Chapter 2
Receptor Binding Assay and Reporter Gene Assay of Medaka

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1. Principle of the assay

1-1. Receptor binding assay

Figure 1 shows the principle of receptor binding assay. Some of procedure for receptor binding assay is as follows.

1-1-1. Procedure

(1) Sample solution (10 μl) and 5 nM [2,4,6,7,16,17-^{3}H] 17β-estradiol (10 μl) were dissolved in Tris-HCl (pH 7.4, 70 μl) containing 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10% glycerol, 10 mg/ml γ-globulin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2 mM leupeptin.
(2) A solution (10 μl) of recombinant estrogen receptor ligand binding domain fused with glutathione *S*-transferase expressed in *E. coli* was added to the solution.
(3) Incubated for 1 h at 25°C.
(4) When test chemicals possessed receptor-binding ability, they competed with [^{3}H] 17β-estradiol for the ligand binding domain of the receptor.
(5) In order to remove free radioligand, 100 μl of dextran-coated charcoal (DCC, 0.2% activated charcoal and 0.02% dextran in PBS (pH 7.4)) was added and incubated for 10 min at 4°C.
(6) After centrifugation or filtration, radioactivity in supernatant was measured using liquid scintillation counter.

The percent ratio (B/B_0 (%)) of standard ligand ([^{3}H] 17β-estradiol) bound to the receptor was represented as the formula below.

$$B/B_0\ (\%) = \frac{(X - NSB)}{(Y - NSB)} \times 100$$

where,

X: amount of standard ligand bound to the receptor in the presence of test chemical
Y: amount of standard ligand bound to the receptor in the absence of test chemical
NSB: amount of standard ligand bound to the receptor nonspecifically

1-1-2. Data analysis

Data were analyzed by using the computer program GraphPad Prism® and IC_{50} value of each chemical was calculated. The binding abilities of test chemicals to the receptor were evaluated by relative binding affinity (RBA), ratio of IC_{50} values to estradiol.

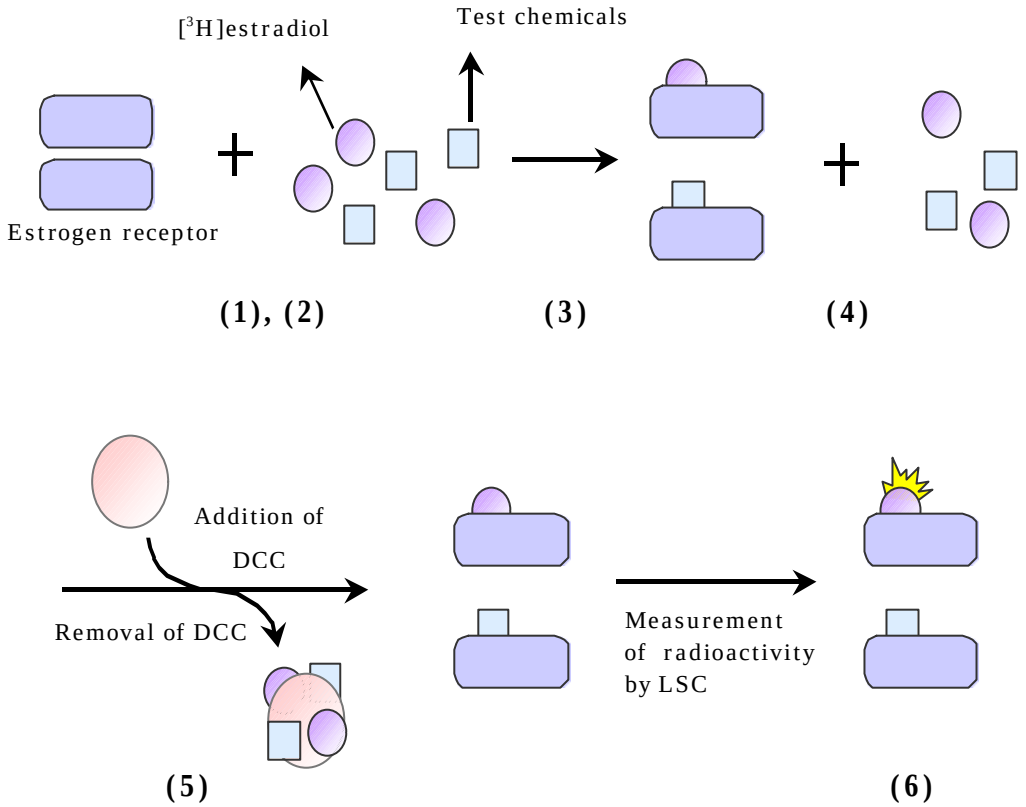

Fig. 1. Principle of receptor binding assay

1-2. Reporter gene assay

Figure 2 shows the principle of reporter gene assay. For procedure of the reporter gene assay, it takes about three days. Each procedure is as follows. This procedure was used in common for both ER and AR.

