. 2005). Therefore, the benefits of such a therapy may not outweigh the perceived risks, such as a possible impact on the prostate gland in terms of hyperplasia or cancer development and/or growth.

Individuals may experience diverse symptoms from adult onset hypogonadism and the threshold at which they experience these symptoms may differ between males making symptomatic diagnosis of adult onset hypogonadism problematic. Strikingly however, the threshold serum T level at which a single individual experiences symptoms associated with androgen decline appears to be consistent (Kelleher et al. 2004). Therefore, it appears that a arbitrary universal threshold of serum T to define individuals with suspected androgen deficiency may not be reliable between individuals.

Despite several cohort studies demonstrating relationships between serum levels of T and grip strength, waist circumference, sexual desire and erectile function (Schiavi et al. 1991; Schaap et al. 2005; Allan et al. 2006; Derby et al. 2006; Travison et al. 2006; Schaap et al. 2007), the specific role of the variance in androgens in these aspects of physiology are not yet fully

understood, partly because of a lack of data on the availability and/or molecular action of androgens on tissues such as muscle, the brain and adipose tissue but also due to the complex inter-relationships of age, the reproductive axis, androgens, muscle, adipose tissue, metabolic status and brain function. In an attempt to provide a more physiological measure of T, bioassays have been developed to measure serum androgens capable of activating the AR and have lead to the coining of the phrase bioactive T (BioT), which has been defined as the fraction of androgens in serum that is capable of activating the androgen receptor within a mammalian cell environment in a bioassay-type system. None of these bioassays, however, extensively define this measure in relation to other measures of serum T and some assays actually extract the hormone from the serum matrix and then measure this extraction in a bioassay thereby creating an assay in mammalian cells for total T, rather than BioT (Chen et al. 2006; Roy et al. 2006). While other studies have demonstrated feasibility of the measurement of serum androgens using mammalian cell-based bioassays, the lack of comparison to alternative measures of serum T such as BT or FT along with small samples sizes in these studies have precluded a definitive comparison of the BioT measure to alternative measures of serum T.

The Florey Adelaide Male Aging Study (FAMAS) is an Australian cohort of 1195 men aged between 35-80 years randomly recruited from the northern and western suburbs of Adelaide. This study focuses on the reproductive, physical and psychological health of males as they age in an Australian population with an aim to determine the conditions that promote healthy aging. This cohort of men has been comprehensively examined for variables associated with the androgen axis and age such as grip strength as a measure of muscle strength (Schaap et al. 2007), waist circumference as a measure of visceral fat, and self reported erectile function and sexual desire. In addition, dietary intake and exercise frequency have been evaluated by questionnaires along with providing serum samples for the measurement of, amongst others, total T, immunoassayed bioavailable T (iBT), SHBG, albumin, total cholesterol, LDLc,

HDLc, Haemoglobin A1c, packed cell volume, LH, FSH, E2, serum triglycerides and blood glucose (Martin et al. 2007). Due to the comprehensive nature of this testing, the serum from each of the FAMAS participants provides an ideal resource with which to test the bioassay developed in chapter 6 and thereby determine the physiological relevance of the measures in the bioassay. Therefore, the aims of this chapter are i) to define the bioassay measure in relation to the physiological parameters grip strength, waist circumference, sexual desire and erectile function in the FAMAS cohort and ii) to define the bioassay measure in relation to other serum measures of T within the FAMAS cohort in order to provide an understanding of the physiological relevance of serum T measurement by serum androgen bioassays.

7.2 Methods

7.2.1 FAMAS cohort recruitment

Recruitment, testing and inclusion and exclusion criteria are outlined in detail in two articles from Martin et al (2007). Briefly, inclusion criteria for the study were that subjects were male, aged between 35-80 years, were willing and able to give written consent and to comply with the protocols which were approved by the Royal Adelaide Hospital Research Ethics Committee. The participants also had to have a connected telephone number in the Adelaide white pages and live within the defined catchment areas. Respondents were excluded from the study if they were of insufficient mental capacity to understand the requirements of the protocols, were living in a residential care facility, had severely limited English or were too ill or incapacitated to attend the clinics.

7.2.2 FAMAS cohort serum measurements

In the clinics which ran between 0830 and 1130, a fasting blood sample was drawn into vacuette tubes by venipuncture in the antecubital fossa and immediately refrigerated and transported to the NATA certified Clinical Biochemistry laboratory of the Institute of Medical and Veterinary Science where all pathology analyses were performed. In the laboratory, the blood was inverted several times, separated into components via centrifugation at 1000 g for 15 minutes and tested for the serum components outlined below.

7.2.2.1 Total T, SHBG and Albumin

The serum levels of total T and SHBG were measured via the Immulite 2000 (DPC Corporation, Los Angeles, USA) and albumin by the Olympus AU5400 (Center Valley, Pennsylvania, USA) platforms by the above laboratory. Surplus serum was separated into several aliquots and stored at -70^{o}C until required for analysis. It has been previously

demonstrated that T and iBT is stable for up to 7 years in serum stored at or below this temperature (Morley et al. 2002). The interassay CV for total T is 9.3% at a concentration of 10.7 nmol/L while the interassay CV for SHBG is 4.0% at 32 nmol/L. The interassay CV for albumin at a concentration of 40 g/L is 1.5%. Free testosterone was calculated based on the method of Vermeulen et al (1999).

7.2.2.2 Immunoassayed BT (iBT)

Measurement of immunoassayed BT was performed according to the method outlined previously (O'Connor et al. 1973). This procedure was performed by the above laboratory on the same serum samples as measured for total T, SHBG and iBT. The inter-assay CV's for the assay at 4.99 and 0.18 nmol/L were 6.2% and 14.2% respectively while the intra-assay CV's are 3.0 and 3.3% at 8.1 and 1.4 nmol/L respectively.

7.2.2.3 Bioactive T (BioT)

The bioassay of serum BioT was performed over a period of 5 days utilising the method outlined in section 6.3.7 on stored FAMAS serum samples. Any serum samples that were above the standard curve were diluted one in four in hormone stripped male serum and re-run through the assay. Comparison of control human serum diluted in this manner revealed no significant difference in the final BioT concentration between the diluted sample and the non-diluted sample in the assay. In all, about 30% of the samples required dilution and re-assay. Twelve of the samples were found to cause bacterial contamination of the cells upon treatment in two runs, presumably due to contamination of the serum with bacteria post-sampling. As bacterial growth is toxic to tissue culture cells, these samples were not included in the BioT analysis. Also excluded from the bioassay were sera from individuals who had undergone a bilateral orchiectomy as their serum testosterone levels were below the detection limits of the assay or they were receiving T replacement therapy (4 participants). In all, BioT measures from 976 individuals were included in subsequent statistical analyses. The inter- and intra assay CV's were 15.0 and 15.2% respectively at 10.8 nmol/L BioT.

7.2.3 FAMAS cohort physiological measures

All physiological measures were performed at the same baseline clinic visit as the collection of the serum described above. These parameters are described below. In addition, participants filled out a survey requesting details of currently diagnosed chronic medical conditions including: anxiety, asthma, angina, depression, diabetes, high cholesterol, hypertension, insomnia, osteoarthritis, rheumatoid arthritis, enlarged prostate, prostate cancer, other cancers and thyroid problems. Data relating to all medications used was also collected. Additionally, participants completed a food frequency questionnaire (Hodge et al. 2000) and an exercise frequency questionnaire as developed by the Australian Institute of Health and Welfare (Armstrong et al. 2000).

7.2.3.1 Waist Circumference

Waist circumference was measured in cm rounded up to the nearest half cm, and was taked as the diameter of a participant's trunk midway between the lower costal margin and the iliac crest at the end of an expiration of breath. This measure was taken 3 times for each participant with the average of the 3 measures being utilised in statistical analyses.

7.2.3.2 Grip strength

Bilateral handgrip peak force was measured with maximal isometric contraction utilising a grip dynamometer (Smedley, Chicago, IL, USA) to assess maximal voluntary contraction. Prior to the measurement, participants were requested to grip the dynamometer three times at 50% of maximum followed by three repetitions of 75% and one at maximum. After a rest of two minutes, subjects were tested for maximum grip 3 times with verbal motivation by the tester. This protocol was performed for both the dominant and the non dominant hand of each individual. The average of three repetitions for each hand was utilised in analyses. Individuals with pathologies compromising this test such as having a history of hand surgery or osteoarthritis in the hand in question were excluded from this testing (22 participants).

7.2.3.3 The international index of erectile function (IIEF)

The International Index of Erectile Function (IIEF) is comprised of 15 questions which quiz participants about 5 domains of sexual function: i) erectile function (EF), ii) sexual desire (SD), iii) intercourse satisfaction (IS), iv) orgasmic function and v) overall satisfaction (OS). The IIEF is comprised of questions A.16 to A.29 of Questionnaire B in the FAMAS with the orgasmic function questions omitted (Appendix C). This questionnaire was designed for utilisation across cultures and to monitor response to treatment for erectile function (Rosen et al. 1997).

7.2.3.4 The sexual desire inventory (SDI)

The sexual desire inventory consists of two domains i) dyadic sexual desire indicating the interest or wish to engage in sexual activity with another person and ii) solitary sexual desire which is the interest to engage in sexual activity with oneself. This is a well validated questionnaire and has a high degree of internal consistency (Spector et al. 1996). Questionnaire B of the FAMAS contains the SDI as questions A.1 to A.14 (Appendix C).

7.2.3.5 The Global Impotence Rating (GIR)

This consists of a single question which correlates well with the IIEF ($r = 0.71$, $p<0.001$) and characterises the individual as having no erectile dysfunction (0 on the scale) to severe erectile dysfunction (3 on the scale) (Feldman et al. 1994). In questionnaire B of the FAMAS, the GIR is question A.15 (Appendix C).

7.2.4 Statistical analyses

Serum T measures, waist circumference, and SHBG values were log transformed to achieve normality as assessed by D'Agonstino Pearson test for all correlations, linear regressions and analysis of covariance. Tests were considered statistically significant if they reached $p <0.05$, with two tailed analyses for all parametric calculations. Statistical analyses were conducted utilising the Graphpad Prism™ computer package and the statistical package for the social

sciences (SPSS). Statistical advice was obtained from Dr Nancy Briggs from the Data Management and Analysis Centre (DMAC) at the University of Adelaide.

7.3 Results

The median age of the FAMAS individuals in which serum BioT was measured is 53 years (range 35-78 years). These men had a median waist circumference of 99.5 cm (range 71-162.5 cm). Serum total T ranged from very low (2.4 nmol/L) to relatively high (37.7 nmol/L) with a median of 12.8 nmol/L (Table 7.1).

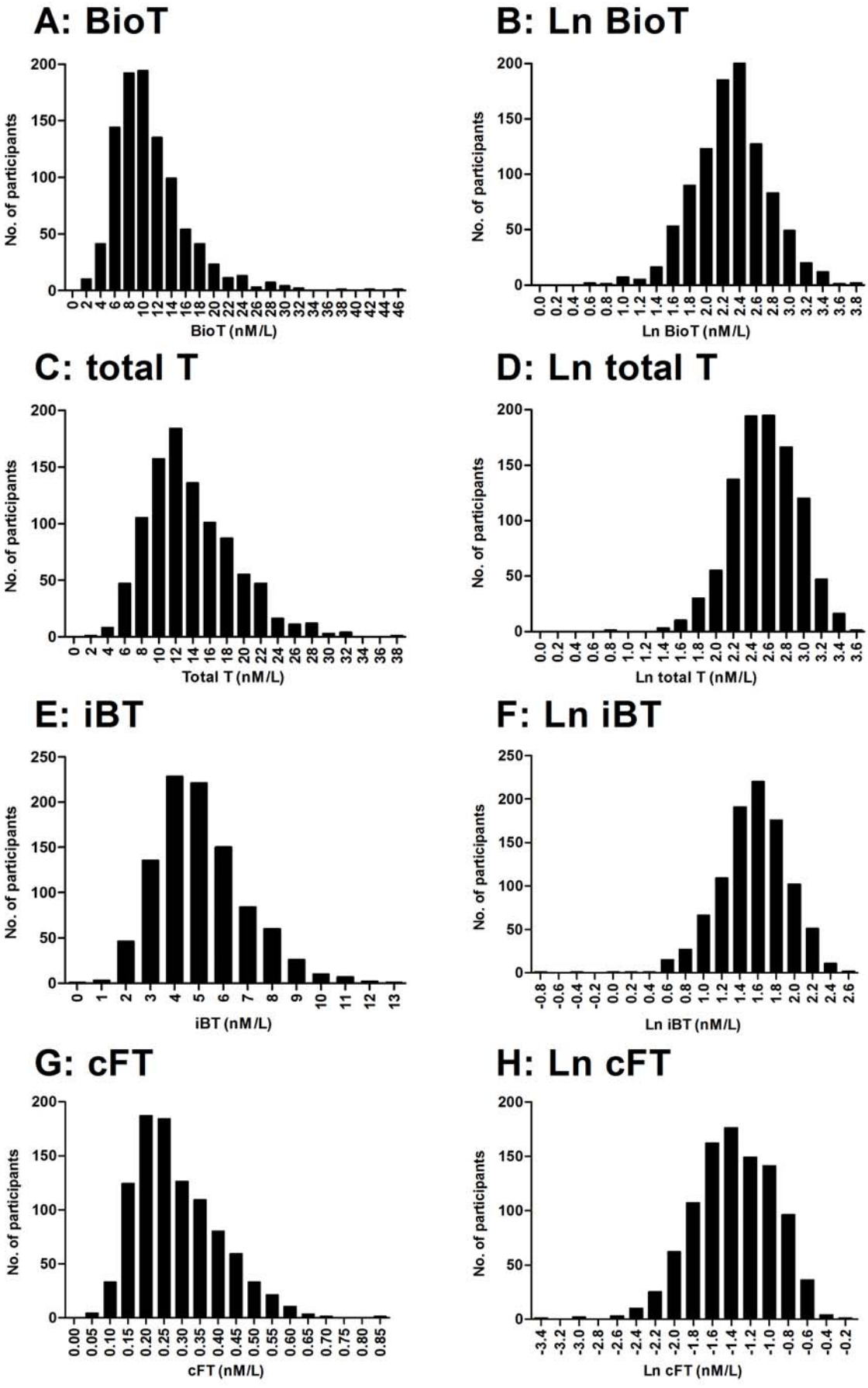
7.3.1 The measurement of bioactive T in 976 FAMAS serum samples reveals a measure that reflects the distribution of serum T measures in this cohort

Serum samples from 996 men were tested in the bioassay with 16 excluded from further analysis due to the individual having undergone a bilateral orchiectomy or the serum samples having bacterial contamination. The BioT measure of each of these samples was extrapolated from a standard curve on each plate created by spiking known quantities of T into hormone stripped male serum from a healthy male volunteer aged 25 years. The distribution of BioT reflects that of the other serum T measures from the same serum samples; a right skewed distribution with positive kurtosis (Figure 7.1A, C, E and G). Log transformation of these variables changed the distribution of these variables so that they did not significantly differ from a normal distribution as assessed by the D'Agostino-Pearson omnibus test (Figure 7.1B, D, F and H).

	N	arithmetic mean	Range	geometric mean	Median	Transformation	K-S distance	p value	Ln arithmetic mean
Age (yrs)	976	53.36 (52.6, 54.11)	35 - 81	52.06 (51.31, 52.82)	54.5				
Waist circ (cm)	976	100.6 (99.88, 101.4)	71 - 162.5	99.94 (99.21, 100.7)	99.5	Logarithmic	0.03539	p > 0.1	4.605 (4.597, 4.612)
serum total T (nmol/L)	975	13.74 (13.42, 14.07)	2.4 - 37.7	12.78 (12.47, 13.10)	12.8	Logarithmic	0.02737	p > 0.1	2.548 (2.523, 2.573)
serum iBT (nmol/L)	975	5.065 (4.949, 5.182)	0.29 - 12.54	4.714 (4.598, 4.834)	4.8	Logarithmic	0.04693	p > 0.05	1.551 (1.526, 1.576)
serum cFT (nmol/L)	975	0.2838 (0.2764, 0.2913)	0.034 - 0.83	0.2595 (0.2524, 0.2668)	0.26	Logarithmic	0.02476	p > 0.1	-1.349 (-1.377,-1.321)
serum BioT (nmol/L)	975	10.99 (10.66, 11.31)	0.72 - 45.38	9.941 (9.663, 10.23)	10	Logarithmic	0.03253	p > 0.1	2.297 (2.268, 2.325)
serum SHBG (nmol/L)	975	35.76 (34.76, 36.76)	6 - 102	32.62 (31.75, 33.51)	32	Logarithmic	0.02503	p < 0.1	3.485 (3.458, 3.512)
serum albumin (g/L)	975	41.42 (41.24, 41.61)	31 - 50	41.32 (41.14, 41.5)	41	not required			

Table 7.1: Characteristics of FAMAS participants and their serum parameters utilised in this chapter. K-S: Komolgorov-Smirnoff D statistic after logarithmic transformation. Ln: logarithmic. The right hand 3 boxes pertain to the transformed variable.

Figure 7.1: BioT has a distribution that reflects serum total T, iBT and cFT. All of the serum T measures in the FAMAS have right skewed distributions. **A** – BioT, **B** – Ln BioT, **C** - total T, **D** – Ln total T, **E** – iBT, **F** – Ln iBT, **G** – cFT, **H** – Ln cFT.Upon log transformation each of the serum measures of T did not significantly differ from a normal distribution. BioT – bioactive T, the measure from the assay. iBT – immunoassayed bioavailable T. cFT – calculated free T utilising the method described in Vermeulen et al., 1999.


A: BioT
No. of participants
BioT (nM/L)
B: Ln BioT
No. of participants
Ln BioT (nM/L)
C: total T
No. of participants
Total T (nM/L)
D: Ln total T
No. of participants
Ln total T (nM/L)
E: iBT
No. of participants
iBT (nM/L)
F: Ln iBT
No. of participants
Ln iBT (nM/L)
G: cFT
No. of participants
cFT (nM/L)
H: Ln cFT
No. of participants
Ln cFT (nM/L)

7.3.2 BioT has the strongest correlation with serum total T in comparison to the other serum T measures from the FAMAS

The strongest correlation of the BioT measure was with serum total T (Pearson r = 0.592, p<0.0001), followed by immunoassayed bioavailable T (iBT) (Pearson r = 0.466, p<0.0001, Fig 7.2B) and calculated free T (cFT)(Pearson r = 0.402, p<0.0001) (Table 7.2). In comparison, total T correlated most strongly with iBT or cFT than BioT (Pearson r = 0.706, p<0.0001 and Pearson r = 0.813, p<0.0001, respectively). The relationship between cFT and iBT was also stronger than the relationship of either of these variables with BioT (Pearson r = 0.720, p<0.0001)(Table 7.2) The associations are also presented as scatterplots in Figure 7.1 for ease of comparison.

