A novel method for detection of dioxins: exonuclease protection mediated PCR assay

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Introduction

The aromatic hydrocarbon receptor (AhR) is a ligand-actived transcription factor that mediates many of the biologic and toxicologic effects of dioxin-like chemicals (DLCs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Numerous AhR-based bioassays for identification and detection of DLCs have been developed in vitro[1]. Such as the chemical-activated luciferase gene expression (CALUX), ethoxyresolufin-O-deethylase (EROD) activity are sometimes represented as the next best system when compared with whole body or in vivo systems[2,3]. However, cell systems can be affected by the toxic chemical itself during the assay, thus confusing problems couldn't be avoided in the assay. Incorporation of metabolism in cell systems with uncertain consequences prolongs assay complexity and time. Thus these drawbacks limit the utility of cell systems for screening purposes. Most cell-free bioassays require radioactivity, such as the gel retardation of AhR binding (GRAB) assay [4], or antibody of AhR or ligand, which are unfeasible for some laboratories [5].

Here a cell-free bioanalysis method, Exonuclease Protection Mediated PCR (EPM-PCR) bioassay, was established for detection of AhR ligands based on the binding of the dioxin:AhR complex to the specific DNA. EPM-PCR can provide indirect detection of ligands by quantification of the specific AhR-binding DNA, no necessary of any DNA labeling and sophisticated equipments. This new bioassay not only has the higher sensitivity and specificity, but it is rapid and easy to perform.

Methods and Materials

Preparation of DRE and molecular probe. **The plasmid pAlterMax (Sigma) were amplified with primers F1(5'- CTCTTCTCACGCAACTCCGTGTAACAAGGGTGAACAC -3') and R1(5'-CGGAGTTGCGTGAGAAGAGCGTTGATATATCCCAATG -3') to generate a 285bp-length PCR products containing two AhR binding sites on the either end. The sequences underline were corresponding to AhR binding site of mouse DRE3 [6]. PCR products were purified and quantified at OD_{260nm} to be referred to as DRE (dioxin-responsive elements). The sense primer (5'ACCTATAACCAGACCGTTCAGC3') and antisense primer (5'TCACCGTCTTTCATTGC CATAC3') were used for real-time PCR.**

Preparation of complex of activated AhR binding to DRE. **Hepatic cytosol of male SD rats was prepared at 4 in HEDGK buffer (25mM Hepes, PH7.5, 1mM EDTA, 1mMDTT, 10%(v/v)glycerol and 80mM KCl) according to Denison [7] and protein concentrations were measured by the method of Bradford using bovine serum albumin as the standard and adjusted to 10~15mg/ml. Hepatic cytosol was incubated with DMSO(20µl/ml) , different concentrations of TCDD (0.01pM~10nM TCDD in DMSO, for 2h at 20 , then mixed with poly(dI/dC)(~1.0µg) and incubated for 15min at 20 and followed by the addition of ~0.5µg DRE, and further incubation for 15min.**

EPM-PCR assay. **The 10µl aliquots of binding reaction mixture described as above were digested with ExonucleaseIII and S1 nuclease (Promega Co.,Madison, WI) to remove the free DRE. Digestion products was as template to be subjected to real-time PCR. The 20ul real-time PCR mixture contained 2ul LightCycler DNA Mastermix(Roche Molecular Biochemicals, Mannheim, Germany),2ul template or distilled H_2O (for negative control), 0.75uM each of primers, and 3.0mM $MgCl_2$. The LightCycler DNA Mastermix contained Taq-polymerase, dNTPs(with dUTP instead of dTTP), PCR buffer, and the Sybr green I. PCR were run in the LightCycler instrument (Roche molecular biochemicals,Mannheim,Germany). Protocol was done by 0s denaturation at 95 , 5s annealing at 51 ,and 10s elongation at 72 for 40 cycles. Fluorescence was detected at the end of every 72 extensive phase. Melting curve analysis was applied to all end PCR products after cycling protocol. The melting step consisted of denaturation at 95 , cooling to 65 for 10s, then ramping to 90 at 0.2 /s, monitoring fluorescence continuously.**

To determine the absolution copy number of the binding DNA in samples, 10-fold dilution series of dioxin probe (1×10^2~1×10^8 copies) were used in the real-time PCR.

