Table of Contents

1. Kit Contents

The PolarScreen™ Androgen Receptor Competitor Assay, Red, Catalog no. PV4293, contains the following:

Component	Composition	Amount	Storage Temp.	Individual Catalog no.
Fluormone™ AL Red	200 nM in 50% methanol/water	200 µl	-20 °C	PV4294
AR-LBD	Rat AR ligand-binding domain in a buffer (pH 7.5) containing protein stabilizing agents and glycerol	425 pmol	-80 °C	P3009
AR Red Screening Buffer	Proprietary buffer (pH 7.5)	20 ml	4 °C	PV4295
DTT, 1 M	in water	1 ml	-20 °C or -80 °C	P2325

2. Materials Required but Not Supplied

The following materials are required but not supplied in the kit:

- Fluorescence polarization instrument with suitable excitation and emission capabilities (see **Instrument Settings**, page 5).
- Pipetting devices for 5–1000 µl volumes, suitable repeater pipetters, or multi-channel pipetters.

- Black, 384-well assay plates. This assay has been optimized for a 40-µl volume in black polypropylene plates. Other plate types may give satisfactory results as well.
- A potent AR ligand, such as testosterone, dihydrotestosterone (DHT), or methyltrienolone (R1881), to serve as a positive control for competition.

3. Introduction

The androgen receptor (AR) is a ligand-dependent transcription factor that mediates the action of androgenic steroid hormones. Invitrogen's PolarScreen™ Androgen Receptor Competitor Assay, Red, is a fluorescence polarization (FP)-based competition assay that provides a sensitive and robust method for high-throughput screening of potential AR ligands. The kit uses the novel, tight-binding fluorescent AR ligand Fluormone™ AL Red and a rat AR ligand-binding domain (AR-LBD) that is tagged with histidine and glutathione-S-transferase (GST) in a homogenous mix-and-read assay format. This kit contains enough reagents for 400 40-µl assays.

3.1. Biology of the Androgen Receptor

Ligand-dependent activation of cytosolic, chaperone-bound androgen receptor results in its translocation to the nucleus and conformational changes in the receptor leading to dissociation of the chaperone proteins, recruitment of coactivator proteins, and specific interactions with DNA response elements at target genes. The genetic programs regulated by AR involve male differentiation and development, including prostate cell proliferation associated with prostate cancer.

AR agonists are used as therapeutics for sex-specific developmental disorders, while AR antagonists are used to treat prostatic carcinoma.

3.2. Assay Overview

Using the PolarScreen™ Androgen Receptor Competitor Assay, Red, you add AR-LBD to Fluormone™ AL Red (the "tracer") in the presence of a test compound in a microtiter plate, and measure the polarization value of the tracer. The shift in polarization value is used to determine the relative affinity of the test compound for AR-LBD.

If the test compound binds to the receptor, it will prevent the formation of the receptor/tracer complex, and the tracer will be free in solution. When the tracer is free in solution, its rotational mobility is greater than when bound to the receptor, resulting in a low polarization value. If the test compound does not bind to the receptor, it will have no effect on formation of the receptor/tracer complex, and the measured polarization value of the tracer will remain high.