		BioT	Total T	iBT	cFT
Ln BioT	**Pearson r**		0.592	0.466	0.402
	N		976	976	976
Ln Total T	**Pearson r**	0.592		0.706	0.813
	N	976		976	976
Ln iBT	**Pearson r**	0.466	0.706		0.72
	N	976	976		976
Ln cFT	**Pearson r**	0.402	0.813	0.72	
	N	976	976	976	

Table 7.2: Correlations of alternate serum T measures with BioT. All correlations were performend on log transformed serum T measures and were significant at p<0.0001.

7.3.3 BioT and total T have weak negative relationships with age

Assessment of the relationship of each of the serum T measures with age revealed weak negative correlations with BioT and total T (Pearson r = -0.073, p<0.05, Pearson r = -0.178, p<0.0001, respectively) (Table 7.3). In comparison, the negative relationship of cFT and iBT with age is stronger in the cohort (Pearson r = -0.382, p<0.0001 and Pearson r = -0.435, p<0.0001, respectively)(Table 7.3). The stronger relationship of iBT and cFT with age has been previously described (Nankin et al. 1986; Gray et al. 1991) and has been attributed to increasing SHBG levels as men age, as is observed in the FAMAS cohort (Pearson r = 0.363, p<0.0001) (Table 7.3).

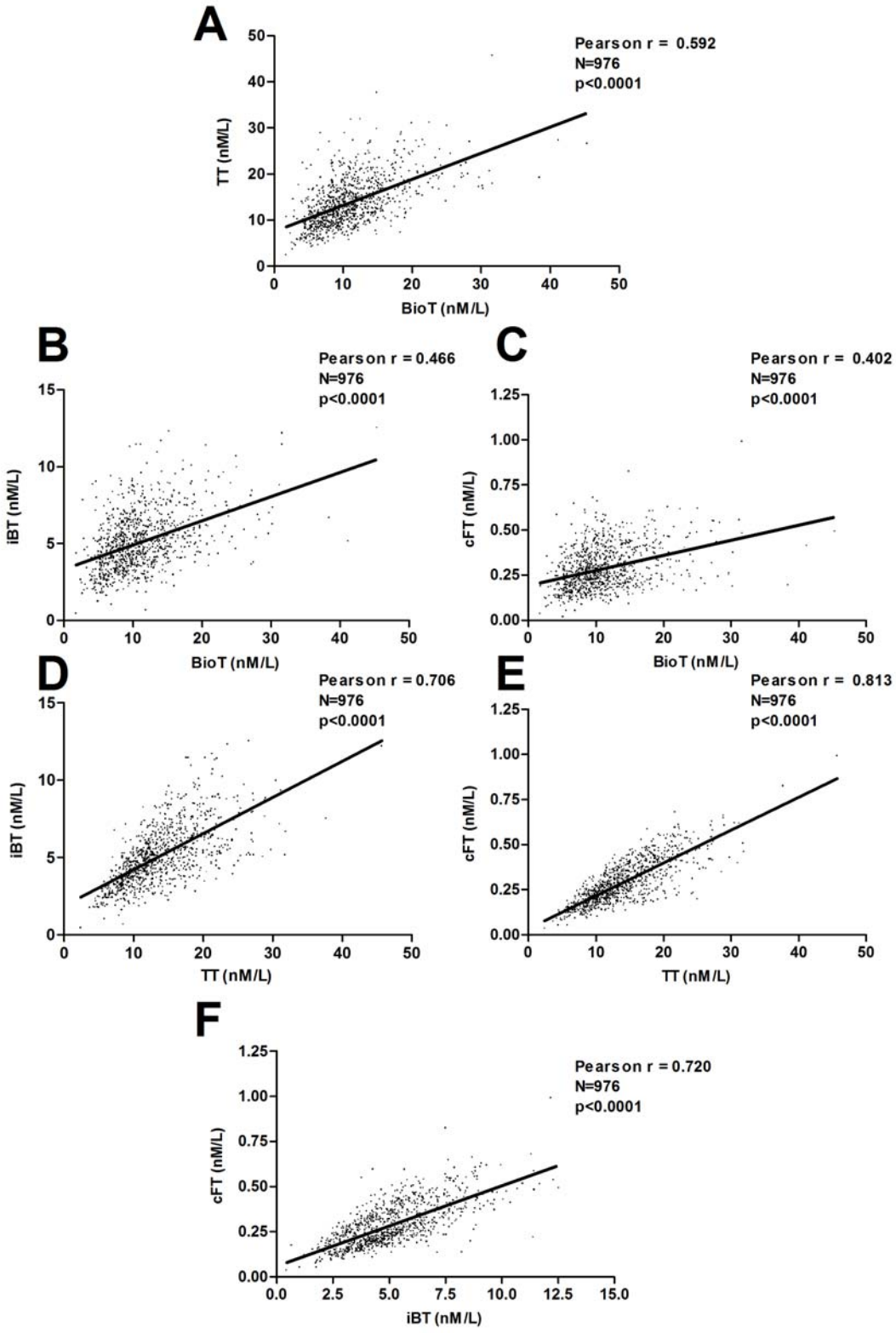


Figure 7.1: Scatterplots of associations between the serum T measures. A: BioT compared to total T, B: BioT compared to iBT, C: BioT compared to cFT, D: TT compared to iBT, E: total T compared to cFT and F: cFT compared to iBT.

		Age
Ln BioT	**Pearson r**	-0.073*
	N	976
Ln Total T	**Pearson r**	-0.178***
	N	975
Ln iBT	**Pearson r**	-0.435***
	N	975
Ln cFT	**Pearson r**	-0.382***
	N	975
Ln SHBG	**Pearson r**	0.363***
	N	975

Table 7.3: Correlations of BioT, total T, iBT, cFT and SHBG with age. Pearson correlations on log transformed values. * $p<0.05$, ***$p<0.0001$

7.3.4 Increase in waist circumference is related negatively with serum T levels in the FAMAS

There are now several descriptions of the negative correlation of serum T with waist circumference (Allan et al. 2006; Derby et al. 2006). Total T and iBT were more strongly related to waist circumference in the FAMAS cohort than cFT or BioT (Pearson r = -0.394, $p<0.0001$, Pearson r = -0.306, $p<0.0001$ compared to -0.239, $p<0.0001$, Pearson r = -0.269, $p<0.0001$, respectively) (Table 7.4).

		Waist circumference
Ln BioT	**Pearson r**	-0.269
	N	976
Ln Total T	**Pearson r**	-0.394
	N	975
Ln iBT	**Pearson r**	-0.306
	N	975
Ln cFT	**Pearson r**	-0.239
	N	975

Table 7.4: Correlations of BioT, total T, iBT and cFT with waist circumference. Pearson correlations on log transformed serum T measures. All correlations were significant at $p<0.0001$.

7.3.5 Regression analysis of serum T measures with grip strength, sexual desire and erectile function.

Negative relationships between age and grip strength (Baumgartner et al. 1999), age and sexual desire and age and erectile function (Schiavi et al. 1991) have previously been described as has the negative relationship between waist circumference and the above physiological parameters (Baumgartner et al. 1999; Bhasin et al. 2007). The associations of the FAMAS serum T measures with age and waist circumference are documented above. Therefore, partial correlations were performed between each of the serum T measures and grip strength, sexual desire and erectile function whilst controlling for age and waist circumference, smoking, number of chronic conditions and alcohol consumption. All of the serum T measures except BioT demonstrated a weak positive correlation with erectile function, with the strongest relationship observed between cFT and the IIEF score of erectile function (Table 7.5). Additionally, total T and iBT also demonstrated a weak positive correlation with IIEF sexual desire in this cohort. A stronger positive correlation was observed with total T and cFT and sexual desire on the dyadic scale of the SDI (Table 7.5). None of the serum measures of T correlated with the measure of erectile function on the GIR, while iBT demonstrated weak positive relationships with all other measures such as intercourse satisfaction and overall satisfaction on the IIEF scale and solitary sexual desire on the SDI (Table 7.5).

Grip strength for the dominant hand correlated weakly but positively with all of the serum T measures except for BioT whilst controlling for age, waist circumference, alcohol consumption, number of chronic conditions and hours of exercise per week (total T 0.127 $p<0.001$; iBT 0.071, $p<0.05$; cFT 0.161, $p<0.001$). These results were paralleled for the non dominant hand correlations although the relationship with iBT was lost (Table 7.6).

7.3.6 Comparison of serum T measures indicates that BioT is most closely related to total T

To determine which portion of serum T the bioassay may therefore be measuring, I examined the relationship between the serum measures of T was examined in order to establish which serum T measure showed the closest relationship with BioT. While all of the serum T measures had distinct means and medians as measured by paired T-test and Wilcoxon signed rank tests respectively, the variances of BioT and total T were not statistically significantly different as assessed by an F test of their variances (Table 7.7). In comparison, all of the other serum measures of T had distinct variances in comparison to each other with a high level of significance (Table 7.7). Examination of the contribution of each of the serum T measures to the variance of the BioT measure revealed that total T contributed the largest portion to the variance in BioT in comparison to cFT or iBT, with a standardised B statistic of 0.812 in comparison to 0.204 or -0.3 for iBT and cFT respectively (Table 7.8).

Variable		Ln BioT	Ln total T	Ln iBT	Ln cFT
IIEF SD	Correlation	0.058	0.085*	0.094**	0.062
	df	886	886	886	886
IIEF EF	Correlation	0.028	0.085*	0.097**	0.099**
	df	886	886	886	886
IIEF IS	Correlation	0.034	0.074*	0.093**	0.081*
	df	886	886	886	886
IIEF OS	Correlation	0.009	0.046	0.087**	0.047
	df	886	886	886	886
SDI dyadic	Correlation	0.056	0.113***	0.088**	0.123***
	df	886	886	886	886
SDI solitary	Correlation	-0.018	0.057	0.08*	0.064
	df	886	886	886	886
GIR	Correlation	-0.02	-0.026	-0.005	-0.042
	df	886	886	886	886

Table 7.5: Correlation matrix of serum T measures with erectile function and sexual desire in the FAMAS cohort. The table summarises the partial correlations of serum T measures with measures of self-reported sexual function.*$p<0.05$, **$p<0.01$, ***$p\leq0.001$. IIEF: international index of erectile function. SD: sexual desire. EF: erectile function. IS: intercourse satisfaction. OS: overall satisfaction. SDI: sexual desire inventory. Dyadic: measures desire for intimacy or sexual intercourse with another. Solitary: desire for sexual relations with oneself. GIR: global impotence rating. Correlations are controlled for age, waist circumference, number of chronic conditions, smoking status and amount of alcohol consumed per day.

Variable		Grip dominant	Grip non-dominant
Ln BioT	Correlation	0.052	0.052
	df	853	853
Ln total T	Correlation	0.127***	0.105**
	df	853	853
Ln iBT	Correlation	0.071*	0.05
	df	853	853
Ln cFT	Correlation	0.161***	0.137***
	df	853	853

Table 7.6: Correlation matrix of serum T measures with grip strength in the FAMAS cohort. The table summarises the partial correlations for each of the serum T measures with grip strength on both the dominant and non dominant hand. *p<0.05, **p<0.01, ***p<0.001. Correlations are controlled for age, waist circumference, alcohol consumption per day, number of chronic conditions and hours of exercise per week.

	Means (paired T test, 2 tailed)	Medians (Wilcoxon signed rank, 2 tailed)	Variance (F Test, 2 tailed)
BioT vs total T	t=20.48, df=977,p<0.0001	p<0.0001	n.s.
BioT vs iBT	t=53.54, df=977, p<0.0001	p<0.0001	p<0.0001
BioT vs cFT	t=238.1, df=977, p<0.0001	p<0.0001	p<0.0001
Total T vs iBT	t=107.2, df=977, p<0.0001	p<0.0001	p<0.0001
Total T vs cFT	t=488.0, df=977, p<0.0001	p<0.0001	p<0.0001
iBT vs cFT	t=298.7, df=977, p<0.0001	p<0.0001	p<0.0001

Table 7.7: The means and medians of the serum measures of T are distinct but the variances of serum total T and BioT are not significantly different. Paired T-tests and F tests were performed on log transformed serum T values while the wilcoxon signed rank test as a non-parametric test was performed on non-log transformed serum T values. The df for each test was equal across the tests, df = 977 and all tests were performed 2-tailed. F test is a measure of the variance ratio.

Source	Type III sum of squares	df	Mean Square	F	Sig.	B	Std. Error
Corrected Model	76.677[a]	3	25.559	204.21	p<0.001		
Intercept	0.822	1	0.822	6.57	p<0.05	-0.491	0.192
total T_Ln	28.602	1	28.6	228.52	p<0.001	0.812	0.054
cFT_Ln	4.71	1	4.71	37.61	p<0.001	0.204	0.045
iBT_Ln	2.61	1	2.61	20.82	p<0.001	-0.3	0.049
Error	121.78	973	0.125				
Total	5360.38	977					
Corrected Total	198.46	976					

a. R squared = 0.386 (adjusted R Squared = 0.384)

Table 7.8: Total T contributes the largest portion to the observed variance in the BioT measure out of all of the serum T measures. The table summarises univariate analysis of covariance on log transformed serum T values. All contributions of the serum T values are significant.

7.4 Discussion

There is now a large body of data relating various physiological parameters in men with serum T levels. The negative relationships observed between age and total T, age and iBT and age and cFT are well established. The results in the present study indicate that BioT is not influenced to the same extent as cFT or iBT by increasing SHBG levels as men age, but rather reflects the relationship of total T with age. It is important to note that the strength of the correlation of BioT with age is weaker than the other three measures, and may be attributable to the larger CV of the bioassay in comparison to the CV of the assays for the other serum T measures.

This is the first comparison of the relationship of serum T measures and grip strength in a cohort study including men aged below 65 years of age. A previous cross sectional study in elderly men aged between 65-88 years reported that serum total T and calculated serum BT (utilising the method of van den Beld (2000)) are positively correlated with grip strength in men aged between 65-88 years when controlling for age, level of education, alcohol use, physical activity, chronic disease and body mass index (Schaap et al. 2005). The stronger relationship between cBT and grip strength in comparison to total T was also found in another study investigating 403 independently living elderly men aged between 73-94 years old where muscle strength (as measured with grip strength) was most closely related to cBT and cFT in comparison to total T whilst controlling for BMI only (van den Beld et al. 2000). Currently there is only one report on a longitudinal study of older men (median age approximately 74 years) suggesting that serum total T and cFT are not associated with greater declines in grip strength over a period of 3 years, but that low baseline levels of serum T and cFT are associated with decreased grip strength (Schaap et al. 2007). It is demonstrated in this study that cFT has the strongest relationship with grip strength followed by total T, and then iBT while controlling for confounding variables such as age, waist circumference, number of

hours of exercise per week and alcohol intake in a cohort involving younger males. Combined with the findings that SHBG increases with age, and the observation that SHBG contributes positively to a frailty phenotype in men aged between 65-88 years (Mohr et al. 2007) these results suggest that SHBG may be a mediator of the relationship of serum T and physical function as men age. The precise relationship between physical function, serum T, SHBG and age remains to be defined and it will be interesting in the future to determine the relative contribution of each of these measures to physical function in the FAMAS cohort as a function of age.

Sexual function is determined by multiple behavioural, interpersonal, psychological and physiological factors. It has been reported that sexual dysfunction is associated with poor physical and emotional health (Laumann et al. 2006) and approximately 70 percent of erectile dysfunction cases are associated with diseases such as chronic alcoholism, diabetes, hypertension and atherosclerosis (Bortolotti et al. 1997). This is the first study to define weak but positive relationships between serum T measures and sexual desire on two scales whilst controlling for the confounding variables of age, waist circumference, smoking status, number of chronic conditions and alcohol consumption. Serum T concentrations show circadian rhythm with a peak in the morning and a nadir in the evening (Gupta et al. 2000). Therefore, the inability of other studies to demonstrate this relationship may be due to an inconsistent blood sampling time, whereas in the present study all serum samples were collected in the morning.

In support of the relationship between serum T levels and sexual desire, treating healthy young men for 4-6 weeks with a GnRH antagonist leads to significant decreases in self-reported sexual desire and intercourse along with a trend for decreased spontaneous erections. In comparison, men who received T replacement did not show any significant decreases in any aspect of physical function (Bagatell et al. 1994). Additionally, a positive relationship

between libido (frequency of sexual thoughts, fantasies or desires) between serum total T and calculated BT has been described (Travison et al. 2006). Finally, studies on androgen supplementation therapy in men with low baseline levels of serum T indicate that restoration of serum T levels to those found in healthy young men results in an increase in sexual desire (Seftel et al. 2004; Reyes-Vallejo et al. 2007; Yassin et al. 2007). Collectively, these results demonstrate a modest functional link between serum T levels and sexual desire in men.

The strongest relationships of serum T levels with aspects of sexual function in this study were with sexual desire rather than erectile function. However, significant weak positive relationships between serum measures of T and erectile function were found in this study with the comprehensive scale of erectile function, the IIEF but not with the one-question GIR. This is the first report of serum T demonstrating a significant relationship with erectile function independent of age, waist circumference, smoking status, number of chronic diseases and alcohol consumption. Previous studies have shown positive correlations between measured FT and erectile function and calculated BT and erectile function as measured by the IIEF without controlling for age (Tsujimura et al. 2003; Armagan et al. 2006). Evidence from studies of hypogonadism in rats and men indicates that there is a threshold serum T level for erectile dysfunction below which symptoms of erectile dysfunction become endemic; in rats this occurred when serum T levels fell to 10-12% of normal levels (Ahn et al. 2002). A threshold for symptoms of androgen deficiency, including decreases in sexual desire, that is unique for each individual has been defined and is generally around the lower end of the reference range in healthy young men (Kelleher et al. 2004). In comparison, a serum T threshold for erectile dysfunction remains elusive. Collectively, this data is suggestive of a link between erectile function and serum T levels but further verification is needed, particularly with respect to potential confounding influences in the complex aetiology of erectile dysfunction.

While the BioT measure generally shows the same direction of relationships as serum T measures, it does not achieve significance with any of the physiological variables. In comparison to the other serum T measures, the BioT measure has a higher inter and intra assay CV and this higher variability may impact on these associations. Future work will be aimed at examining the relationships of each of the serum T measures with other physiological parameters such as body composition on dual x-ray absorptiometer scans and aspects of cognitive function such as visuospatial ability, memory, visuomotor tracking and attention or depression scales to determine if BioT is more predictive in relation to alternative measures of serum T.

