RAPID AND QUANTITATIVE DETECTION OF AH RECEPTOR IN HUMAN BLOOD CELLS USING THE AHRC PCR™ KIT

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Introduction

The immune system is a sensitive target for the toxic effects of dioxin exposure. The activation of the aryl hydrocarbon receptor (AhR) by 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD) has been shown to suppress T cell and B cell-mediated immune responses [1,2]. In addition, activated AhR has been reported in unexposed populations of lymphocytes, monocytes, and macrophages suggesting an important role for the receptor in the physiological processes of these cells [3,4].

The AhR is a ligand-activated transcription factor that is a member of the basic helix-loop-helix-PAS family of proteins. The AhR and its binding partner, the aryl hydrocarbon receptor nuclear translocator (ARNT), are members of the PAS family of proteins, named for Per, ARNT, and Sim proteins, and share homology in this domain [5,6].

The inactive, unliganded AhR is complexed in the cytoplasm with heat shock protein 90 and an immunophilin-like protein termed XAP2, AIM, or Ara9, depending on the discovering laboratory and host species. Upon binding ligand, the activated AhR dissociates from the complex and binds ARNT in the nucleus. The transformed AhR/ARNT complex is a heteromer that is then able to bind a DNA response element specific for dioxins, i.e., the dioxin-responsive element ("DRE"). Binding of the Ah receptor to the DRE initiates a cascade of biochemical effects leading to toxicological consequences [2].

The AhRC PCR kit was designed to provide an economical, sensitive and consistent method to quantitatively measure dioxins in environmental or food samples. The AhR mediates most, if not all, of the harmful effects associated with exposure to these compounds. How tightly or loosely these compounds bind to the AhR is one of the determining factors of their toxicity. The advantages of the AhRC PCR assay result, in part, from the high degree of specificity that is required for the activated receptor to bind the DRE and the unmatched sensitivity of polymerase chain reaction (PCR). Once bound by the receptor in this selective manner, the DRE is readily amplified and detected using real-time PCR. In this study, we examine the feasibility of using the AhRC PCR assay to detect activated AhR in human blood cells.

Methods and Materials

The human monocyte cell-line U937 (ATCC Catalog No. CRL-2367) was maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies) at 37°C in a humidified atmosphere of 5% CO_2.

The AhRC PCR kit was provided by Hybrizyme Corporation (Raleigh, NC, USA, www.hybrizyme.com) and contained reagents for preparing cellular extracts and for determining

the amount of activated receptor present. Each kit contained the following: Capture Strips, Capture Reagent, Assay Buffer, Wash Buffer, Extraction Reagent, DRE-probe, Mutant-probe, Competitor-probe, Glass Vials, and PCR Reagent.

Cell extract was prepared from U937 cells grown in suspension cultures. Cells were collected from culture media by centrifugation at 1,000 x g for 5 min. The cells were washed in phosphate-buffered saline and the cell pellet resuspended in 2 volumes of extraction reagent. Subsequent steps were performed at 4°C. The cells where disrupted using a Branson Model 450 Sonifier with an attached microtip set for 10 seconds at maximum output and a 40% duty cycle. The supernatant was cleared at 100,000 x g for 60 minutes and stored at –80°C. Liver cytosol from Hartley guinea pigs was prepared essentially as described substituting Hybrizyme extraction reagent for HEDG buffer [7].

The AhRC PCR assay was performed as described in the Users Guide. Briefly, 100 µL of cell extract was placed in a glass vial and DRE-probe added. The vials were incubated at room temperature for 60 minutes. The extract (50 µL) from each vial was transferred to a microwell of a capture strip and incubated for an additional 30 minutes at room temperature. The capture strip was washed using a Biotech ELx50 Strip Washer. PCR reagent was added to each well and results determined using an ABI Prism 7000 real-time thermocycler (Figure 1).

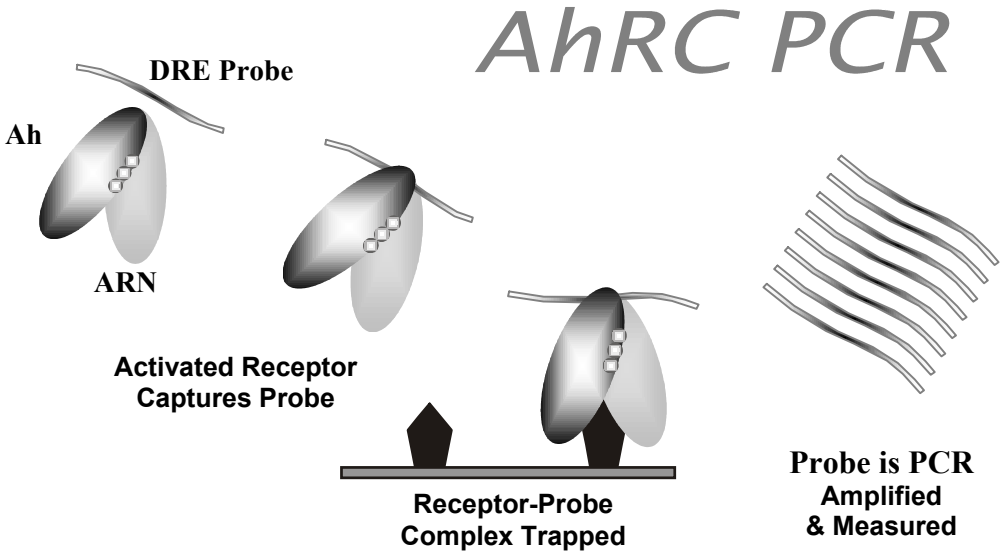

Figure 1. Schematic of AhRC PCR Assay.
Following the addition of DRE-probe to the cellular extract, the activated receptor complex composed of AhR and ARNT binds to the DRE sequence of the probe. The DRE-probe is designed to include both a DRE consensus sequence for AhR/ARNT binding and a primer recognition sequence for PCR amplification. The complex is trapped onto a microwell and the cellular material and unbound DRE-probe is washed away. The receptor-bound DRE-probe is then amplified and measured by PCR.