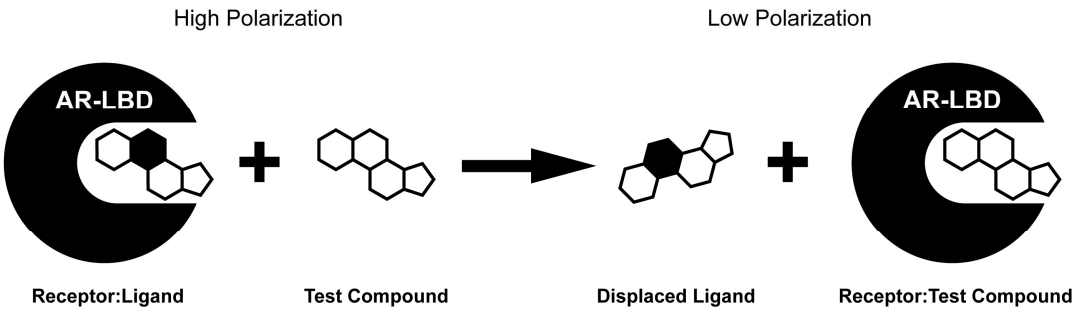

Figure 1. Nuclear Receptor PolarScreen™ Competition Assay Schematic

3.3. Fluorescence Polarization Theory

For detailed information on fluorescence polarization theory and techniques, see the **Fluorescence Polarization Technical Resource Guide** at http://www.invitrogen.com/fpguide.

4. Guidelines and Recommendations

4.1. Receptor/Tracer Binding Affinity

We recommend performing competition experiments using the EC_{80} of the receptor. This concentration has been determined to be approximately 10 nM AR-LBD when using 2 nM Fluormone™ AL Red. Sample receptor/tracer binding data is provided in Figure 2.

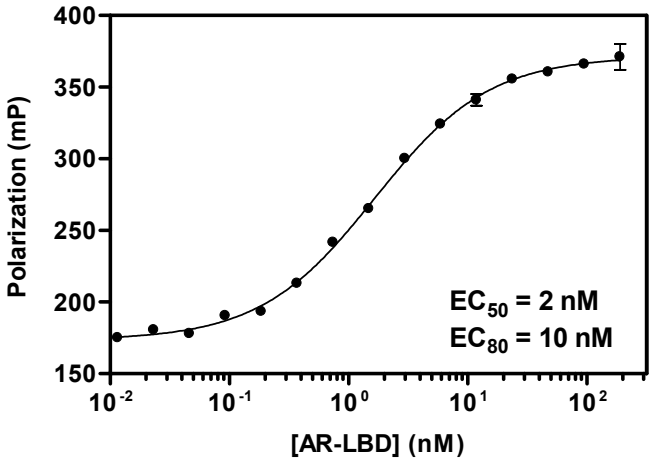

Figure 2. AR-LBD/Fluormone™ AL Red Binding Curve. Data points represent the mean polarization value of 2 nM Fluormone™ AL Red (±1 standard deviation, n=3) at the indicated concentration of AR-LBD.

4.2. Determining the IC_{50}

You can determine the IC_{50} value for a given test compound by sequentially adding Fluormone™ AL Red and AR-LBD at final concentrations of 2 nM and 10 nM, respectively, to a dilution series of the test compound. See **Assay Pharmacology**, page 6, for examples of IC_{50} curves.

Note that many test compounds have low solubility in aqueous solutions. Be careful when preparing serial dilutions of these compounds in aqueous solutions to prevent precipitation or carry-over on plastic pipette tips.

4.3. Reagent Handling

AR-LBD

Store AR-LBD at -80 °C. For best results, thaw on ice for 10 minutes before use. Keep on ice once thawed and perform all dilutions while on ice. In concentrated stock solutions, AR-LBD is unstable at temperatures >4°C. Never vortex the AR-LBD stock or dilutions. Do not expose this reagent to more than 4 freeze-thaw cycles.

Fluormone™ AL Red

Store Fluormone™ AL Red at -20 °C. Thaw on ice for 30 minutes prior to use. This reagent is stable for at least 8 freeze-thaw cycles. Because of the hydrophobic nature of Fluormone™ AL Red, we recommend preparing dilutions in glass rather than plastic containers.

AR Red Screening Buffer

Thaw AR Red Screening Buffer at room temperature upon receipt. Mix well before first use, as the buffer is viscous and may not have thawed evenly. The buffer is stable at room temperature for at least 4 weeks. For long-term storage, keep at 4 °C.

4.4. Solvent Tolerance

Up to 2% DMSO, 2% methanol, 1% ethanol, or 16% glycerol may be present in the assay without a significant reduction in the assay dynamic range (ΔmP). However, we always recommend including a compound's vehicle solvent in each of the

control conditions. Note that while glycerol may not affect the ΔmP, it may affect polarization values. Figure 3 contains sample data illustrating the effect of different solvents on the assay ΔmP.