The bioassay presented in this thesis is an artificial system that involves long term culture of specific tissue culture cells with male serum. Whilst matrix effects of male serum on the cells are controlled in this study design with the use of hormone stripped male serum to create the standard curves and also with a novel short treatment time in comparison to other bioassays, there are several aspects of the design that do not reflect the physiological process of androgen entry and action within cells. One such difference is the static treatment of the cells with serum, which may be the cause of the measure reflecting total T rather than a physiological portion of T. In support of this, BioT as measured by two other bioassay systems involving 24 hours of treatment with serum demonstrated strong correlations with serum total T levels as measured with RIA ($r=0.93$, $p<0.0001$, $n=22$)(Raivio et al. 2001) or gas chromatography mass spectrometry ($r = 0.99$, $p<0.05$, $n=15$)(Chen et al. 2006). One other assay has reported a good correlation of bioassay values with total T levels in 30 serum samples measured by radioimmunoassay but utilised extracted serum samples and failed to report a correlation coefficient (Roy et al. 2006). The data presented here represent the first and most comprehensive report of the validation of a bioassay on a large number of serum samples, and is also the first report investigating the relationships between BioT and the alternate measures of serum androgens, iBT and cFT.

In conclusion, the findings in this chapter demonstrate that serum T measures correlate positively but weakly with measures of androgenic action such as grip strength, sexual desire and erectile function in men whilst controlling for potential confounders of this relationship and that the BioT measure most closely reflects the measure of serum total T, highlighting the limitations of static assay systems which aim to represent physiologically relevant levels of T in serum.

Chapter 8: General discussion

The actions of androgens are critical for male development and for the maintenance of physiological and psychological characteristics in adult males. However, many aspects of the mechanism of androgenic action in males are still unclear, such as the portion of serum androgens capable of mediating genomic effects via the AR within tissues or the specific structural features of the AR required for the sensitive activation of gene transcription in androgen target tissues. To this end, this thesis utilised two distinct but complementary approaches to investigate androgen signalling in males. The first approach involved the investigation of the structural features of the AR required for the activation of genes in response to androgens, whereas the second approach investigated the fraction of androgens in serum capable of mediating transcriptional activation via the AR.

8.1 The AR requires AF5 for sensitive transcriptional activation in response to androgens

The findings in this thesis demonstrate that amino acids 500 to 535 in the AF5 domain in the NTD of the AR are required for the N/C interaction and p160 binding. Additionally, advanced prostate cancer mutations identified in this 35 amino acid region result in enhanced activity of the receptor in an environment containing high cellular levels of coactivators. These findings imply that this region of the receptor is important for inter-domain and intermolecular communication and are supported by the findings in this thesis that the entire AF5 domain of the receptor is required for mediating sensitive transcriptional activation in response to androgens via mediation of p160 coactivator binding and the N/C interaction. Previous observations have highlighted the mutually exclusive nature of p160 and N/C interactions but the findings in this thesis allow the proposal of a model where these two interactions are required in a sequential, co-operative manner to mediate sensitive AR transcriptional activation in response to androgens. The observations that mutations resulting in decreased or absent N/C interactions result in complete or partial androgen insensitivity syndrome

highlights the important role of this interaction in male development and physiology (Jaaskelainen et al. 2006). Observations of p160 knockout mice also demonstrate that p160 coactivators are essential for male physiology. The NCOA1 knockout mouse is partially resistant to steroid hormones and has decreased growth of the prostate gland and the testes (Xu et al. 1998), while NCOA2 knockout mice have impaired spermatogenesis, leading to hypofertility (Gehin et al. 2002). Furthermore, the male double NCOA1 and NCOA2 knockout mouse has more severe defects in spermatogenesis with the testis phenotype of these mice reflecting that found in elderly males (Mark et al. 2004). Together with the findings in this thesis, it would appear that the AF5 region is required for the determination of a sensitive response to androgens via mediation of N/C and p160 interactions. The interactions allow high levels of AR transcriptional activation in response to androgens in tissues which require this sensitivity for efficient function, such as the male reproductive tissues. Modulation of the AF5 region via mutations may therefore contribute to prostate cancer progression by causing increased sensitivity of the AR to androgens. This increased sensitivity may allow continued prostate cell growth in a castrate environment such as that encountered during CAB, thereby leading to biochemical and metastatic disease relapse.

8.2 Static mammalian cell based androgen bioassays measure total T

The finding in this thesis that 5 minutes of serum treatment results in a more specific bioassay for serum T via decreasing activity caused by androgenic precursors allowed the adoption of an assay that specifically measures serum T. The subsequent comparison of the bioassay results with other measures of serum T identified that the assay measures a fraction of T in serum that most closely relates to total T. Furthermore, the assay measure does not associate with any parameter previously associated with androgenic action which indicates that current bioassays for measuring bioactive T may not measure the physiological portion of T in serum. Indeed, one of the key limitations to understanding androgenic action in males is our current

inability to precisely define the fraction of serum T that is capable of mediating cellular effects, thereby leading to physiological androgenic action. A model of steroid bioavailability to cells has been proposed which involves four main effectors. These effectors are i) influx of hormone into the cell, ii) dissociation of the hormone from serum binding proteins, iii) the flow of the hormone past the cell membrane whilst in circulation and iv) metabolism of the hormone once it enters the cell (Pardridge 1987; Mendel 1989). Each of these effectors are dependent on each other.

Static treatment is an invariable hallmark of all currently described androgen serum bioassays, generally involving treatment with serum for 24 hours. It is demonstrated in this thesis that contact with serum or hormone for 24 hours resulted in greater AR activity at lower concentrations of hormone compared with 5 minutes of treatment. These findings imply that the longer period of serum contact used in all previous static assay systems may lead to an overestimation of the proportion of hormone capable of entering cells *in vivo*. Relating to the general model of steroid bioavailability, this long period of serum contact on cells compromises the main effector of the flow of hormone past the cell membrane (effector iii) thereby leading to greater dissociation of hormone from serum binding proteins (effector ii) and a greater influx of hormone into the cell that would occur *in vivo* (effector i). Indeed, one androgen bioassay involving a 24 hour treatment time reported a very strong correlation with total T (r=0.99) in female serum (Chen et al. 2006).

In contrast to all previous androgen bioassay investigations, the current studies were able to comprehensively document the relationship of measured BioT with traditional measures of serum T and other calculated parameters such as iBT or cFT. The findings in this thesis that BioT measured from only 5 minutes of human serum treatment relates most closely to total T demonstrates that this androgen bioassay does not represent a measure of biologically available or physiologically relevant T. In conclusion, the findings in this thesis combined

with the observation that all previously developed androgen bioassays involve 16 hours or more of treatment of cells with serum, means that it is entirely possible that all currently reported serum androgen bioassays measure total T, albeit with a more complex and variable method than current immunoassay-based methods of measuring total T.

8.3 Future Studies

8.3.1 Investigation of AR coregulator and N/C interactions utilising full length AR molecules

The current studies demonstrate the importance of inter-domain interactions for sensitive AR signalling. Therefore, further studies investigating AR-coregulator and AR N/C interactions must utilise full length receptors in order to define further the contacts that these receptors make and the nature of these interactions during AR transcriptional activation. Such studies could be performed utilising emerging FRET technologies involving real time imaging of cells upon ligand addition. Before the utilisation of these technologies it must be ensured that tagging the receptor with reporter proteins does not interfere with the native function of the receptor. Furthermore, the utilisation of stable cell lines expressing each of the AR NTD deletion variants investigated in this thesis will provide an ideal platform for the investigation via chromatin immunoprecipitation of the role of coregulator binding in the AR NTD during transcriptional activation, and would ease identification of protein interaction partners with each of these domains utilising co-immunoprecipitation and mass spectrometry.

8.3.2. Investigation of the functional consequences of advanced prostate cancer mutations in the AR AF5 region

Previously, our laboratory has shown that the E231G mutation in the NTD of the mouse confers increased coactivation of the receptor in comparison to wtAR (Han et al. 2001), similar to the phenotype of the mutations in the current study. Currently in our laboratory we are investigating the transcriptional profile induced by overexpression of the E231G AR variant in mouse prostate in comparison to wtAR. Preliminary evidence indicates that the

E231G mutation induces a unique transcriptional profile in comparison to wtAR overexpressing mice (unpublished data). It is anticipated that results gained from this study will be verified in a similar manner to determine if the unique E231G transcriptional profile is a result of the specific E231G mutation or a general response to the disruption of AR NTD function. Such a study is essential in order to further define the involvement of the AR, and in particular the AR NTD, in prostate cancer development and progression and to further define the role of AF5 in the modulation of sensitive AR signalling.

8.3.3 Investigation of alternate strategies to measure androgen bioactivity

Verification of the postulation that the bioassay measure produced from a 24 hour serum treatment of the cells will even more closely measure total T than a 5 minute treatment is required. Furthermore, the limitation of the current bioassay approach involving long static treatments with hormone requires further optimisation. A system involving placing culture cells in a column and allowing serum to pass through in a defined time with a contact period of serum not exceeding 9 seconds may be one way to address this issue. Additionally, dissociation of the hormone from serum binding proteins and influx of the hormone into the cell may be affected adversely if the level of AR exceeds physiological levels found in androgen target tissues. Finally, the careful choice of a cell line with regards to the levels of coregulators and metabolic enzymes involved in steroid metabolism and catabolism will be required as the result may only be applicable to a particular cell type rather than a whole tissue.

Another inherent limitation of the transactivation assay system utilised in all currently reported androgen serum bioassays is that traditional luciferase assays measure accumulated activity over a long time period, in this case 24 hours after treatment. Consequently, assay results will not only reflect the portion of androgen in serum, but also reflect the ability of the cells to sustain androgenic action after the treatment period has ceased as well as the ability of the cells to transcribe luciferase mRNA and metabolise luciferase protein. Therefore, instead

of providing a snapshot of the cells at a particular time point, the assay provides a history of the androgenic action of the cells and luciferase metabolism over the time period. Consequently, approaches such as chromatin immunoprecipitation assays, real time PCR, more sophisticated BRET or FRET technology, or the use of more labile luciferase proteins may allow snapshots of the cells to be taken at shorter time periods following androgen exposure. If these technologies can be developed into a high throughput approach, they may allow an understanding of the actions of androgens over a period of time from the moment of treatment with serum to the activation of transcription of androgen regulated genes. This approach would have the potential to more closely estimate the proportion of androgens capable of crossing cell membranes and mediating activity via the AR *in vivo*.

8.3.4 Measurement of androgenic metabolites in male serum and urine

While current bioassays of androgens in serum focus on the actions of the AR signalling pathway, there is evidence that the actions of androgens prior to binding to the AR will affect male physiology such as the metabolic conversion of androgens within target cells. For example, it was recently demonstrated that polymorphisms in genes involved in androgen inactivation via glucuronidation are associated with serum levels of androstenediol-17G, and with urinary T and DHT. An investigation of over 2000 men revealed an association of a polymorphism in a gene involved in androgen inactivation (UGT2B15) and BMI, body fat and trunk fat (Swanson et al. 2007). Another study demonstrated higher serum T in young men with a polymorphism in another gene involved in androgen inactivation (*UGT2B7)* and increased cortical bone size (Estany et al. 2007). In elderly males, serum levels of the glucuronidated metabolic product of androstenediol glucuronide and 3α-diol glucuronide have been demonstrated to be predictors of bone mineral density and prostate volume (Vandenput et al. 2007). These results indicate that the measurement of androgenic metabolites via liquid or gas chromatography combined with mass spectrometry may provide a unique measure of androgen action in males and measurement of these metabolites or polymorphisms in our cohort may provide useful insights into androgen function in males as they age.

8.4 Conclusion

The findings in this thesis challenge several assumptions regarding the actions of androgens from their availability to cells to their ability to mediate sensitive AR signalling in males. The findings in chapters 6 and 7 demonstrate that the measurement of serum BioT with static mammalian cell-based androgen bioassays does not correlate more strongly with physiological parameters associated with androgenic action such as grip strength or sexual desire than other measures of serum T. This finding further defines the essential factors governing steroid bioavailability to cells due to the extensive optimisation of the assay described in chapter 6. Additionally, chapter 7 describes the relationships between different fractions of serum T and physiological traits associated with androgenic action and demonstrates that no one serum T measure will be optimal with respect to all aspects of physiological androgen action. It is possible that in the process of better defining AR structure and function, we may be able to design a better method to measure bioactive T. To this end, the finding in this thesis that the AF5 region of the AR mediates maximal sensitivity of AR signalling via p160 and N/C interactions demonstrates the importance of the AR NTD in mediating the genomic effects of androgens. The findings of this thesis challenge the assumption that the AR is a modular protein and provides evidence for the importance of intra-domain communication in the AR signalling pathway upon the actions of androgens. As sensitive AR signalling is essential for the physical reproductive health of males, the findings of this thesis provide a framework for future studies investigating the consequences of the decline in serum T as men age and how the cellular actions of androgens may be perturbed in disorders associated with male aging such as prostate cancer development and progression, disturbances in libido, erectile dysfunction and the development of frailty.

Appendices

Appendix A1: Cloning primers

Construct Name	Sense Primer Name – Sequence (5' to 3')	Antisense Primer Name – Sequence (5' to 3')	PCR conditions (°C/seconds)	Restriction Endonucleases
CHAPTER 3				
pCMV-ARP502L	N166	J12-502AS – CATGCCGCCAAGGTACCACAC	95/300 then [95/60, 65/60, 72/120] 25 times	
	PCR product from above	HindIIIAS	95/300 then [95/60, 65/60, 72/120] 25 times	*Bsu36I/HindIII*
pCMV-ARS513G	N166	J12-513-AS – ACAAGTGGGACCGGGATAGGG	95/300 then [95/60, 65/60, 72/120] 25 times	
	PCR product from above	X4AS	95/300 then [95/60, 65/60, 72/120] 25 times	*Bsu36I/HindIII*
pCMV-ARD526G	p367-Ex1 - CCCTGGATGGGTAGCTACTCCG	HindIIIAS -	95/300 then [95/60, 65/60, 72/60] 25 times	
	N166 -	PCR product from above	95/300 then [95/60, 60/60, 72/60] 30 times	*Bsu36I/HindIII*
pCMV-ARM535A	N166	J7-535-AS – CTCCAAACGCCTGTCCCCGTA	95/300 then [95/60, 65/60, 72/120] 25 times	
	PCR product from above	X4AS	95/300 then [95/60, 65/60, 72/120] 25 times	*Bsu36I/HindIII*
pCMV-ARM535V	N166	J6-535-AS - CTCCAAACGCACGTCCCCGTA	95/300 then [95/60, 65/60, 72/120] 25 times	
	PCR product from above	X4AS	95/300 then [95/60, 65/60, 72/120] 25 times	*Bsu36I/HindIII*
pCMV-ARΔ35αα	N111 CAGAATCTGTTCCAGAGCGTGC	D35aaAS – CACAGATCAGGCAGGTGCGACTCTGGTACGCAGCTG	95/300 then [96/20, 55/15, 70/90] 40times	
	D35aaS – CACCGCACCTGATGTGCGTTTGGAGACTGCCAGGGACGTC	X4AS – ACACACTACACCTGGCTC	95/300 then [96/20, 60/15, 70/90] 40 times	
	N111 – CAGAATCTGTTCCAGAGCGTGC	X4AS- ACACACTACACCTGGCTC	94/120 then [96/15, 55/30, 68/180] 40 times	*BstEII/TthIII1*

Construct Name	Sense Primer Name – Sequence (5' to 3')	Antisense Primer Name – Sequence (5' to 3')	PCR conditions (°C/seconds)	Restriction Endonucleases
pVP16-AR(1-538)Δ35αα	N111- CAGAATCTGTTCCAGAGCGTGC	pVd35AS – ATTCTAGACTACTCCAAACGCACATCAGGTG CGGTGAA	95/300 then [96/20, 55/15, 70/90] 40times	
	pVd35S – TTCACCGCACCTGATGTGCGTTTGGAGTAGT CTAGAAT	pVP16AS1 – ACCTCTACAAATGTGGTATGGCT	95/300 then [96/20, 60/15, 70/90] 40 times	
	N111 – CAGAATCTGTTCCAGAGCGTGC	pVP16AS1 – ACCTCTACAAATGTGGTATGGCT	94/120 then [96/15, 55/30, 68/180] 40 times	*Bsu36I/XbaI*
pVP16-AR(1-538)M535A	N111- CAGAATCTGTTCCAGAGCGTGC	pVM535A – ATTCTAGACTACTCCAAACGCGCGTCCCCGTA	96/180 then [96/20, 55/15, 72/60] 40 times	*Bsu36I/XbaI*
pVP16-AR(1-538)M535V	N111- CAGAATCTGTTCCAGAGCGTGC	pVM535V – ATTCTAGACTACTCCAAACGCACGTCCCCGTA	96/180 then [96/20, 55/15, 72/60] 40 times	*Bsu36I/XbaI*
pVP16-AR(1-538)P502L, S513G, D526G	N166 – TAGCCCCCTACGGCTACACT	ptmutAS1 - TTATCTAGATTACTCCAA ACGCATGTCCCCGTA	95/300 then [96/20, 55/30, 72/1] 40 times	*Bsu36I/XbaI*
pGL4.14-ARR3-Luc	PB3LucS - TATTGCTAGCGCTCAGATCCAAGCTGGAGCT	PB3LucAS – TATTGATATCTACCAACAGTACCGGAATGCC A	95/300 then [95/20, 53/15, 72/150] 40 times	*NheI/EcoRV*
ARE stable cell line diagnosis	pGL4S3	pGL4AS3	96/180 then [96/20, 68/15, 72/60] 35 times	N/A
AR stable cell line diagnosis	pCMVS3 – ATTGACGCAAATGGGCGGTA	IRESp3AS - CGGGCCAGGTGAATATCAAA	96/180 then [96/20, 68/15, 72/60] 35 times	N/A
CHAPTER 4				
pCMV-ARΔAF1 (deletion 38-360)	pCMVS2 – GTGACGTATGTTCCCATAGTAACGCCAA	ARdAF1AS - AGCCAGTGGAAAGTTGTAGTACGGGTTCTGG ATCACTTCGCGCA	96/300 then [96/20, 55/30, 72/90] 40 times	
	ARdAF1s – TGCGCGAAGTGATCCAGAACCCGTACTACA ACTTTCCACTGGCT	244 - ACCACCACACGGTCCATACAACT	96/300 then [96/20, 55/30, 72/90] 40 times	
	pCMVS2 - GTGACGTATGTTCCCATAGTAACGCCAA	244 – ACCACCACACGGTCCATACAACT	96/300 then [96/20, 58/30, 72/120] 40 times	*SnaBI/BstEII*