Results and Discussions

Scheme of EPM-PCR: **Figure 1 shows the strategy of detection of the AhR ligands using EPM-PCR. The Ah receptor is a soluble intracellular protein that enhances the transcription of a number of genes. After activated by TCDD or other ligands, transformed AhR recognizes a specific DNA motif (DRE). TCDD:AhR complex interacts with DRE in an one-to-one ratio, so the dioxin probe here with two DREs on either ends can bind two TCDD:AhR complexes per probe at saturation. Incubated with the activated AhR complex, both 3' ends of dioxin probe were protected by ligand:AhR:DRE complex and resisted to ExoIII cleavage. This meant that not only binding sites but the sequences between two sites were protected against ExoIII in presence of AhR ligands. With Exo III and S1 nuclease treated, free DNA was deleted at all. Only receptor protein-binding DNAs were remained and amplified by PCR.**

Detection and quantification of TCDD by EPM-PCR bioassay: **In the presence of TCDD, AhR was transformed and receptor-DRE complex was generated that protected receptor-binding DNA against ExoIII digestion. After ExoIII/S1 digestion free DRE were removed and receptor-binding DRE remained and quantified by real-time PCR. C_T values in real-time PCR decreased with the increasing concentration of TCDD (1fM~1nM). Calibration curve was obtained using different copies ranging from 10^2 to 10^8 of DREs as the template in real-time PCR. It showed a linear relation (r=-0.99) between the log of template copy number ([log copy number]) and C_T over the range of DNA copies examined.**

Sensitivity of EPM-PCR bioassay: **Corresponding to the calibration curve, the linear relationship of TCDD concentration to bound DRE copies was obtained. It showed the ability of a chemical to stimulate AhR transformation and DNA binding in vitro was dose-dependent. The minimal detection limit, whose fluorescent signal is 10-fold higher than SD of the mean baseline emission, is 1×10^{-15} M TCDD. We also tested the high concentration (20nM, 50nM and100nM) of TCDD with this system. The C_T values had no significant difference ($P<0.05$). Thus we concluded that the maximal amount of bound DNA was produced at 10nM TCDD and the concentration of half-maximal respond (EC_{50}) was estimated to be 1×10^{-12} M.**

Compared with CALUX**: EPM-PCR is approximately 7 times more sensitive than the CALUX (EC_{50} of 1×10^{-12}M compared to 2×10^{-11}M, respectively). The minimal detection limit is 100 times lower (1×10^{-15} M as compared to 1×10^{-13} M for the CALUX assay) [8]. And it has higher reproducibility (CV of 6%~9% compared to 15%~30%, respectively). Moreover, EPM-PCR bioassay is as rapid as the CALUX with ~5 h and easy for analysis.**

Besides TCDD, EPM-PCR assay was also used to examine the ability of other DLCs to directly stimulate AhR transformation and DNA binding in vitro (data not shown). Dose-response curves demonstrated the rank order in potency was TCDD>TCDF>PCB> BA, which was consistent with their toxic equivalency factors (TEFs).

By applying of ExoIII and S1 nuclease digestion in the AhR-binding DNA bioassay, EPM-PCR provides the sensitive and specific detection and quantification of DLCs. Compared with other existing methods for DLCs detection, EPM-PCR bioassay has advantages, such as non-radioactivity, sensitivity and simpleness. It is useful to screen the positive from large-scale of samples or measure novel AhR agonists. However, it doesn't provide structural information of the agonists like GC/MS analysis. So conjunct of the structure-based approach is necessary for identification of an unknown AhR agonist.

It's very important that the other AhR ligands than DLCs must be removed from environmental or other kinds of samples before EPM-PCR assay.