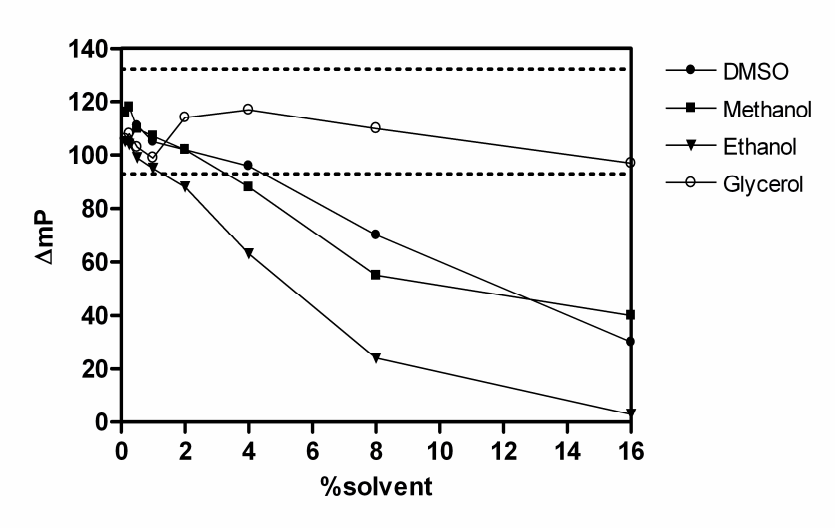

Figure 3. AR Red Assay Solvent Tolerance. Dotted lines represent ±3 standard deviations from the mean ΔmP value in the absence of added solvent. ΔmP is determined by the difference in polarization between the receptor/tracer complex and maximum competition by 10 µM testosterone.

4.5. Note on Reagent Order of Addition

We recommend adding Fluormone™ AL Red and AR-LBD separately to the assay plate, to prevent formation of the receptor/tracer complex prior to introduction of the test compound. Pre-formation of the receptor/tracer complex will increase the incubation time required for the assay to reach equilibrium.

Though not recommended, if you want to add a pre-formed receptor/tracer complex to the assay plate, prepare the complex on ice and dispense it to the plate as soon as possible. The resulting assay may require additional time to come to equilibrium, which must be determined by the user.

4.6. Incubation Conditions

Equilibration Time

The assay has a stable read window of 4–20 hours, where the maximum ΔmP value is stable and excellent Z'-factor values are achieved (see Figure 4). If adequate mixing is not performed at the start of the incubation period, the minimum time required for the assay to reach equilibrium may increase.

Incubation Time (hours)	ΔmP	Z'-Factor
4	114	0.79
6	125	0.83
8	129	0.84
10	128	0.83
12	128	0.84
20	117	0.81

Figure 4. Effect of Incubation Time on Assay Performance. Sample data represents mean values from 3 separate experiments (n=24). ΔmP was determined by the difference in polarization between the receptor/tracer complex and competition by 10 µM testosterone. Z'-factor was calculated using the method of Zhang *et al*. (1999) and is an indication of the robustness of the assay. Values > 0.5 are generally considered good, while a value of 1 indicates a theoretically ideal assay with no variability.

Temperature

Assay performance is sensitive to changes in temperature. We recommend that you perform assays in a temperature-controlled environment (20–22°C). The stability of the assay deteriorates significantly at temperatures >25°C. All sample data presented in this protocol was generated using incubations at 22°C.

4.7. Instrument Settings

The excitation/emission spectra of Fluormone™ AL Red are shown in Figure 5. We recommend using excitation/emission wavelengths of 535 nm/590 nm and excitation/emission bandwidths of 25 nm/20 nm (indicated by the shaded bands in the figure). Other filter combinations may also perform satisfactorily.

We recommend allowing the instrument to automatically determine optimal gain settings based on wells that contain fully bound Fluormone™ AL Red. Gain settings determined by this method may then be fixed for subsequent assays performed in the same manner. This assay was developed using a Tecan Ultra plate reader with the indicated filter sets.