Construct Name	Sense Primer Name – Sequence (5' to 3')	Antisense Primer Name – Sequence (5' to 3')	PCR conditions (^{o}C/seconds)	Restriction Endonucleases
pCMV-ARΔAF5 (deletion 360-535)	133 – CAGGAAGCAGTATCCGAAGGCA	dAF5AS – TGGCAGTCTCCAAACGGCGACTCTGGTACGCA	96/180 then [96/30, 60/30, 72/150] 40 times	
	dAF5S – TGCGTACCAGAGTCGCCGTTTGGAGACTGCCA	X4AS – ACACACTACACCTGGCTC	96/180 then [96/30, 50/30, 72/150] 40 times	
	133 – CAGGAAGCAGTATCCGAAGGCA	X4AS – ACACACTACACCTGGCTC	96/180 then [96/30, 60/30, 72/150] 40 times	*BsmI/TthIII1*
pCMV-ARΔcAF5 (deletion 360-494)	133 – CAGGAAGCAGTATCCGAAGGCA	dcAF5AS – GTACCACACATCAGGTGCGGTGCGACTCTGGTACG	96/180 then [96/30, 60/30, 72/150] 40 times	
	dcAF5S – CGTACCAGAGTCGCACCGCACCTGATGTGTGGTAC	X4AS – ACACACTACACCTGGCTC	96/180 then [96/30, 50/30, 72/150] 40 times	
	133 – CAGGAAGCAGTATCCGAAGGCA	X4AS – ACACACTACACCTGGCTC	96/180 then [96/30, 60/30, 72/150] 40 times	*BsmI/TthIII1*
pVP16-AR(1-538)ΔAF5	N111	dAF5AS – TGGCAGTCTCCAAACGGCGACTCTGGTACGCA	96/180 then [96/20, 50/30, 72/60] 40 times	
	dAF5S – TGCGTACCAGAGTCGCCGTTTGGAGACTGCCA	pVP16AS1	96/180 then [96/20, 50/30, 72/60] 40 times	
	N111	pVP16AS1	95/180 then [96/20, 50/30, 72/150] 40 times	*BclI/XbaI*
pVP16-AR(1-538)ΔcAF5	N111	dcAF5AS – GTACCACACATCAGGTGCGGTGCGACTCTGGTACG	96/180 then [96/20, 50/30, 72/60] 40 times	
	dcAF5S – CGTACCAGAGTCGCACCGCACCTGATGTGTGGTAC	pVP16AS1	96/180 then [96/20, 50/30, 72/60] 40 times	
	N111	pVP16AS1	95/180 then [96/20, 50/30, 72/150] 40 times	*BclI/XbaI*

Construct Name	Sense Primer Name – Sequence (5' to 3')	Antisense Primer Name – Sequence (5' to 3')	PCR conditions (°C/seconds)	Restriction Endonucleases
pCMV-ARΔ39-100	pCMVS2 – GTGACGTATGTTCCCATAGTAACGCCAA	ARd39as - CAGGACCAGGTAGCCTGTGGGGCCCGGGTTCTGGATCACTT	96/300 then [96/20, 62/15, 72/90] 40 times	
	ARd39s – AAGTGATCCAGAACCCGGGCCCCACAGGCTACCTGGTCCTGGAT	N222D - GTCAGAAATGGTCGAAGTGCC	96/300 then [96/20, 68/15, 72/90] 40 times	
	pCMVS2 – GTGACGTATGTTCCCATAGTAACGCCAA	N222D - GTCAGAAATGGTCGAAGTGCC	96/300 then [96/20, 62/15, 72/120] 40 times	*EagI/AflII*
pCMV-ARΔ101-160	N111 – CAGAATCTGTTCCAGAGCGTGC	ARd101as – GTGGGGCCCAGCAGGGACAAGCCTCTACGATGGGCTTGGGGA	96/300 then [96/20, 62/15, 72/90] 40 times	
	ARd101s – TCCCCAAGCCCATCGTAGAGGCTTGTCCCTGCTGGGCCCCAC	N233O – CTAGGCTCTCGCCTTCTAGC	96/300 then [96/20, 62/15, 72/90] 40 times	
	N111 – CAGAATCTGTTCCAGAGCGTGC	N233O – CTAGGCTCTCGCCTTCTAGC	96/300 then [96/20, 60/15, 72/120] 40 times	*NarI/BsmI*
pCMV-ARΔ161-220	N111 – CAGAATCTGTTCCAGAGCGTGC	ARd161as – GAAGTGCCCCCTAAGTAATTCGTGGATGGGGCAGCTGAGTCAT	96/300 then [96/20, 62/15, 72/90] 40 times	
	ARd161s – ACTCAGCTGCCCCATCCACGAATTACTTAGGGGGCACTTCGACCA	N233O – CTAGGCTCTCGCCTTCTAGC	96/300 then [96/20, 62/15, 72/90] 40 times	
	N111 – CAGAATCTGTTCCAGAGCGTGC	N233O - CTAGGCTCTCGCCTTCTAGC	96/300 then [96/20, 60/15, 72/120] 40 times	*NarI/BsmI*
pCMV-ARΔ221-297	N122 – CAAGCCCATCGTAGAGGCCCCAC	ARd221as - AGCAGTATCTTCAGTGCTCTTGTCCTTGGAGGAAGTGGGAGC	96/300 then [96/20, 62/15, 72/90] 40 times	
	ARd221s – CTCCCACTTCCTCCAAGGACAAGAGCACTGAAGATACTGCTGAGTATTCCCCTT	120727as – TCGCCAGGTCCCCATAGCGGCACT	96/300 then [96/20, 62/15, 72/90] 40 times	
	N122 – CAAGCCCATCGTAGAGGCCCCAC	120727as - TCGCCAGGTCCCCATAGCGGCACT	96/300 then [96/20, 60/15, 72/120] 40 times	*AflII/RsrII*

Construct Name	Sense Primer Name – Sequence (5' to 3')	Antisense Primer Name – Sequence (5' to 3')	PCR conditions (°C/seconds)	Restriction Endonucleases
pCMV-ARΔWXXLF	120727s – GCACTGGACGAGGCAGCTGCGTACCAG	dWXXLFas - CAACTGGCCTTCTTCGGCTGTGGATGAGGAAG	96/180 then [96/30, 50/30, 72/150] 40 times	
	dWXXLFs – CAGCCGCCGCTTCCTCATCCACAGCCGAAGAA	X4AS – ACACACTACACCTGGCTC	96/180 then [96/30, 50/30, 72/150] 40 times	
	120727s – GCACTGGACGAGGCAGCTGCGTACCAG	X4AS – ACACACTACACCTGGCTC	96/180 then [96/30, 55/30, 72/150] 40 times	*BsmI/TThIII1*
CHAPTER 5				
pcDNA3.1-RlucAR	RlucS1 – TGCTATTGTTGAAGGTGCCAA	RlucASAS1 – TGGCACAACATGTCGCCATA	96/300 then [96/20, 60/30, 72/360] 40 times	*Xho1/XbaI*
			Subcloned from pCMV-AR3.1	*AflII/Bsu36I*
pcDNA3.1-ARRluc	EYFPS1 – TCACATGGTCCTGCTGGAGT	EYFPAS1 - ACGCTGAACTTGTGGCCGT	96/300 then [96/20, 60/30, 72/360] 40 times	*BamHI/XhoI*
			Subcloned from pCMV-AR3.1	*AflII/Bsu36I*
pcDNA3.1-EYFPAR			Subcloned from pcDNA3.1-RlucAR	*XhoI/XbaI*
pcDNA3.1-AREYFP			Subcloned from pcDNA3.1-ARRluc	*BamHI/XhoI*
CHAPTER 6				
pIRESpuro3-AR	ARLBDS2 - ATAACCGGTACTCTGGGAGCCCGGAAGCT	ARLBDAS2 - TATGGATCCTCACTGGGTGTGGAAATAGATG	95/180 then [95/20, 55/30, 72/120] 40 times	*AgeI/BamHI*
	ARDBDS2 – TAAGCTAGCCGTTTGGAGACTGCCAGG	ARDBDAS2 - TAAACCGGTCATCCCTGCTTCATAACA	96/180 then [96/20, 61/15, 72/60] 40 times	*NheI/AgeI*
	ARNTDS2 - ATGGAAGTGCAGTTAGGGCT	ARNTDAS2 - TATGCTAGCCATGTCCCCGTAAGGTCCGGA	96/300 then [96/20, 55.5/30, 72/240] 40 times	*EcoRV/NheI*
			Subcloned from pCMV-AR3.1	*AflII/BstBI*

Appendix A2: Sequencing primers

AR sense sequencing primers

Primer Name	**Primer sequence (5' to 3')**	**Position (*αα*)**
N111	CAGAATCTGTTCCAGAGCGTGC	24 to 31
133	CAGGAAGCAGTATCCGAAGGCA	196 to 202
N133D	AACGCCAAGGAGTTGTGTAAG	233 to 239
144	GCACTGAAGATACTGCTGAGT	301 to 308
120727s	GCACTGGACGAGGCAGCTGCGTACCAG	351 to 359
N155	CATCCTGGCACACTCTCTTCAC	430 to 437
N166	TAGCCCCCTACGGCTACACT	475 to 481
HindIIIs	TGGAGATGAAGCTTCTGGGTGTC	560 to 567
pCMVS1	GGAGGTCTATATAGCAGAGCTCGTT	Nucleotides -88 to -64 of pCMV-AR3.1
pCMVS2	GTGACGTATGTTCCCATAGTAACGCCAA	Nucleotides -504 to -477 of pCMV-AR3.1
pCMVS3	ATTGACGCAAATGGGCGGTA	Nucleotides -149 to -130 of pcDNA3.1-AR
111(c)	GCCTGTTGAACTCTTGTGAGC	Nucleotides -70 to -57 of pCMV-AR3.1
pMS1	AGCATAGAATAAGTGCGACA	Nucleotides -80 to -61 of pM-AR(1-538)
pVP16S1	TCTGGATATGGCCGACTTCGA	Nucleotides -76 to -56 of pVP16-AR(1-538)

AR antisense sequencing primers

Primer Name	**Primer sequence (5' to 3')**	**Position (*αα*)**
pcDNA3.1AS1	ACACCTACTCAGACAATGCGAT	Nucleotides +180 to +201 of MCS of pcDNA3.1
pVP16AS1	ACCTCTACAAATGTGGTATGGCT	Nucleotides +47 to +25 of pVP16-AR(1-538)
pMAS1	AGCAAGTAAAACCTCTACAAAT	Nucleotides +57 to +36 of pM-AR(1-538)
IRESp3AS	CGGGCCAGGTGAATATCAAA	In IRES sequence +330 to +311 from pCMV
211D	GCTGTGAAGGTTGCTGTTCCTC	108 to 115
N222	GTCTTTAAGGTCAGCGGAGCA	174 to 180
N222D	GTCAGAAATGGTCGAAGTGCC	226 to 232
N233	CTGGACTCAGATGCTCCAACG	251 to 258
120727as	TCGCCAGGTCCCCATAGCGGCACT	401 to 409

244	ACCACCACACGGTCCATACAACT	442 to 449
X4AS	ACACACTACACCTGGCTC	678 to 684
X7AS	GGTAGAAGCGTCTTGAGCAGG	849 to 855
2977as	CAGGCAGAAGACATCTGAAAG	Nucleotides +61 to +41 of pCMV-AR3.1

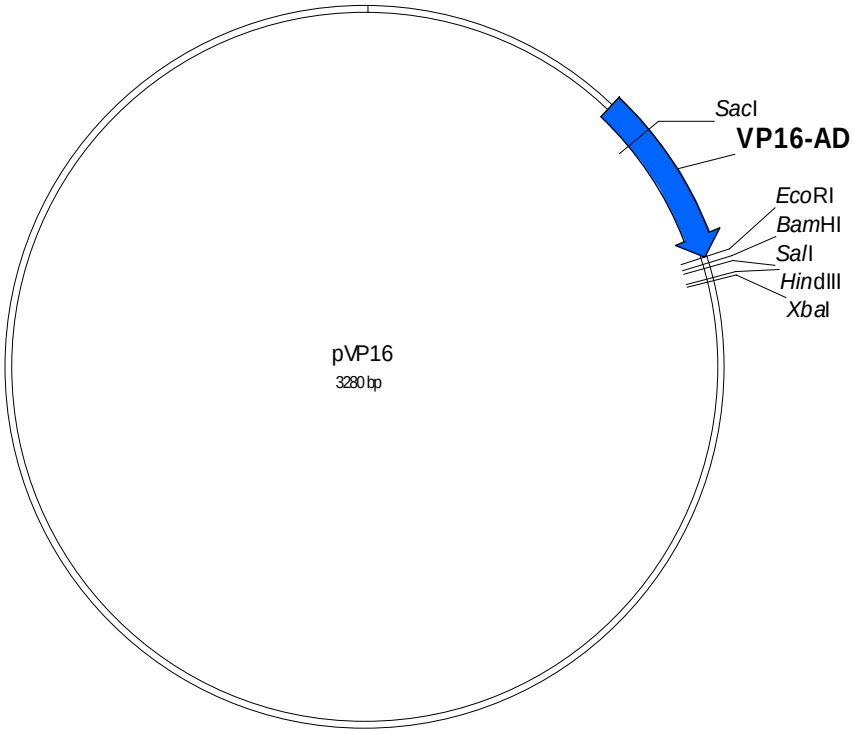
ARR3-Luc sequencing primers

Primer Name	**Primer sequence (5' to 3')**	**Direction**
PB3LucS	TATTGCTAGCGCTCAGATCCAAGCTGGAGCT	Sense
PB3LucAS	TATTGATATCTACCAACAGTACCGGAATGCCA	Antisense
pGLPB3LUCS1	GAGGTGAACATCACGTACGCGGAA	Sense
LucS	AGGTATTGGACAGGCCGCAAT	Sense
LucAS	AGGGCGTAGCGCTTCATGGCT	Antisense

