PolarScreen™ Androgen Receptor Competitor Assay, Green Protocol

Part # P3018

839-0308268 052704

TABLE OF CONTENTS

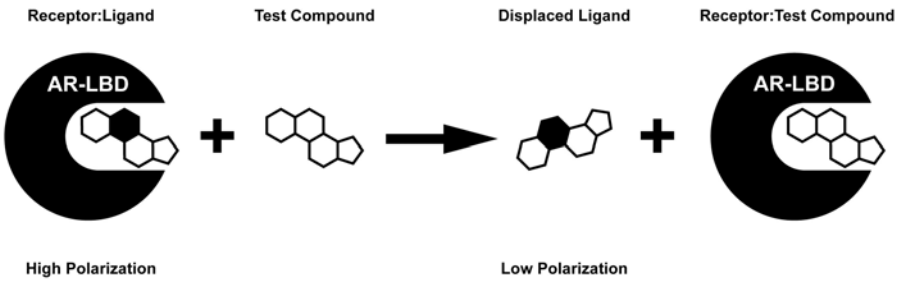
1.0 INTRODUCTION

The androgen receptor (AR) is a ligand-dependent transcription factor that mediates the action of androgenic steroids. Ligand-dependent activation of cytosolic, chaperone-bound AR results in receptor dimerization, translocation to the nucleus, and binding of the receptor to specific DNA sequences. The genetic programs regulated by AR include gametogenesis, male-specific brain function programs, and prostate cell proliferation associated with prostate cancer. New AR ligands are needed to advance therapeutic applications based on the importance of AR in cellular regulation. AR agonists are used as therapeutics for sex-specific developmental disorders, while AR antagonists are applied to prostate cancer ablation treatments.

Invitrogen's PolarScreen™ AR fluorescence polarization (FP) assay provides a sensitive and efficient method for high-throughput screening of potential AR ligands. The kit uses the rat AR ligand-binding domain tagged with His and GST [AR-LBD (His-GST)], and a novel, tight-binding, selective fluorescent androgen ligand (Fluormone™ AL Green) in a homogenous mix-and-read assay format. This kit contains enough reagents for 400 assays in 40 µL volumes.

2.0 ASSAY THEORY

Androgen receptor [AR-LBD (His-GST)] is added to a fluorescent androgen ligand (Fluormone™ AL Green) to form an AR-LBD (His-GST)/Fluormone™ AL Green complex resulting in a high polarization value. This complex is then added to individual test compounds in microwell plates. Competitors displace the fluorescent Fluormone™ AL Green ligand from the AR-LBD (His-GST)/Fluormone™ AL Green complex, causing the fluorescent ligand to tumble rapidly during its fluorescence lifetime, resulting in a low polarization value. Noncompetitors will not displace the fluorescent ligand from the complex, so the polarization value remains high. The shift in polarization value in the presence of test compounds is used to determine relative affinity of test compounds for AR-LBD (His-GST).



If you would like more information on fluorescence polarization theory and techniques, please see our online Fluorescence Polarization Applications Guide at **www.invitrogen.com/fpguide**

If you would like more information about this and other products, please visit **www.invitrogen.com/drugdiscovery**

3.0 DESCRIPTION

3.1 Materials Supplied

Description	Composition	Amount	Cat. no.
Fluormone™ AL Green	200 nM in 20 mM Tris, 90% methanol	100 µL	PV3010
Purified Androgen Receptor Ligand Binding Domain tagged with His and GST [AR-LBD (His-GST)], Recombinant Rat	Buffer (pH 7.5) containing protein stabilizing agents and glycerol	425 pmol	P3009
AR Green Assay Buffer	Buffer (pH 7.5) containing protein stabilizing agents and glycerol	25 mL	P3011
1 M DTT	in water	1 mL	P2325

3.2 Storage and Stability

Description	Storage Temp.	Notes	Cat. no.
Fluormone™ AL Green	–20°C		PV3010
Purified Androgen Receptor Ligand Binding Domain tagged with His and GST [AR-LBD (His-GST)], Recombinant Rat	–80°C	Avoid repeated freeze-thaw cycles. Do not expose this reagent to more than 4 freeze-thaw cycles. The reagent is unstable at room temperature and must remain on ice once thawed.	P3009
AR Green Assay Buffer	Room Temperature		P3011
1 M DTT	–80°C or –20°C		P2325

3.3 Materials Required but Not Supplied

- Fluorescence polarization instrument with suitable 485 nm excitation and 535 nm emission interference filters and a sensitivity of 0.5 nM in polarization mode
- Pipetting devices for 5-1000 µL volumes, suitable repeater pipetters, or multi-channel pipetters
- Black multiwell plates for use in the fluorescence polarization instrument
- FP One-Step Reference Kit (Invitrogen Cat. no. P3088)
- An androgenic steroid, such as dihydrotestosterone or methyltrienolone, is required for the positive control

4.0 ANDROGEN RECEPTOR FP ASSAY CONSIDERATIONS

This kit contains enough reagents to perform 400 assays in 40 µL well volumes. The amount of receptor and Fluormone™ AL Green required per well has been predetermined to bind ~80% of Fluormone™ AL Green. The concentrations determined give the optimal signal range and sensitivity to competitors. These conditions result in a high Z'-factor value of 0.7 (384-well format, n = 144).