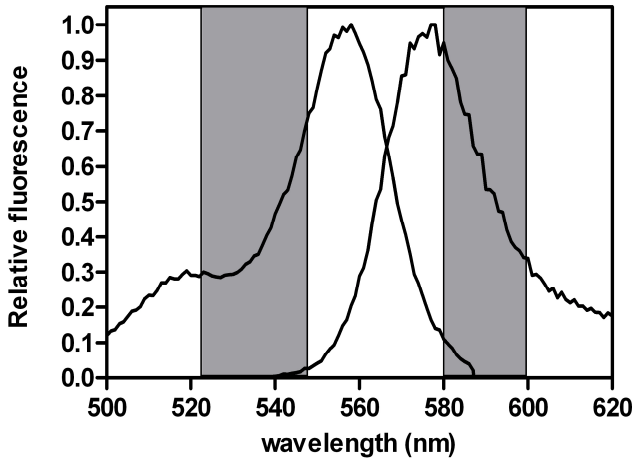

Figure 5. Fluormone™ AL Red Excitation/Emission Spectra.

5. Procedure

5.1. Preparing the Reagents

Before proceeding with the assay, prepare the reagents as described in this section.

Complete AR Red Screening Buffer

For each milliliter of AR Red Screening Buffer, add 2 µl of 1 M DTT and mix thoroughly (2 mM final concentration of DTT). Prepare only enough Complete AR Red Screening Buffer for assays to be performed in one day. Prolonged storage of the buffer with DTT results in oxidation of the DTT and subsequent destabilization of the AR-LBD protein. Complete AR Red Screening Buffer may be kept at room temperature and used for preparation of all reagents except for the 4X AR-LBD, which should be prepared on ice.

2X Test Compound, Solvent Control, and Control Competitor

Dilute test compound to a 2X concentration in Complete AR Red Screening Buffer and mix well. Also prepare a solvent control containing an equivalent amount of the test compound's vehicle solvent in buffer. Include this solvent control as part of the Negative Control. For positive control of competition, prepare a 2X solution of a known AR ligand (we recommend a final 1X concentration of 10 µM testosterone, DHT, or R1881) in buffer. Keep these solutions at room temperature.

4X Fluormone™ AL Red

In a glass container, dilute the 200 nM stock solution of Fluormone™ AL Red to 8 nM in Complete AR Red Screening Buffer. For example, to prepare 1 ml of 4X Fluormone™ AL Red, add 40 µl of the 200 nM stock solution to 960 µl of Complete AR Red Screening Buffer. Vortex well. Keep this solution at room temperature.

4X AR-LBD

On ice, dilute the AR-LBD stock solution to 40 nM in Complete AR Red Screening Buffer. The concentration of the AR-LBD stock is indicated on the tube and its Certificate of Analysis. ***Never vortex the AR-LBD stock or dilutions***. Mix by pipetting or gentle inversion. Keep this solution on ice until needed for dispensing.

5.2. Reagent Volumes

The following table summarizes the reagent amounts and order of addition for each assay condition.

Note: We recommend adding Fluormone™ AL Red and AR-LBD separately to prevent formation of receptor/tracer complex prior to introduction of the test compound/control. See **Note on Reagent Order of Addition** on page 4 for more information.

Assay	Reagent Additions	Purpose
Test Compound	1. 20 µl 2X Test Compound 2. 10 µl 4X Fluormone™ AL Red 3. 10 µl 4X AR-LBD	Assess competition by test compound of interest using a single point or dilution series
Positive Control	1. 20 µl 2X Control Competitor 2. 10 µl 4X Fluormone™ AL Red 3. 10 µl 4X AR-LBD	Represents 100% competition (minimum mP value) by a known, potent androgen receptor ligand. We recommend using 10 µM testosterone, DHT, or R1881 as the Control Competitor.
Negative Control	1. 20 µl 2X Test Compound Solvent Control 2. 10 µl 4X Fluormone™ AL Red 3. 10 µl 4X AR-LBD	Represents 0% competition (maximum mP value) and accounts for possible interference from a compound's vehicle solvent
Buffer Blank	1. 40 µl Complete AR Red Screening Buffer	Serves as a blank for subtraction of background fluorescence