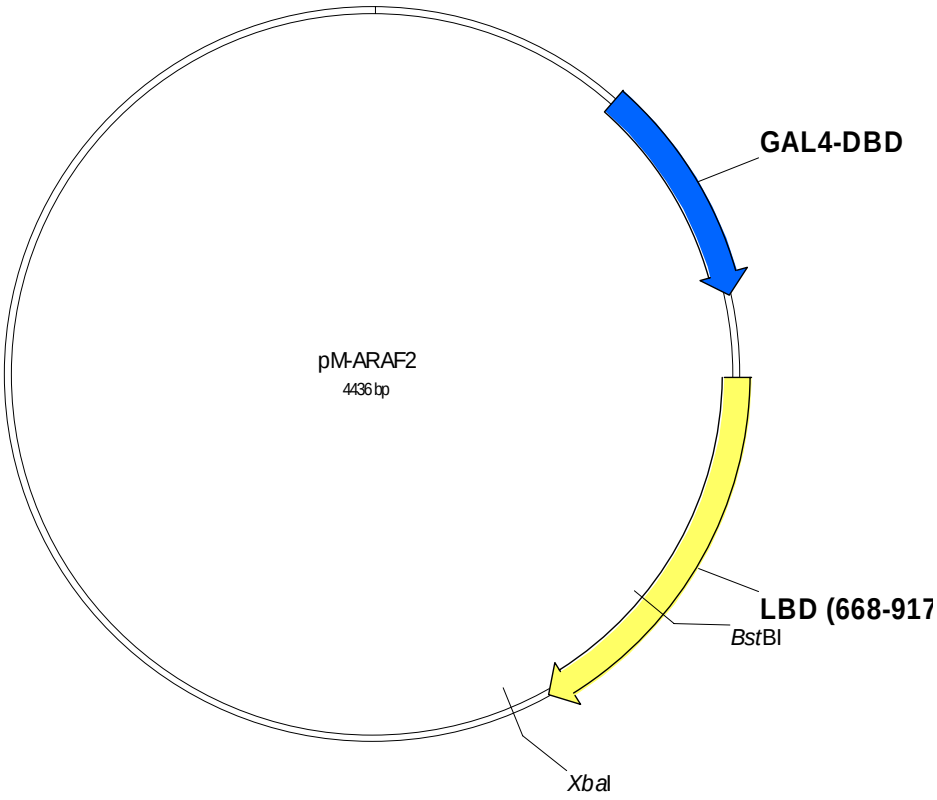
Appendix B: Plasmids

Appendix B1 Plasmids for mammalian 2-hybrid interaction assays

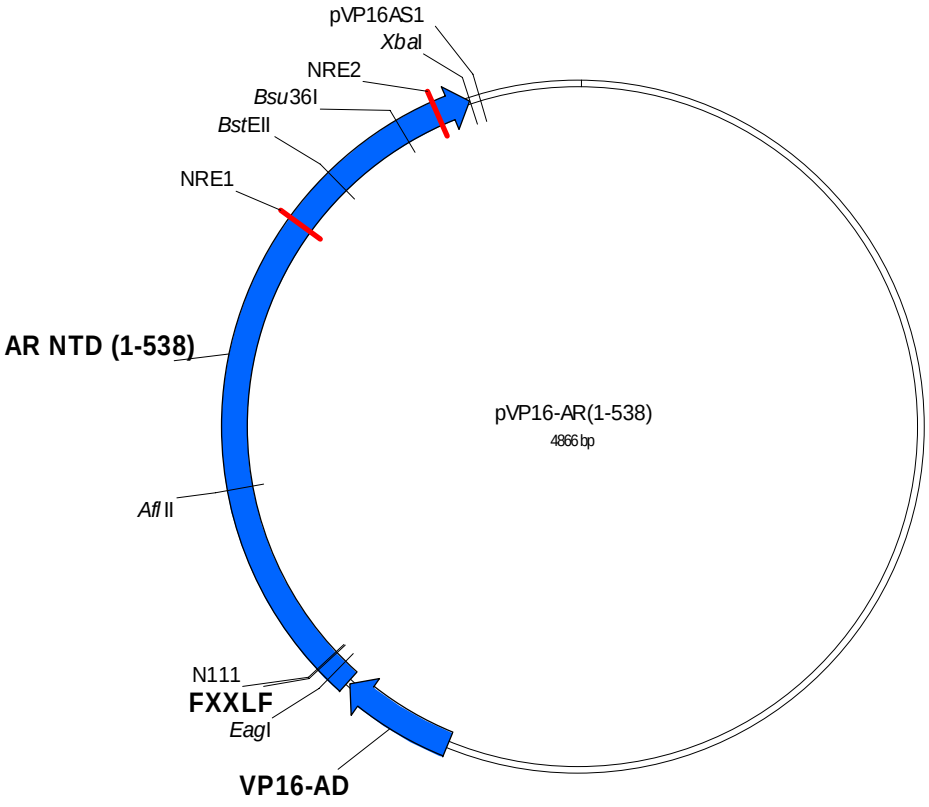
pM



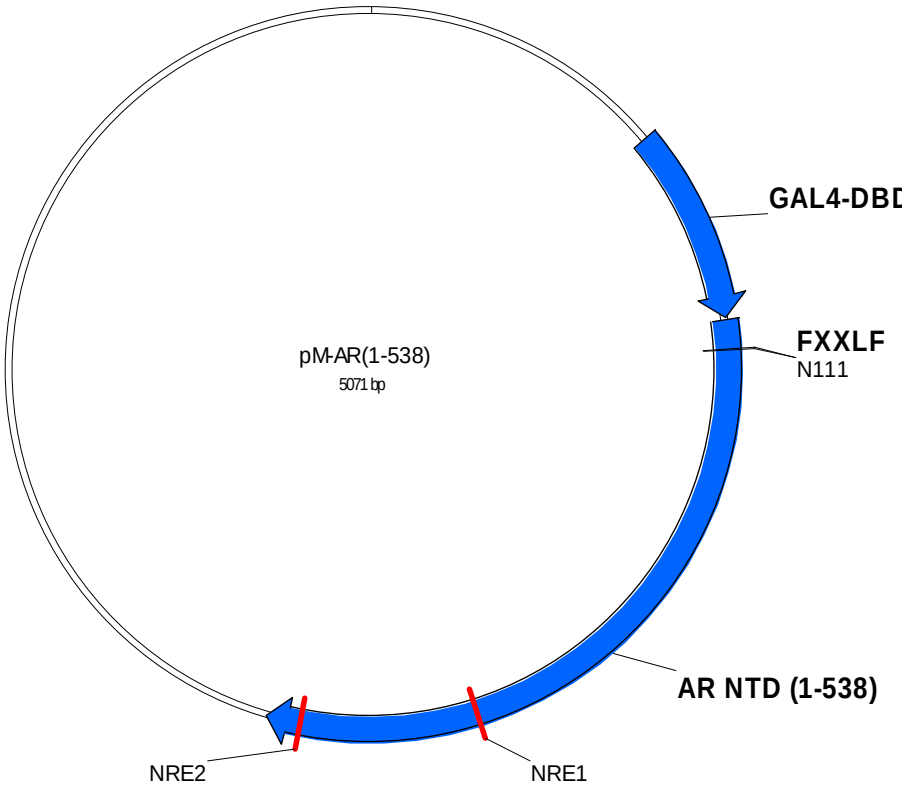
pVP16



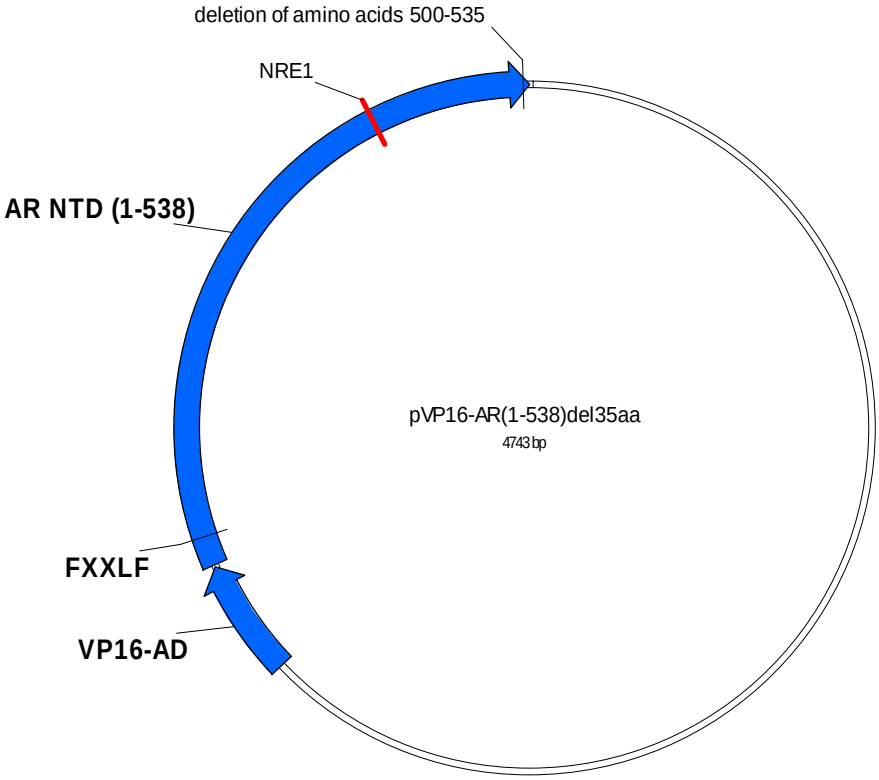
pM:ARAF2



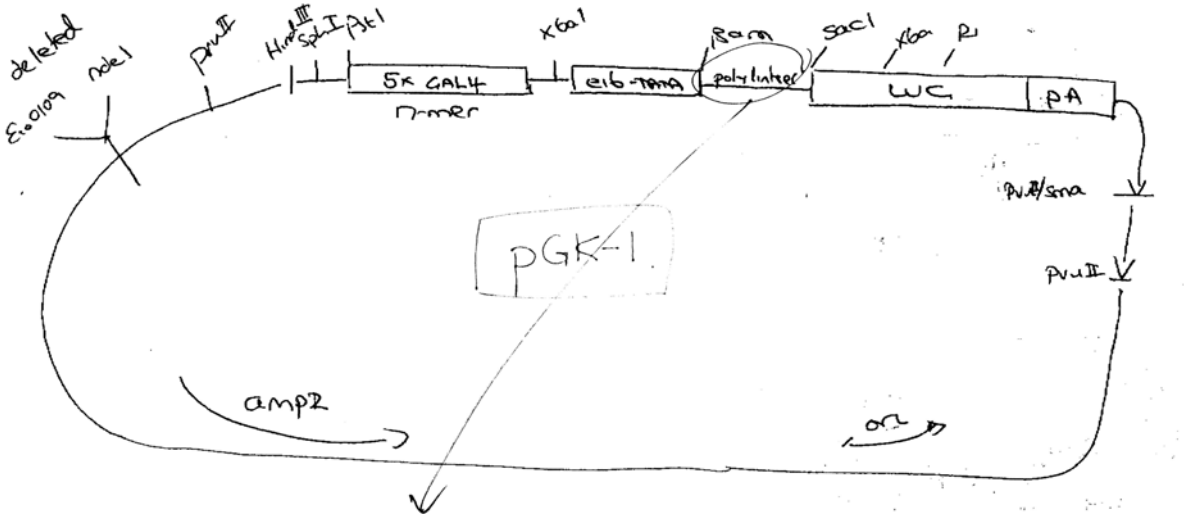
pVP16:AR(1-538)



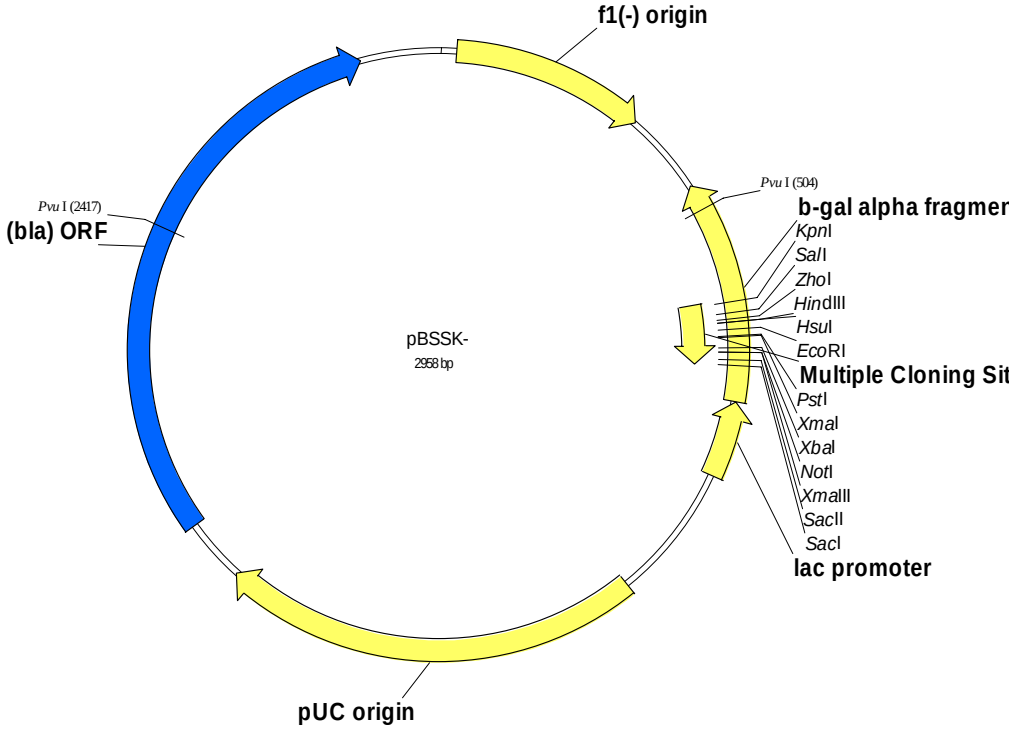
pM:AR(1-538)



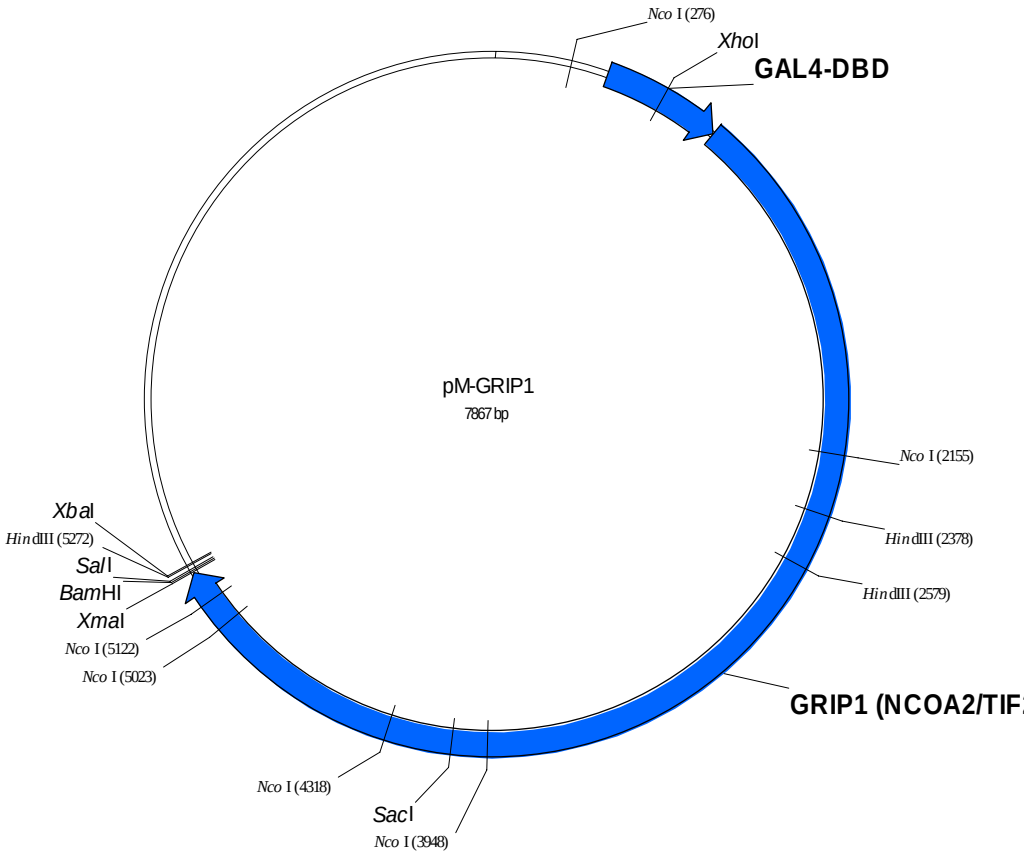
pVP16:AR(1-538)Δ35αα


deleted
EcoRI
NdeI
PvuII
HindIII
SphI
PstI
5x GAL4
17mer
XbaI
eib-TATA
polylinker
BamHI
SacI
XbaI
BglII
WC
pA
PvuII/SmaI
PvuII
pGK-1
ampR
ori

pGK1

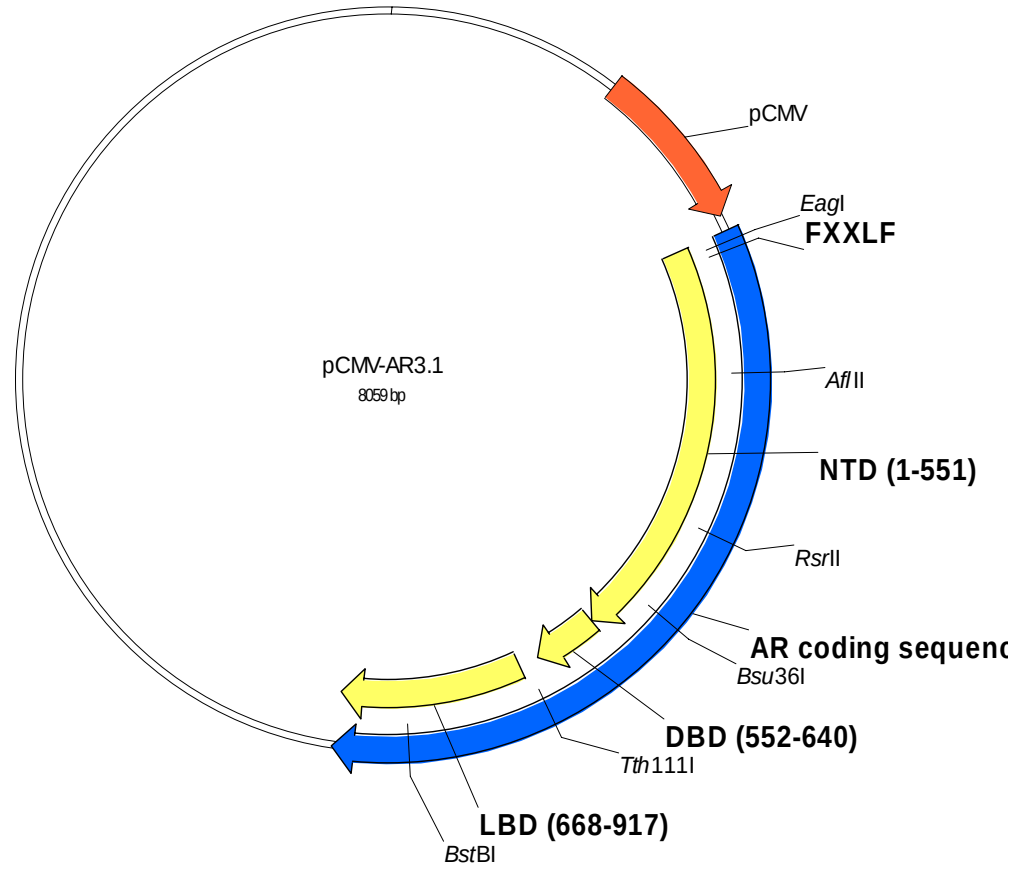

f1(-) origin
Pvu I (504)
b-gal alpha fragmer
KpnI
SalI
ZhoI
HindIII
HsuI
EcoRI
Multiple Cloning Sit
PstI
XmaI
XbaI
NotI
XmaIII
SacII
SacI
lac promoter
Pvu I (2417)
(bla) ORF
pBSSK-
2958 bp
pUC origin

pBS(sk-)