Results and Discussions

A calibration curve was generated from a four-fold serial dilution of the DRE-probe ranging from 150 million to 36 copies per well. Less than 100 copies of DRE-probe could be quantitatively detected using this technology. The Ct value correlates with the amount of input DNA (DRE-robe) present (Figure 2).

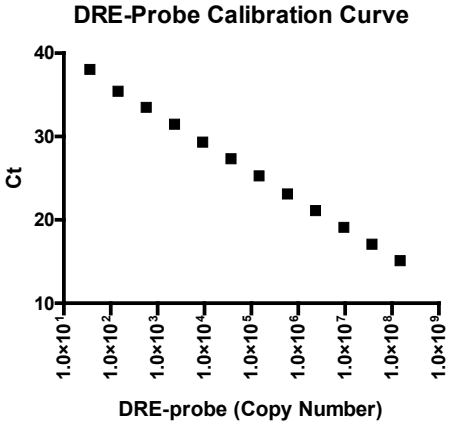

Figure 2. DRE-probe Calibration Curve. Newly synthesized DNA is quantified using TaqMan® fluorescence chemistry. In real-time PCR, the quantification occurs at each cycle, yielding a primary growth curve (fluorescence vs. cycle number). The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle Threshold (Ct).

The human monocyte cell line U937 has been shown to contain significant AhR activity in the absence of ligand and was used in this study [4]. Utilizing the AhRC PCR assay we were able to measure activated AhR in cell extracts diluted more than 600-fold from the original preparation (Figure 3). The ability to measure such small quantities of AhR suggested that similar measurements could be performed on peripheral blood cells prepared from only a few ml of blood.

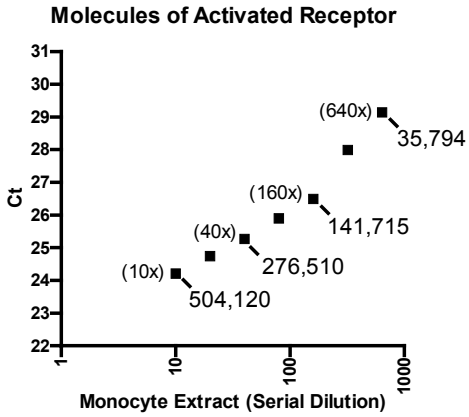

Figure 3. Molecules of Activated Receptor Present in Monocyte Extract. The quantity of receptor is directly proportional to the amount of DRE-probe measured.

To examine the specificity of the DNA binding, 100-fold excess of mutant-probe or competitive-probe were added to the reaction. The mutant-probe contained a DRE sequence that had been altered to prevent receptor binding, while the competitor-probe contained a functional DRE

sequence but did not contain the primer recognition sequences necessary for PCR amplification. Approximately 500,000 molecules of activated receptor were detected in the control reaction. The addition of excess mutant-probe had essentially no effect on binding of the DRE-probe while addition of the competitor-probe completely inhibited the reaction (Table 1). These results indicated that the activated complex being measured was specifically binding to the DRE.

Table 1. Binding Specificity of the AhR Extract.

Control Reaction	Excess Mutant-probe	Excess Competitor-probe
498,349	489,815	Non Detectable

10-fold dilution of the monocyte extract was used.

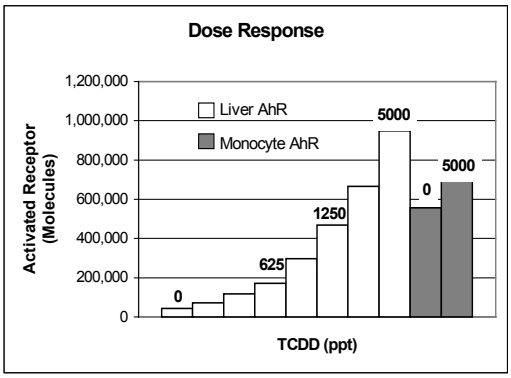

Figure 4. Dose Response of Guinea Pig Liver Cytosol and Monocyte U937 to TCDD.
Standards consisting of 0, 78, 156, 313, 625, 1250, 2500 and 5000 ppt 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were prepared in methanol. Standard (5µL) was added to 100µL of cytosol, incubated for 1 hour at room temperature, and analyzed by AhRC PCR.

The activation of AhR from guinea pig liver cytosol generated a characteristic dose-response curve with approximately a 25-fold induction of DRE binding (Figure 4). However, only the maximum dose of TCDD resulted in a detectable increase in receptor activation (20%) when using cytosol from the monocyte cell line U937. This observation could be attributed to the instability of the AhR/heat shock protein complex resulting in AhR activation or the fact that cytoplasmic extract from monocytes contain a significant DRE binding activity in the absence of ligand. A similar result has been demonstrated in nuclear extracts prepared from U937 cells [4].

Further studies will be necessary to demonstrate similar results using peripheral blood cells collected from humans and animals. It is the goal of these studies to develop a rapid and simple blood test for determining dioxin exposure by measuring AhR or a panel of activated transcription factors from a small sample of blood.

References

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