4.1 General Assay Design

Controls: As a positive control, an androgenic steroid such as dihydrotestosterone or methyltrienolone (IC_{50} = 25 ± 5 nM) may be included on each plate. In addition, include four control wells that contain one of each of the following: 1X Fluormone™ AL Green, 1X AR-LBD (His-GST), 1X AR-LBD (His-GST)/Fluormone™ AL Green complex, and AR Green Assay Buffer alone.

References: We recommend using Low Polarization Reference (Invitrogen Cat. no. P3089) and High Polarization Reference from (Invitrogen Cat. no. P3090) the FP One-Step Reference Kit (Invitrogen Cat. no. P3088) with suitable 485 nm excitation and 535 nm emission interference filters to determine if the instrument is measuring polarization values accurately.

Temperature: The AR assay is sensitive to changes in temperature. We recommend that assays be conducted in a temperature-controlled environment (20-22°C). The stability of the assay deteriorates significantly at temperatures >25°C, such that a read window of <3 hrs is expected. Note that the mP shift of the assay is also dependent on temperature, with a loss of 10-15 mP observed per °C.

Instrumentation: Fluormone™ AL Green concentrations greater than 1 nM may be required in polarization instruments lacking wavelength-specific dichroic mirrors or sensitivity less than 0.5 nM in polarization mode.

AR Handling: For best results, thaw AR-LBD (His-GST) on ice for 10 minutes before use. **Never** vortex AR-LBD (His-GST). Keep AR-LBD (His-GST) on ice. In concentrated stock solutions, AR-LBD (His-GST) is unstable at temperatures >4°C.

Solvent tolerance: The AR competition assay can tolerate up to 2% DMSO, 2% MeOH or 1% EtOH in the standard protocol.

4.2 Competition Experiments

IC_{50} value calculations: When constructing AR competition curves, AR-LBD (His-GST)/Fluormone™ AL Green complex is added to a dilution series of the test compound. The polarization value is plotted against the concentration of test compound. The concentration of the test compound that results in half of the maximum shift in polarization value equals the IC_{50} of the test compound.

Experimental Design: The fluorescence polarization competition experiments should be designed so that the AR-LBD (His-GST)/K_d ratio is at least 1, yielding a starting polarization value representing at least 50% of the maximal shift (*i.e.*, add enough AR-LBD (His-GST) to bind at least 50% of the Fluormone™ AL Green). The K_d of the Fluormone™ AL Green with AR-LBD (His-GST) equals 20 ± 10 nM. We recommend using 25 nM AR-LBD (His-GST) to achieve ~80% saturation with 1 nM Fluormone™ AL Green.

5.0 PROCEDURE

5.1 Prepare Reagents

5.1.1 Complete AR Green Assay Buffer

Prepare only enough buffer for assays to be performed in one day. Prolonged storage of AR Green Assay Buffer with DTT results in oxidation of the DTT and subsequent destabilization of the AR-LBD (His-GST) protein. For each milliliter of AR Green Assay Buffer (Invitrogen Cat. no. P3011), add 2 µL 1 M DTT (Invitrogen Cat. no. P2325) and mix thoroughly.

5.1.2 2X Test Compound of Interest

Dilute the compound of interest to a 2X concentration in Complete AR Green Assay Buffer (**Section 6.1.1**) into the multiwell plate. The concentration of solvent in this 2X preparation should not exceed 4% DMSO, 4% MeOH or 2% EtOH.

Note: Many steroid compounds have low solubility in aqueous solutions. Care should be taken when preparing serial dilutions of these compounds in aqueous solutions to prevent precipitation or carry-over on plastic tips.

5.1.3 2X AR-LBD (His-GST)/Fluormone™ AL Green Complex

Note: The concentration of AR-LBD (His-GST) (nM) may vary depending on the lot number. This dependence should be taken into consideration when preparing the 2X AR-LBD (His-GST)/Fluormone™ AL Green Complex containing 50 nM AR-LBD and 2 nM Fluormone™ AL Green.