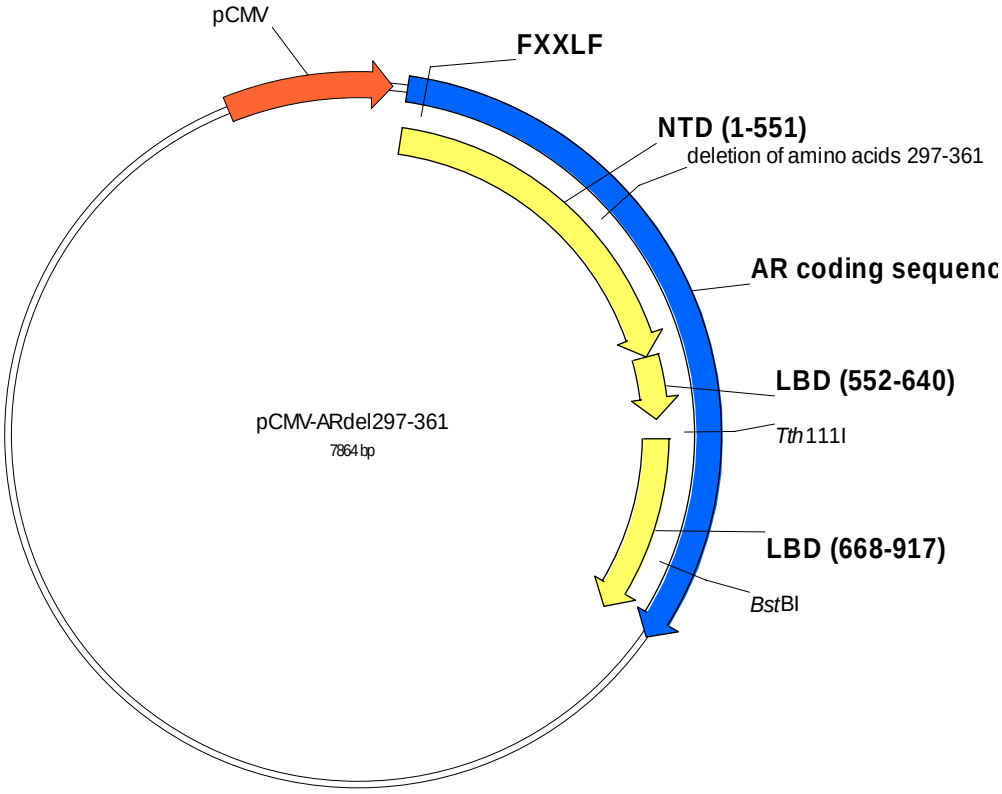
pM-GRIP1

Appendix B2 Plasmids for transactivation assays

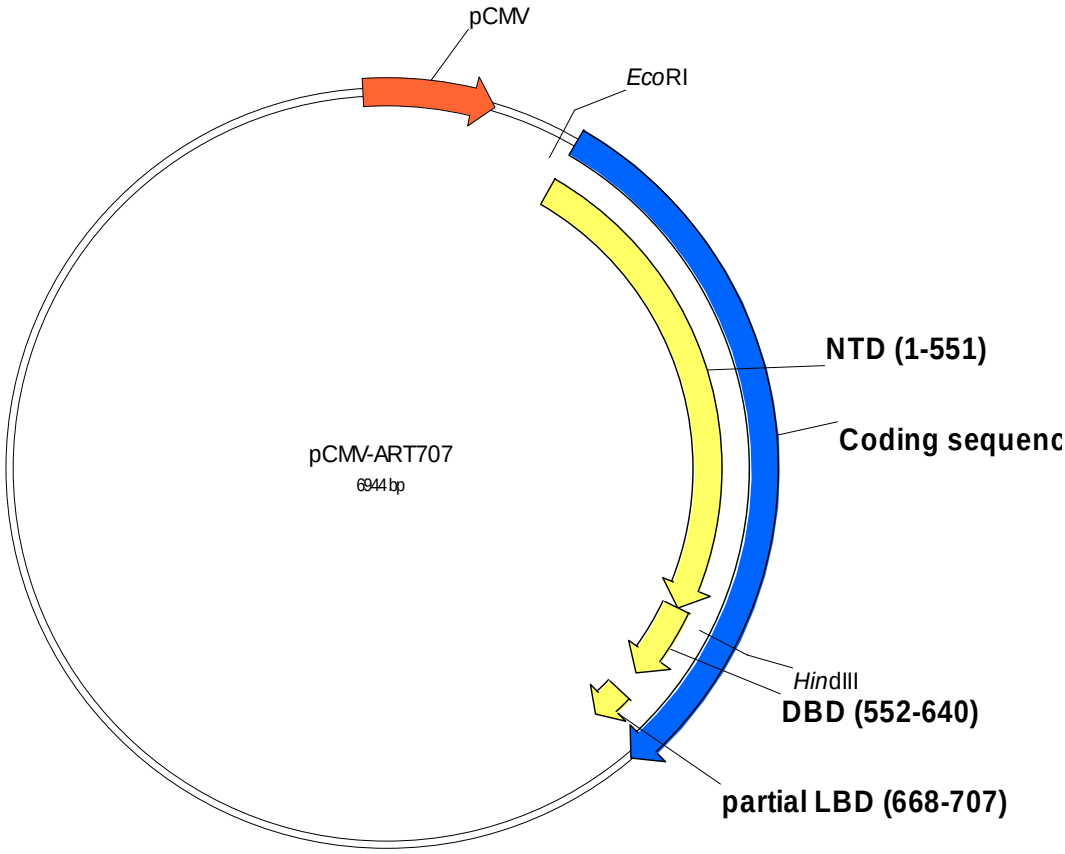


pCMV-AR3.1

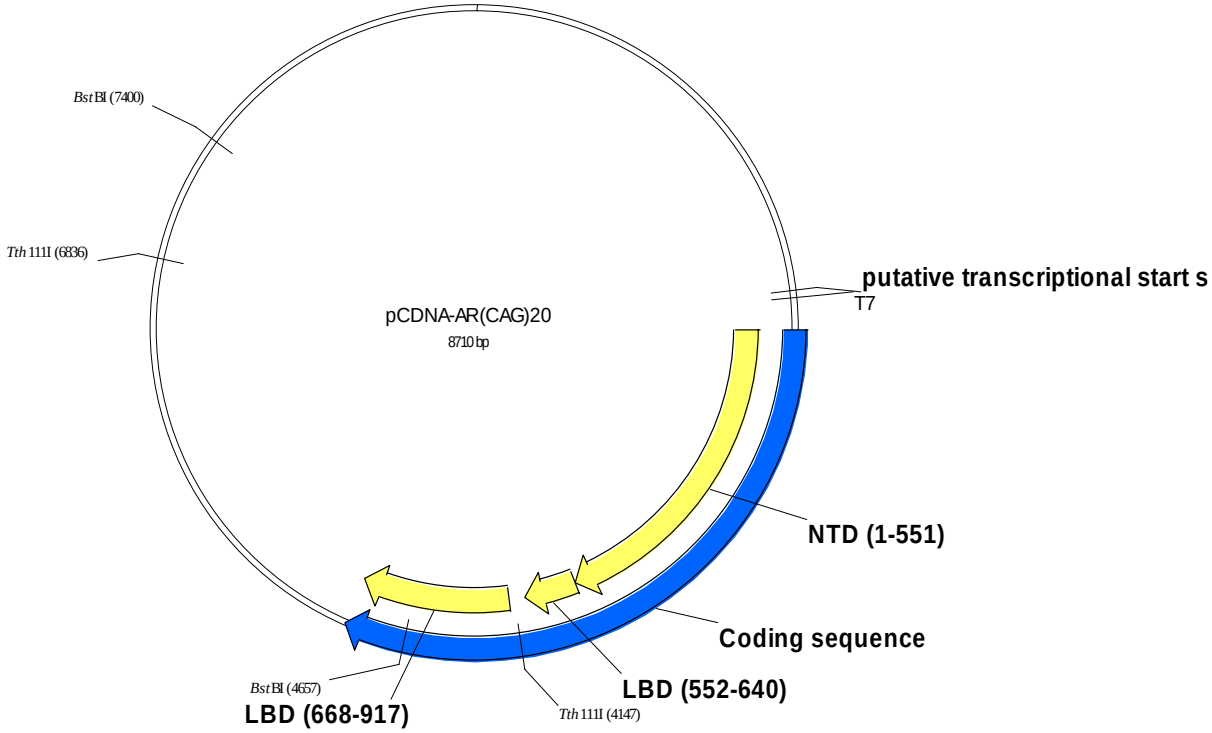
Plus other pCMV-AR3.1 point mutation variants: pCMV-ARP502L, pCMV-ARS513G, pCMV-ARD526G, pCMV-ARM535A, pCMV-ARM535V, pCMV-ARE895Q