5.3. Performing the Assay

1. In a microtiter plate, pipet the reagents into each well in the order listed in the table above.
2. Mix the plate on a plate shaker for ~30 seconds, taking care not to splash liquid out of the wells.
3. Cover the assay plate to protect the reagents from light and evaporation, and incubate in a temperature-controlled environment at 20–22°C for 4–20 hours (see **Equilibration Time**, page 4).
4. Measure the polarization of each well using a fluorescence polarization plate reader. See page 5 for instrument guidelines. You may want to take multiple readings during incubation to determine whether competition by the test compound of interest has reached binding equilibrium.

6. Assay Pharmacology

Serial dilutions of various test compounds (1% final DMSO concentration) were prepared in 384-well plates. Fluormone™ AL Red and AR-LBD were then added to each sample well. The assay was incubated for six hours at 22°C prior to measuring polarization and calculating IC_{50} values. The resulting data is presented in Figure 6. Error bars represent 1 standard deviation from the mean of n=4 for each data point. Curves were fit using a sigmoidal dose-response equation with varying slope:

$$Y = mP_{100\%} + (mP_{0\%} - mP_{100\%})/[1 + 10^{((LogIC50 - X) \times Hill\ Slope)}]$$

Where: Y = mP, X = Log [inhibitor], $mP_{100\%}$ = 100% competition, and $mP_{0\%}$ = 0% competition

Curve fitting was performed using Prism® software from GraphPad™ Software, Inc.

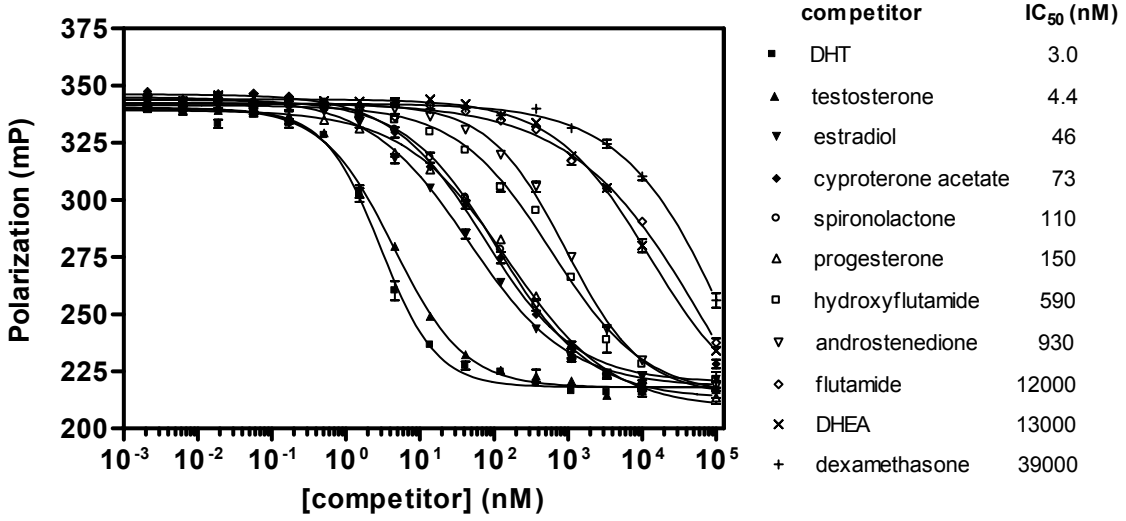

Figure 6. Relative Affinity of Selected Ligands for AR-LBD in the AR Red Assay.

7. References

Zhang, Ji-Hu, Chung, T.D.Y., and Oldenburg, K.R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays, *J. Biomol. Screen 4*, 67–73.