Figure 1: **Scheme of EPM-PCR. Following ligand (TCDD) binding, the cytosolic TCDD:AhR complex dimerizes with the AhR nuclear translocator(Arnt) protein and binds to dioxin probe containing two specific recognition sites, DREs. The binding of DRE to transformed AhR renders them resistant to ExoIII cleavage. The free dioxin probes in the mix are degraded by ExoIII and S1 nuclease completely. The enzyme digestion mixture is subjected to PCR to generate an amplifying signal.**

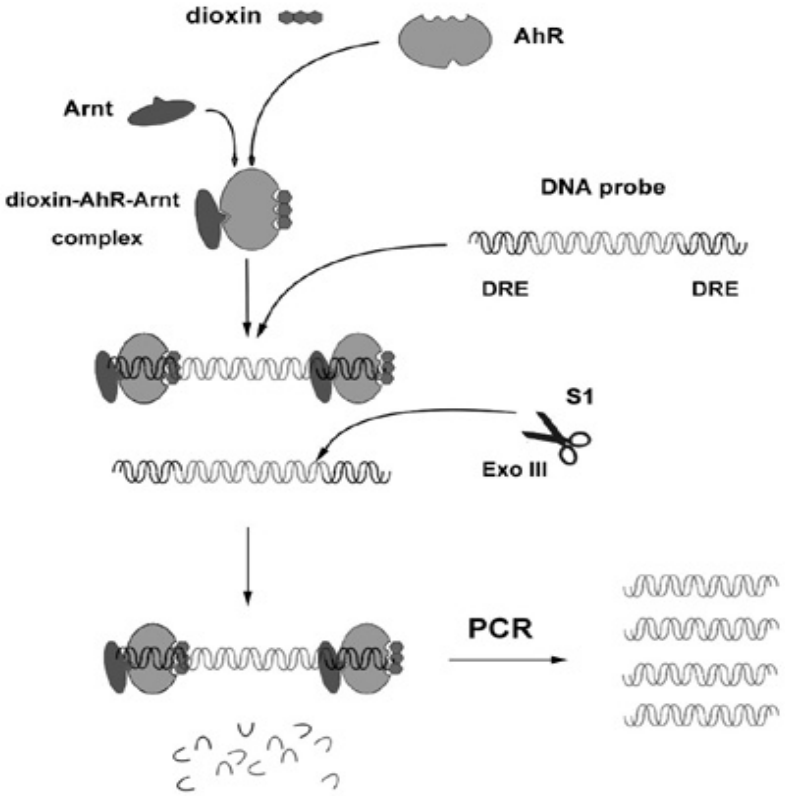
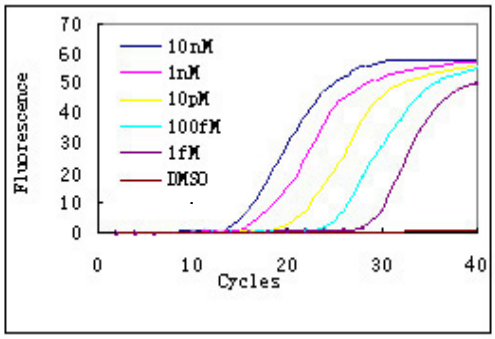
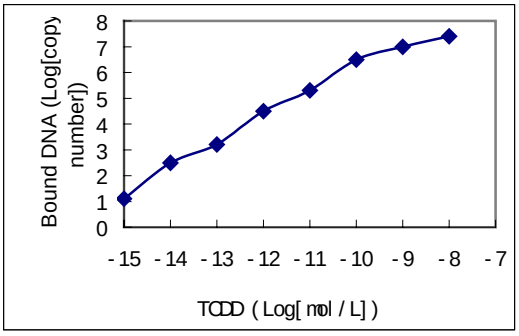


Figure 2: **Effect of TCDD:AhR-binding DRE relative to different TCDD concentrations on real-time PCR. Linear calibration curve from 1×10^2 to 10^8 copies of DRE. (A) Kinetics of fluorescence signal at different concentration of TCDD. The curves from right to left indicated DMSO and different concentrations of TCDD (1fM, 100fM, 10pM, 1nM and 10nM, respectively). The crossing point of the noise band with the amplification curve is the threshold cycle (C_T). (B) Dose-effect curves for the activity by TCDD of the EPM-PCR bioassay.**





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