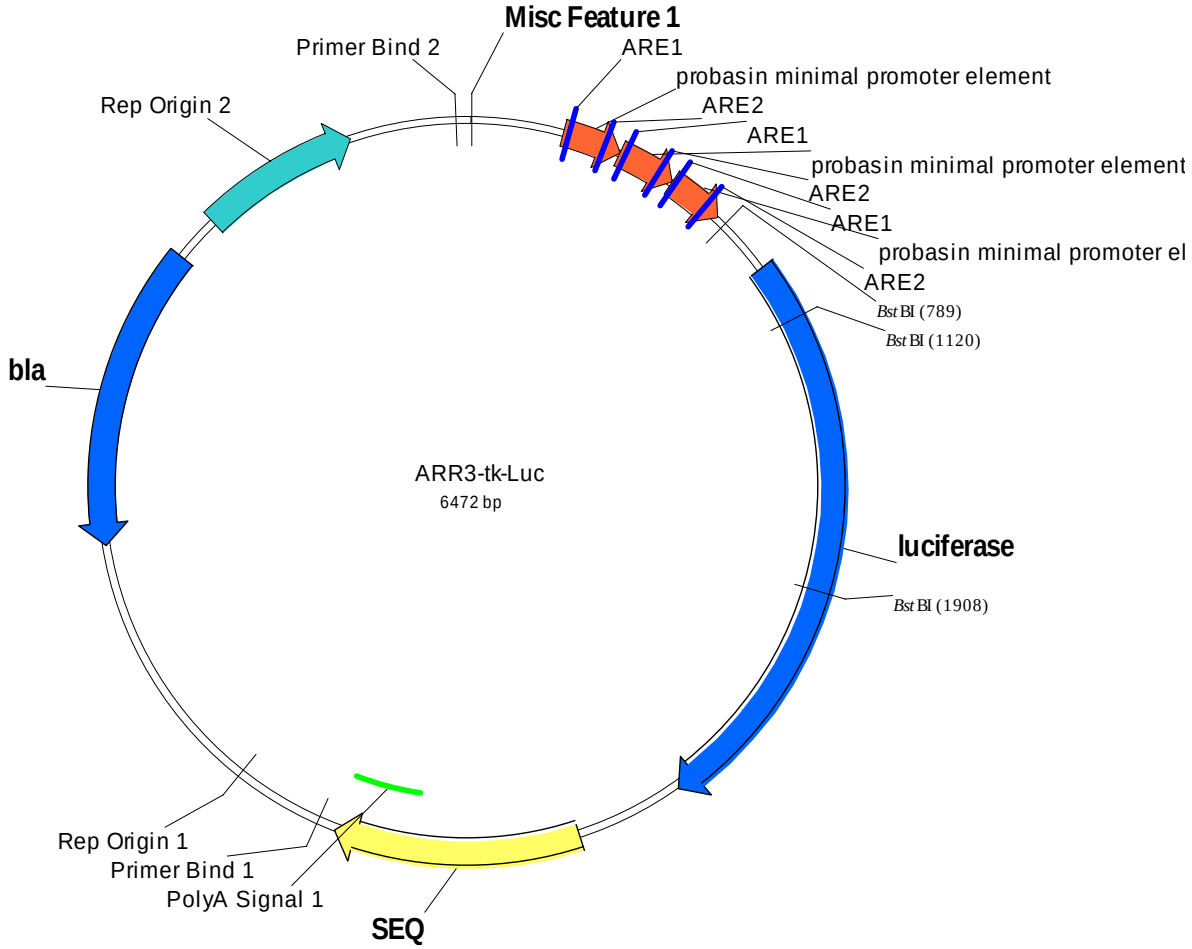
pCMV-ARΔ297-361



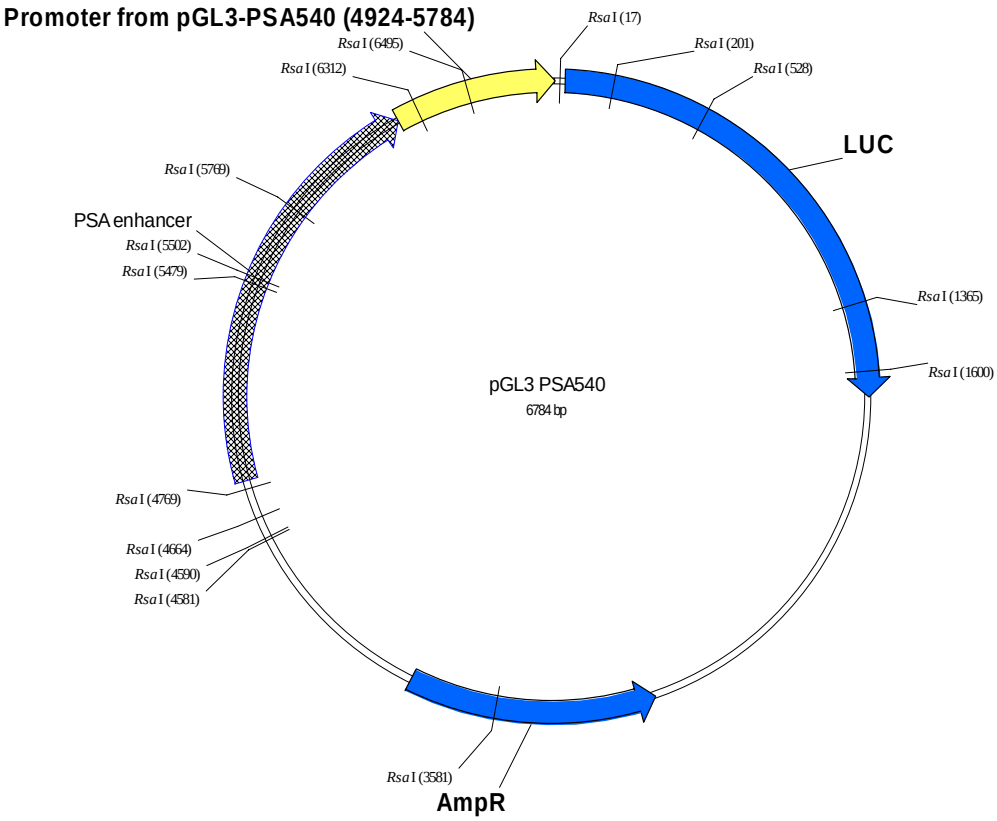
pCMV-ART707



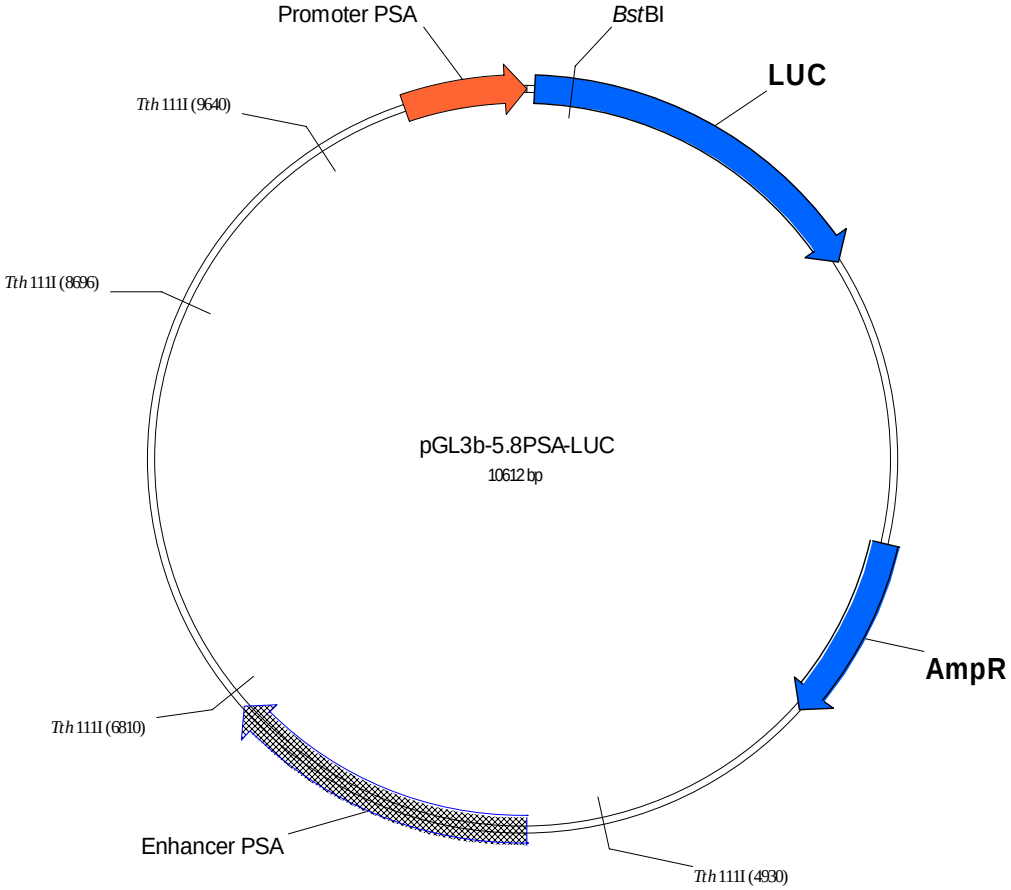
pcDNA-AR(CAG)21



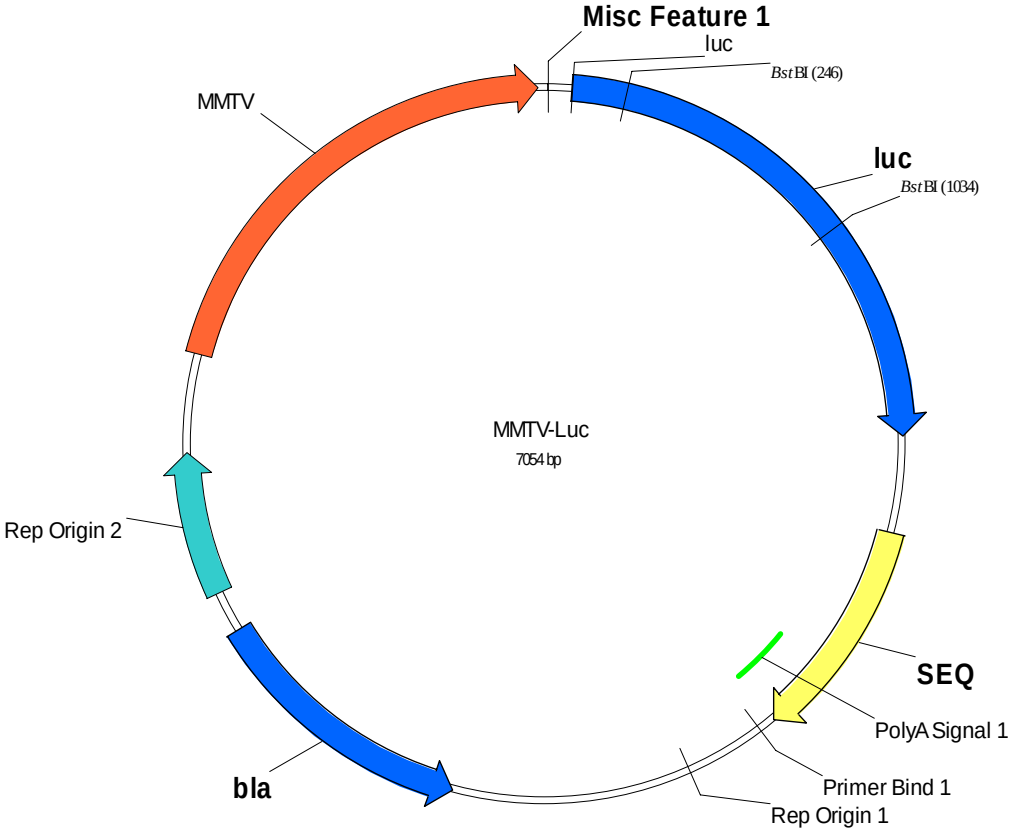
ARR3-tk-Luc



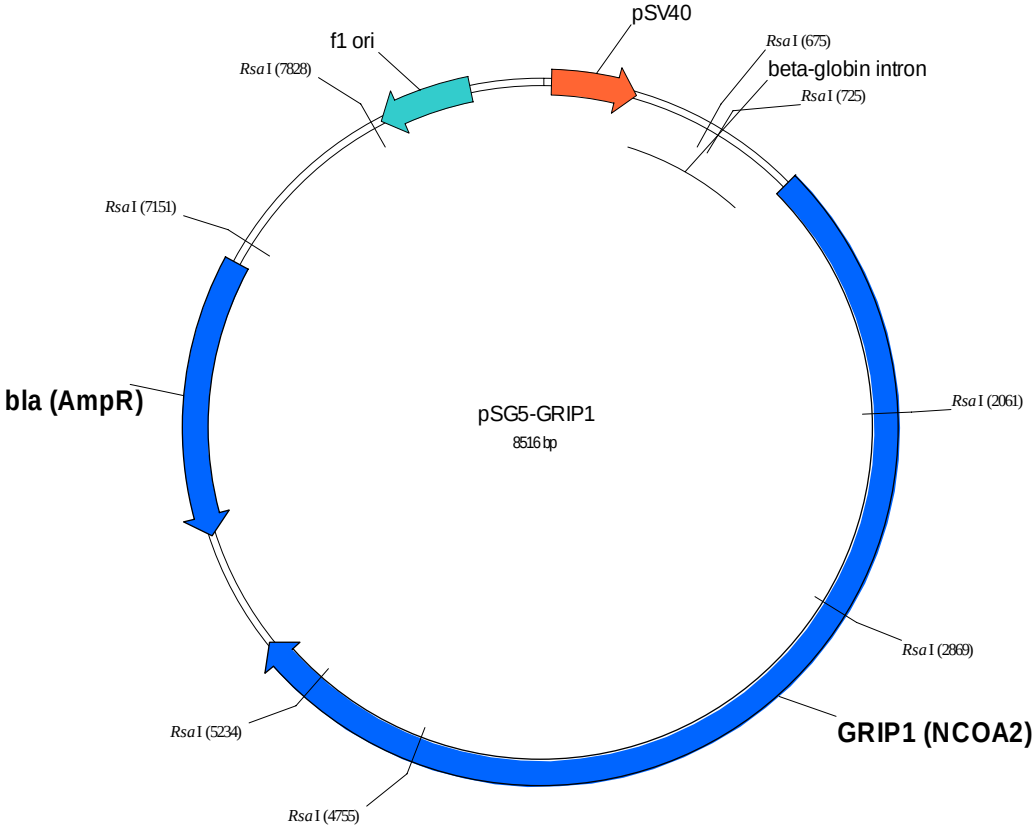
pGL3-PSA540



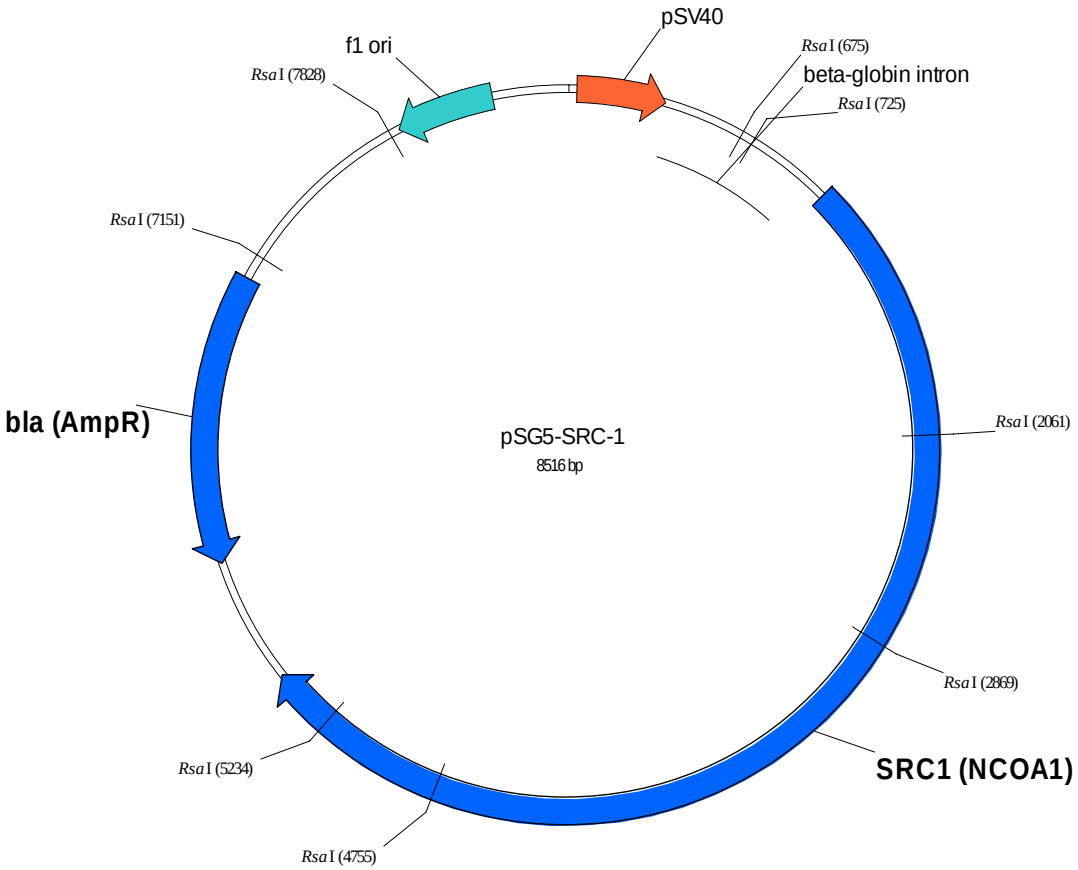
pGL3-5.8PSA



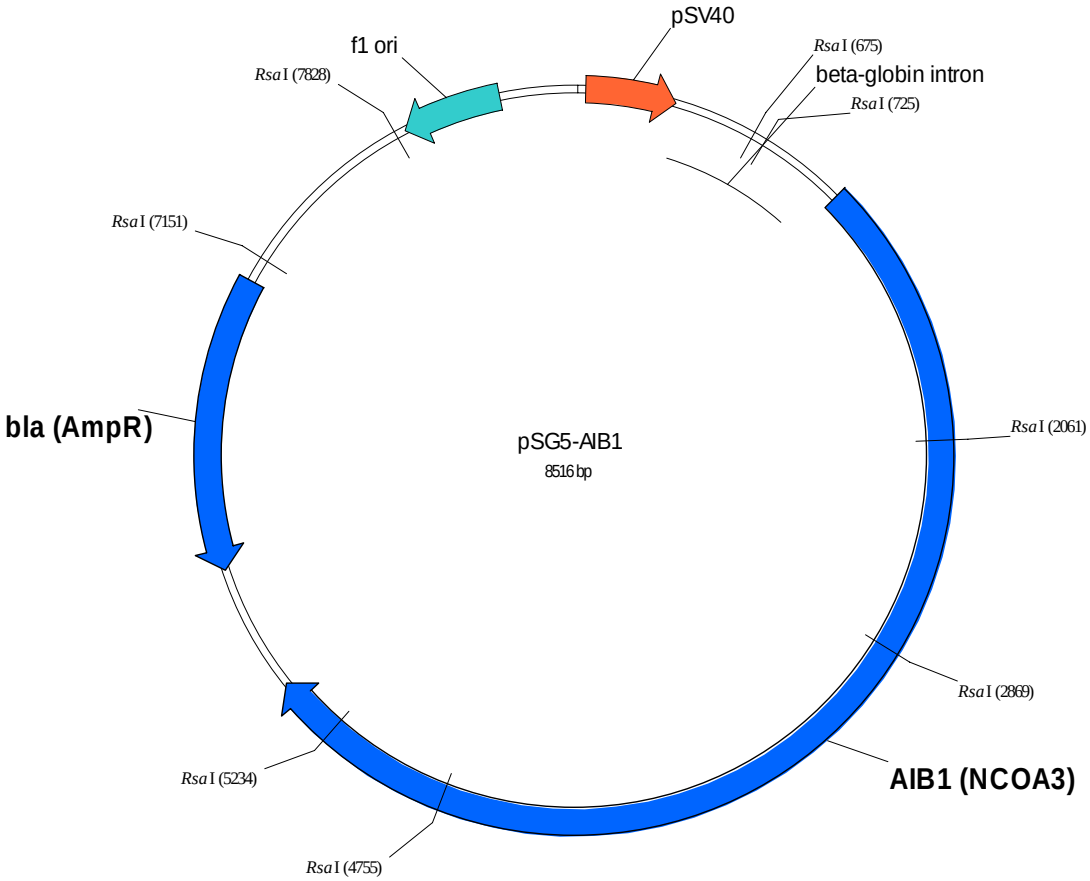
pGL3-MMTV-Luc



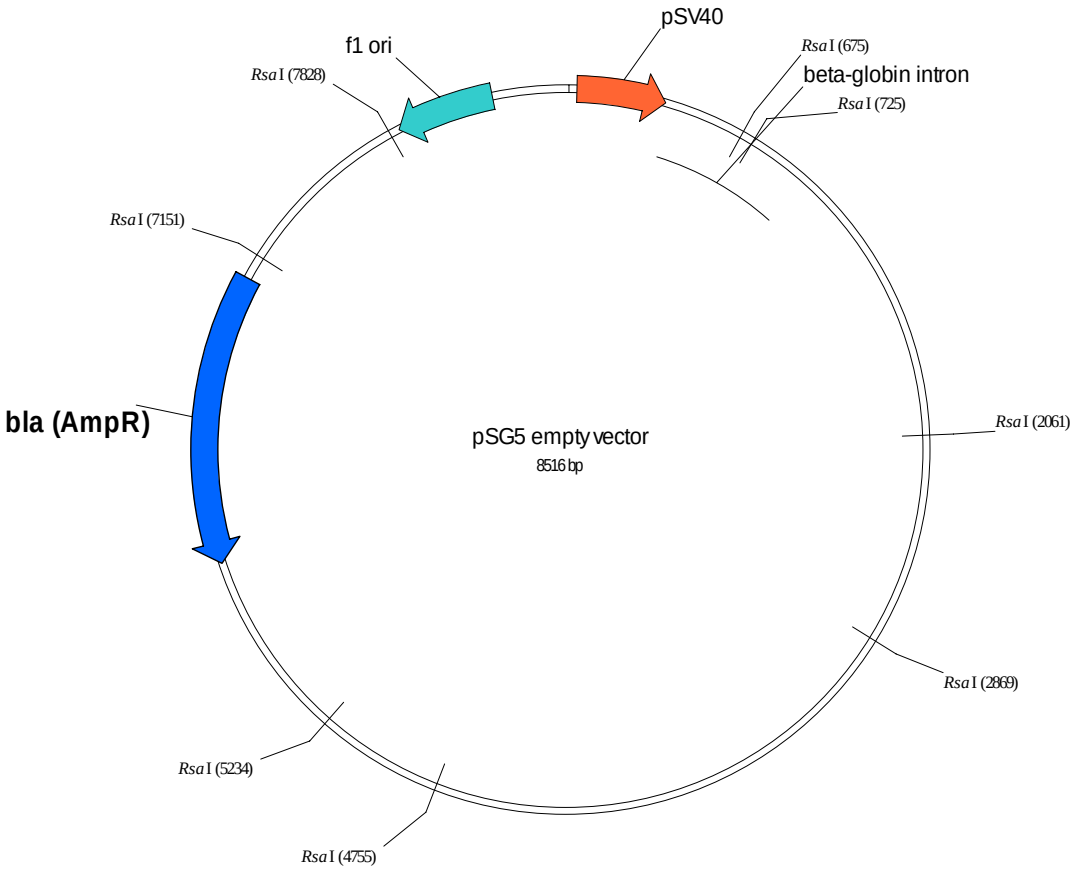
pSG5-GRIP1 (NCOA2)



pSG5-SRC-1 (NCOA1)



pSG5-AIB1



pSG5

Appendix B3: Plasmids constructed for this thesis for mammalian 2-hybrid interaction assays

The following plasmids are variants of the pVP16-AR(1-538) and pM-AR(1-538) plasmids depicted above:

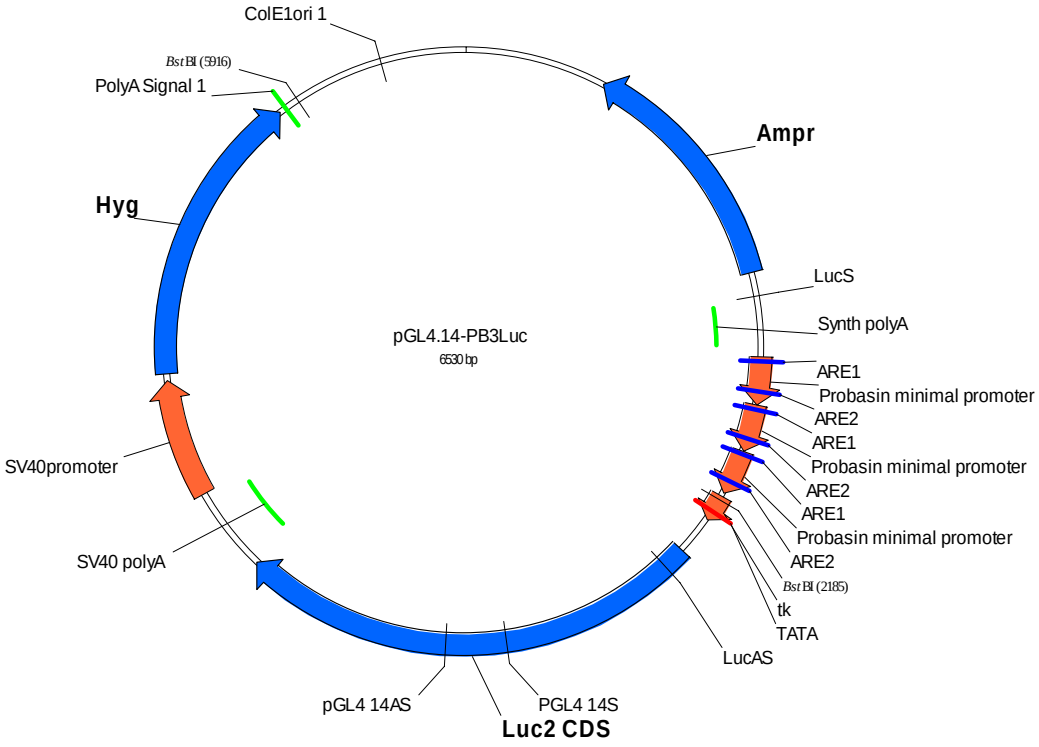
pVP16:AR(1-538)ΔAF1
pM:AR(1-538)ΔAF1
pVP16:AR(1-538)ΔAF5
pM:AR(1-538)ΔAF5
pVP16:AR(1-538)ΔcAF5
pM:AR(1-538)ΔcAF5

Appendix B4: Plasmids constructed for this thesis for transactivation assays

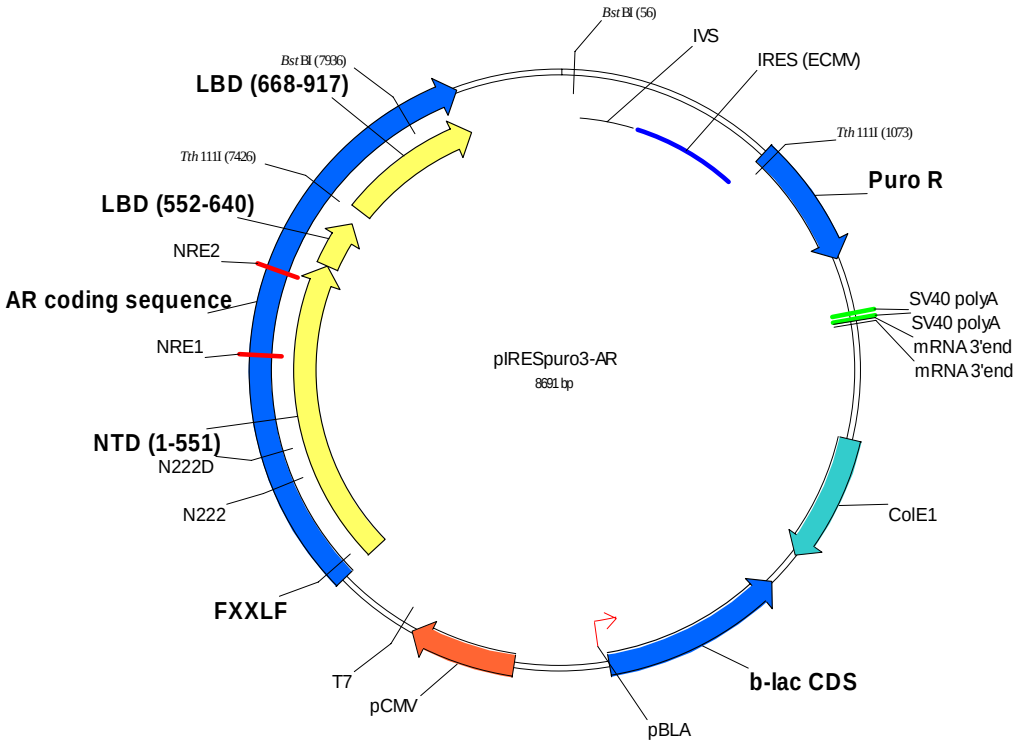
The following plasmids are variants of the pCMV-AR3.1 plasmid depicted above:
pCMV-ARΔAF1
pCMV-ARΔ39-100
pCMV-ARΔ101-160
pCMV-ARΔ161-220
pCMV-ARΔ221-297

pCMV-ARΔAF5
pCMV-ARΔcAF5
pCMV-ARE895QΔAF1
pCMV-ARE895QΔAF5
pCMV-ARE895QΔcAF5

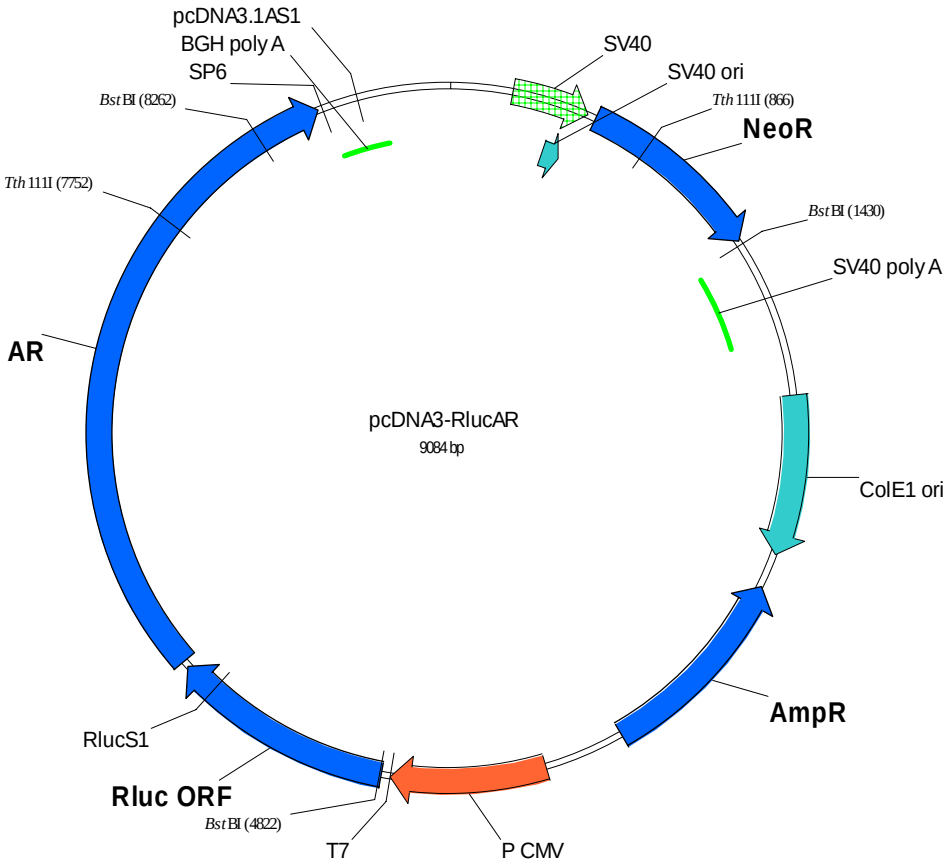
The following plasmids are variants of the pCMV-ART707 plasmid depicted above:
pCMV-ART707ΔAF1
pCMV-ART707ΔAF5
pCMV-ART707ΔcAF5