1. Using the concentration (nM) provided on the tube or the Certificate of Analysis for the AR-LBD (His-GST) protein (Invitrogen Cat. no. P3009), calculate the appropriate quantity of the concentrated AR-LBD (His-GST) to make 20 µL (per well) of a 50 nM AR-LBD (His-GST) solution. Dilute AR-LBD (His-GST) and Fluormone™ AL-Green (Invitrogen Cat. no. PV3010) in Complete AR Green Assay Buffer (**Section 5.1.1**) to make a 2X AR-LBD (His-GST)/Fluormone™ AL Green Complex containing a final concentration of 50 nM AR-LBD (His-GST) and 2 nM Fluormone™ AL Green.
2. The 2X AR-LBD (His-GST)/Fluormone™ AL Green Complex can be added directly to the assay wells as described in **Section 5.2**.

Note: The 2X AR-LBD (His-GST)/Fluormone™ AL Green Complex should be kept on ice and dispensed within 30 minutes of preparation. On average, for every additional 30 minutes the 2X AR-LBD (His-GST)/Fluormone™ AL Green complex is incubated on ice, the time to reach equilibrium increases by 2 hours. For applications requiring more than 30 minutes to dispense, the AR-LBD (His-GST) and Fluormone™ AL Green may be dispensed individually. This individual dispensing should be done at a 4X concentration [100 nM AR-LBD (His-GST) and 4 nM Fluormone™ AL Green]. We recommend dispensing the AR-LBD (His-GST) last.

5.2 Standard Protocol

1. Dispense 20 µL 2X Test Compound in the microwell plate.
2. Add 20 µL 2X AR-LBD (His-GST)/Fluormone™ AL Green Complex to the same plate and mix.
3. Cover the AR Green Assay plates to protect the reagents from light. Incubate the AR Green Assay plates at 20-25°C for 4-8 hours. The polarization values vary less than 10% from maximum values if read within this time period.
4. Measure polarization value of each well.

6.0 RESULTS AND DISCUSSION

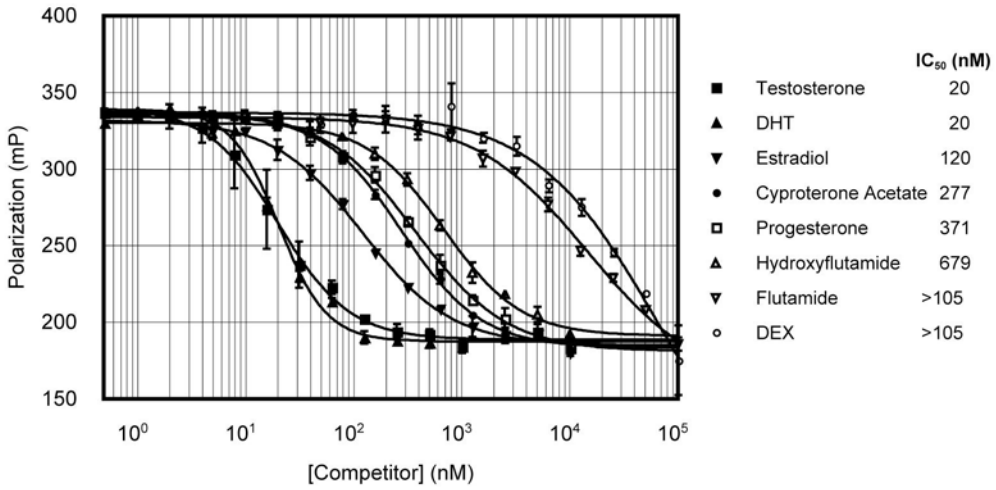
Below is an example of competition data generated on a 384-well plate. The concentration of the test compound that results in a half-maximal shift in polarization value equals the IC_{50} of the test compound, which is a measure of the relative affinity of the test compound for the androgen receptor ligand binding domain. Error bars represent 1 standard deviation from the mean of 3 independent competition curves from assays incubated for 5 hours before reading.

This curve was plotted using the following equation:

$$\mathbf{Y = mP_{100\%} + (mP_{0\%} - mP_{100\%})/1 + 10^{((LogIC50 - X) \times Hillslope)}}$$

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

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



PURCHASER SHALL USE THE PRODUCTS PURCHASED HEREUNDER (the "Products") SOLELY FOR THE PURPOSE OF CONDUCTING INTERNAL RESEARCH AT ITS ORGANIZATION ("RESEARCH"). Purchaser will not sell, transfer, disclose or otherwise provide access to the Products to any third party. Purchaser agrees that Purchaser shall not have the right to authorize any third party to use or sell any Products or derivatives thereof. Unless a license has been executed between Purchaser and Invitrogen Corporation explicitly providing otherwise, Purchaser will not: (i) reformulate or create derivatives or the Products; or (ii) use the Products for providing services to a third party (e.g. screening or profiling); (iii) use the Products in a diagnostic process; (iv) use the Products in a quality control or quality assurance process for the manufacture of a product for sale; or (v) use the Products as a component of a kit.