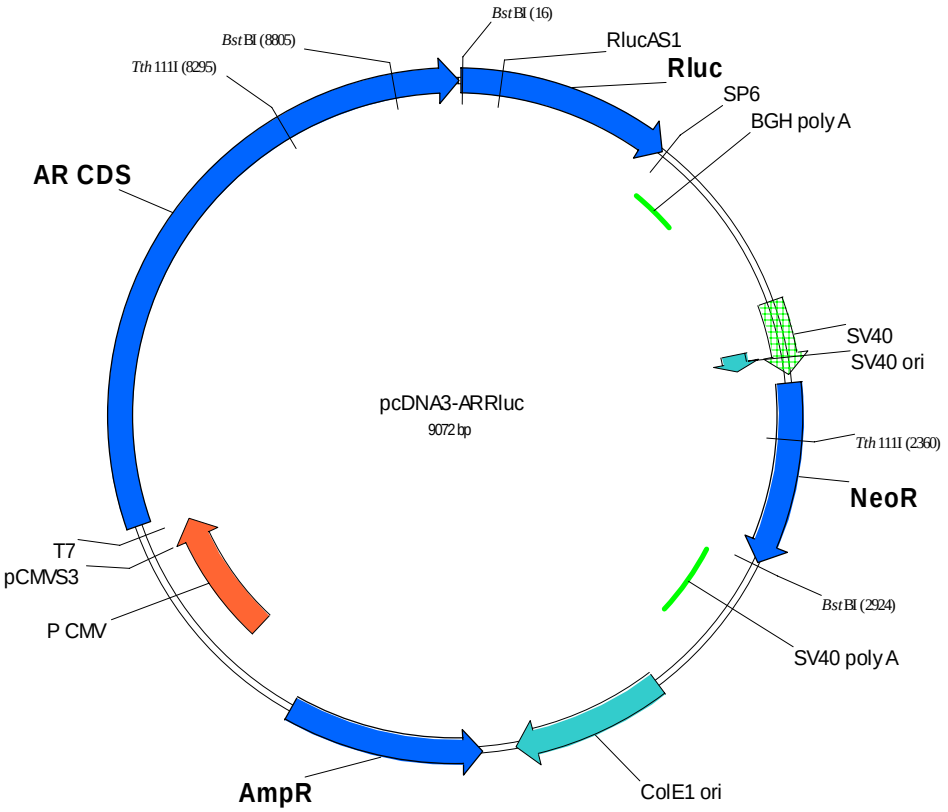
pGL4.14-ARR3-Luc



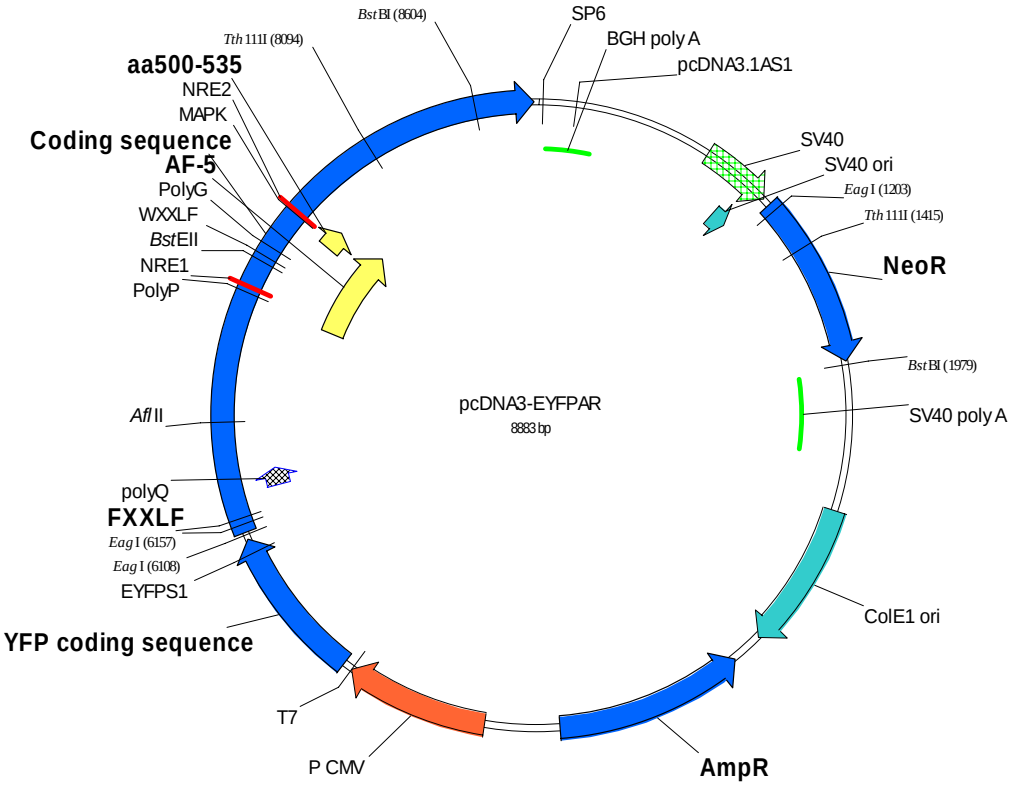
pIRESpuro3-AR



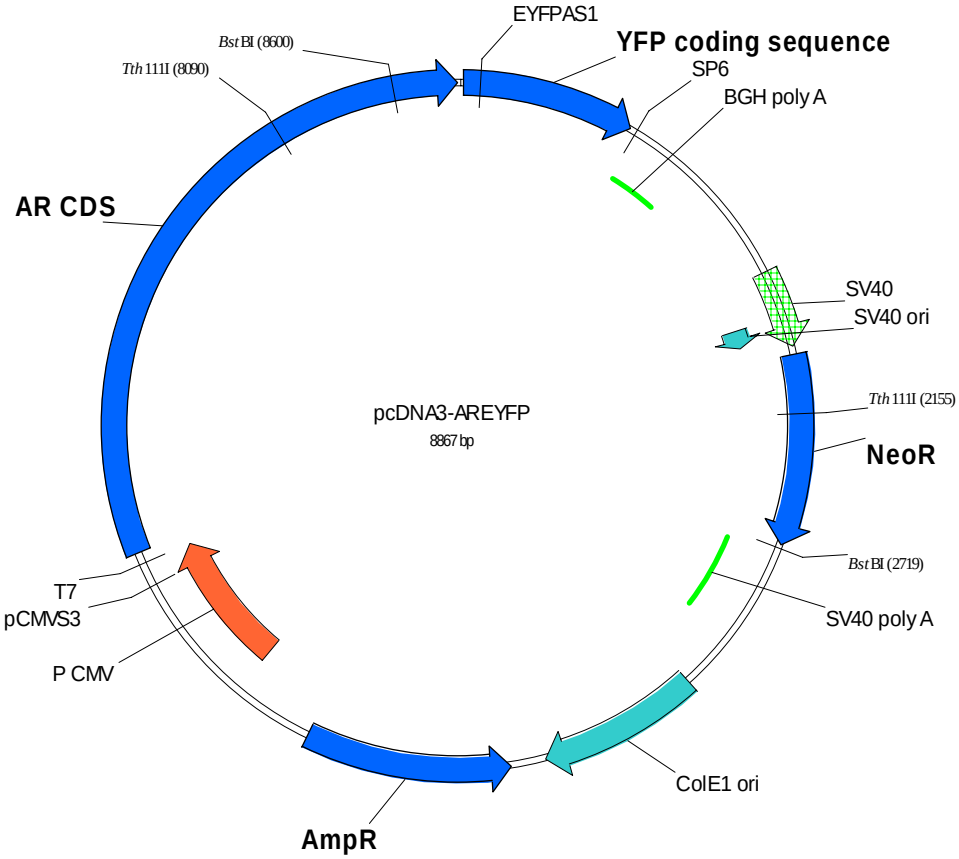
pcDNA3.1-RlucAR



pcDNA3.1ARRluc



pcDNA3.1EYFPAR



pcDNA3.1AREYFP

Appendix C: FAMAS questionnaire B

Florey Adelaide
MALE AGEING STUDY

QUESTIONNAIRE B

Please read the following instructions before answering the questions.

1. Please complete all the questions as per instructions.
   Eg: For questions like this, place a tick in the box that most closely corresponds to your answer.

   ☑ ☐ 0 Not at all

   ☐ 1 some of the time

   For questions like this, circle the number on the scale that most closely corresponds to your answer.

   (No desire) 0 1 2 3 4 5 6 7 8 (Strong desire)

2. Your answers will remain strictly confidential. Results of the study may be published in a medical journal but no information that may lead to the identification of any individual will be released.
3. This questionnaire should take approximately 15 minutes to complete.
4. If you have any problems or questions whilst filling out this questionnaire please direct them to the clinic staff.

SEXUAL HEALTH AND WELL BEING

The first 14 questions ask about your level of sexual desire. By desire, we mean INTEREST IN or WISH FOR sexual activity. For each item, please tick the box that best shows your thoughts and feelings. Please answer each question and be as honest as possible. Your answers will be private.

During the past month, how often would you have liked to engage in sexual activity with a partner (for example, touching each other's genitals, giving or receiving oral stimulation, intercourse etc.)?

- ❑ 0 Not at all
- ❑ 1 Once a month
- ❑ 2 Once every 2 weeks
- ❑ 3 Once a week
- ❑ 4 Twice a week
- ❑ 5 3 to 4 times a week
- ❑ 6 Once a day
- ❑ 7 More than once a day

During the past month, how often have you had sexual thoughts involving a partner?

- ❑ 0 Not at all
- ❑ 1 Once or twice a month
- ❑ 2 Once a week
- ❑ 3 Twice a week
- ❑ 4 3 to 4 times a week
- ❑ 5 Once a day
- ❑ 6 A couple of times a day
- ❑ 7 Many times a day

The next 7 questions ask you to indicate your level of sexual desire on a scale of 0 to 8, with 0 being "no desire" and 8 being "strong desire". Please *circle* ***the number that best corresponds to your level of desire.***

When you have sexual thoughts, how strong is your desire to engage in sexual behaviour with a partner?

(No desire) 0 1 2 3 4 5 6 7 8 (Strong desire)

When you first see an attractive person, how strong is your sexual desire?

(No desire) 0 1 2 3 4 5 6 7 8 (Strong desire)

When you spend time with an attractive person (for example at work or school), how strong is your sexual desire?

(No desire) 0 1 2 3 4 5 6 7 8 (Strong desire)

When you are in romantic situations (such as candle lit dinner, a walk on the beach, etc.) how strong is your sexual desire?

(No desire) 0 1 2 3 4 5 6 7 8 (Strong desire)

How strong is your desire to engage in sexual activity with a partner?

(No desire) 0 1 2 3 4 5 6 7 8 (Strong desire)

How important is it for you to fulfil your sexual desire through activity with a partner?

0 1 2 3 4 5 6 7 8

(Not at all important) (Extremely important)

Compared to other people of your age and sex, how would you rate your desire to behave sexually with a partner?

0 1 2 3 4 5 6 7 8

(Much less desire) (Much more desire)

The next 4 questions ask about your level of desire to behave sexually by yourself.

During the last month, how often would you have liked to behave sexually by yourself (for example, masturbating, touching your genitals etc.)?

- ❑ 0 Not at all
- ❑ 1 Once a month
- ❑ 2 Once every 2 weeks
- ❑ 3 Once a week
- ❑ 4 Twice a week
- ❑ 5 3 to 4 times a week
- ❑ 6 Once a day
- ❑ 7 More than once a day

How strong is your desire to engage in sexual behaviour by yourself?

(No desire) 0 1 2 3 4 5 6 7 8 (Strong desire)

How important is it for you to fulfil your desires to behave sexually by yourself?

0 1 2 3 4 5 6 7 8

(Not at all important) (Extremely important)

Compared to other people of your age and sex, how would you rate your desire to behave sexually by yourself?

0 1 2 3 4 5 6 7 8

(Much less desire) (Much more desire)

How long could you go comfortably without having sexual activity of some kind?

- ❑ 0 Forever
- ❑ 1 A year or two
- ❑ 2 Several months
- ❑ 3 A month
- ❑ 4 A few weeks
- ❑ 5 A week
- ❑ 6 A few days

- ❑ 7 One day
- ❑ 8 Less than one day

The following questions ask about your ability to get and maintain erections.

Impotence means being unable to get and keep an erection that is rigid enough for satisfactory sexual activity. How would you describe yourself? *(tick one box only)*

- ❑ 0 Always able to get an keep an erection good enough for sexual intercourse
- ❑ 1 Usually able to get and keep an erection good enough for sexual intercourse
- ❑ 2 Sometimes able to get and keep an erection good enough for sexual intercourse
- ❑ 3 Never able to get and keep an erection good enough for sexual intercourse

In answering the remaining questions, the following definitions apply:

***Sexual Intercourse** is defined as vaginal penetration (entry) of the partner
****Sexual Activity** includes intercourse, caressing, foreplay and masturbation
*****Ejaculate** is the ejection of semen from the penis (or the sensation of this)
******Sexual Stimulation** includes situations such as love-play with a partner, looking at erotic pictures, etc.

Over the past 4 weeks, how often were you able to get an erection during sexual activity**?

- ❑ 0 No sexual activity
- ❑ 5 Almost always or always
- ❑ 4 Most times (much more than half the time)
- ❑ 3 Sometimes (about half the time)
- ❑ 2 A few times (much less than half the time)
- ❑ 1 Almost never or never

Over the past 4 weeks, when you had erections with sexual stimulation****, how often were your erections hard enough for penetration?

- ❑ 0 No sexual stimulation
- ❑ 5 Almost always or always
- ❑ 4 Most times (much more than half the time)
- ❑ 3 Sometimes (about half the time)
- ❑ 2 A few times (much less than half the time)
- ❑ 1 Almost never or never

The next 3 questions ask about the erections you may have had during sexual intercourse*

Over the past 4 weeks, when you attempted sexual intercourse*, how often were you able to penetrate (enter) your partner?

- ❑ 0 Did not attempt intercourse
- ❑ 5 Almost always or always
- ❑ 4 Most times (much more than half the time)
- ❑ 3 Sometimes (about half the time)
- ❑ 2 A few times (much less than half the time)
- ❑ 1 Almost never or never

Over the past 4 weeks, during sexual intercourse*, how often were you able to maintain your erection after you had penetrated (entered) your partner?

- ❑ 0 Did not attempt intercourse
- ❑ 5 Almost always or always
- ❑ 4 Most times (much more than half the time)
- ❑ 3 Sometimes (about half the time)
- ❑ 2 A few times (much less than half the time)
- ❑ 1 Almost never or never

Over the past 4 weeks, during sexual intercourse*, how difficult was it to maintain your erection to completion of intercourse?

- ❑ 0 Did not attempt intercourse
- ❑ 5 Extremely difficult
- ❑ 4 Very difficult
- ❑ 3 Difficult
- ❑ 2 Slightly difficult
- ❑ 1 Not difficult

Over the past 4 weeks, how many times have you attempted sexual intercourse*?

- ❑ 0 No attempts
- ❑ 1 1-2 attempts
- ❑ 2 3-4 attempts
- ❑ 3 5-6 attempts
- ❑ 4 7-10 attempts
- ❑ 5 11+ attempts

Over the past 4 weeks, when you attempted sexual intercourse*, how often was it satisfactory for you?

- ❑ 0 Did not attempt intercourse
- ❑ 5 Almost always or always
- ❑ 4 Most times (much more than half the time)
- ❑ 3 Sometimes (about half the time)
- ❑ 2 A few times (much less than half the time)

❑ 1 Almost never or never

Over the past 4 weeks, how much have you enjoyed sexual intercourse*?

❑ 0 No intercourse
❑ 5 Very highly enjoyable
❑ 4 Highly enjoyable
❑ 3 Fairly enjoyable
❑ 2 Not very enjoyable
❑ 1 Not enjoyable

Over the past 4 weeks, when you had sexual stimulation**** or intercourse*, how often did you have the feeling of orgasm with or without ejaculation***?

❑ 0 No sexual stimulation or intercourse
❑ 5 Almost always or always
❑ 4 Most times (much more than half the time)
❑ 3 Sometimes (about half the time)
❑ 2 A few times (much less than half the time)
❑ 1 Almost never or never

The next 2 questions ask about sexual desire. Sexual desire is defined as a feeling that may include wanting to have a sexual experience (eg, masturbation or intercourse*), thinking about sex, or feeling frustrated due to lack of sex..

Over the past 4 weeks, how often have you felt sexual desire?

❑ 5 Almost always or always
❑ 4 Most times (much more than half the time)
❑ 3 Sometimes (about half the time)
❑ 2 A few times (much less than half the time)
❑ 1 Almost never or never

Over the past 4 weeks, how would you rate your level of sexual desire?

❑ 5 Very high
❑ 4 High
❑ 3 Moderate
❑ 2 Low
❑ 1 Very low or none at all

Over the past 4 weeks, how satisfied have you been with your overall sex life?

- ❑ 5 Very satisfied
- ❑ 4 Moderately satisfied
- ❑ 3 About equally satisfied and dissatisfied
- ❑ 2 Moderately dissatisfied
- ❑ 1 Very dissatisfied

Over the past 4 weeks, how satisfied have you been with your sexual relationship with your partner?

- ❑ 5 Very satisfied
- ❑ 4 Moderately satisfied
- ❑ 3 About equally satisfied and dissatisfied
- ❑ 2 Moderately dissatisfied
- ❑ 1 Very dissatisfied

Over the past 4 weeks, how would you rate your confidence that you could get and keep an erection?

- ❑ 5 Very high
- ❑ 4 High
- ❑ 3 Moderate
- ❑ 2 Low
- ❑ 1 Very low

Could you please answer the following question?

Would you describe your sexuality as..?

- ❑ 1 Exclusively heterosexual
- ❑ 2 Bisexual
- ❑ 3 Exclusively homosexual
- ❑ 4 Rather not say

What is your date of birth?

_ _ _ _ _ _ _ _
d d m m y y y y

That concludes the survey.
Thank you very much for your time.

Please make sure that you have answered all the questions.

If you have any problems or questions about completing this questionnaire, please ask the clinic staff.

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