Day 1

(1) Cells were co-transfected with both receptor expression and reporter plasmids (3 μg each) in serum-free medium.
(2) After incubation for 4.5 h, the serum-free medium was replaced with serum-containing medium.

Day 2

(3) Cells were harvested and sample solution (1 x 10^{-5}～1 x 10^{-11} M) was exposed to the cells in 96 well plate.

(4) When test chemicals induced transcriptional activation mediated by binding to hormone receptor, firefly luciferase was produced according to their estrogenicities in the cells.

Day 3

(5) The medium was wasted and cells were lysed and enzymatic activity of luciferase was measured.

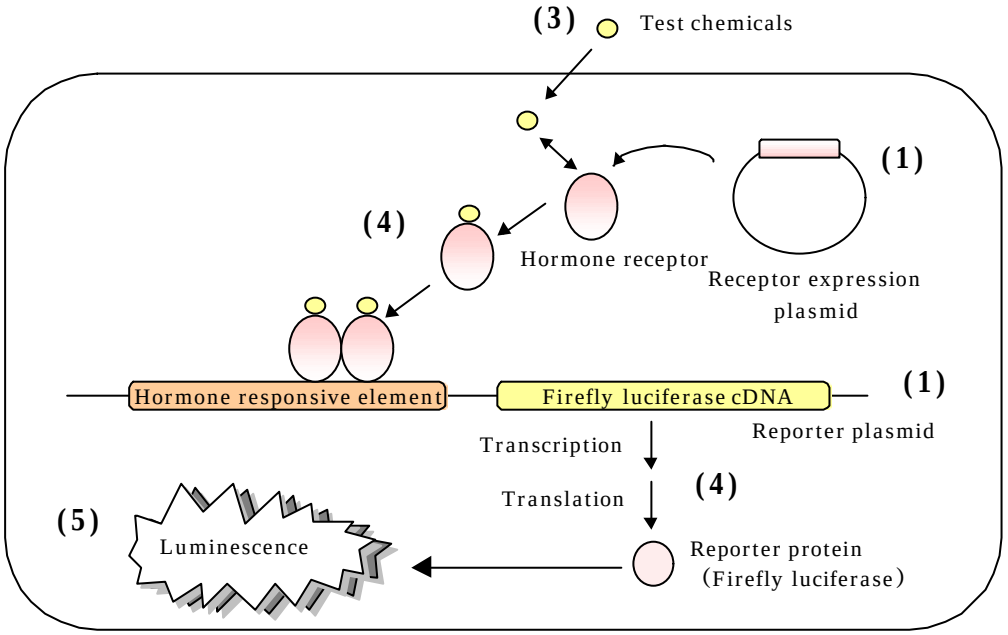

Fig. 2. Principle of reporter gene assay

2. Medaka estrogen receptor (meER a and meER b) binding assay and reporter gene assay

2-1. Objective

To evaluate the binding affinities and estrogenic activities of endocrine-disrupting chemicals to medaka estrogen receptor, receptor-binding assay and reporter gene assay were carried out.

2-2. Principle

Nuclear hormone receptors function as ligand-induced transcriptional factors. It is thought that exhibition of most endocrine-disrupting effects of chemicals, for instance, modulation of gene expression, are mediated by binding to hormone receptors, especially estrogen receptor, and it is very important to demonstrate the binding and gene transactivational potencies of endocrine disrupting chemicals. Receptor binding assay is a simple and convenient method to elucidate the binding properties of many chemicals. In this assay, binding affinities of several endocrine-disrupting chemicals to recombinant Medaka estrogen receptors α and β expressed in *E. coli* are measured. Reporter gene assay shows hormonal activities mediated by binding to target hormone receptors. Results are presented by dose-responsive sigmoid curves in both assays and estrogenicities of chemicals can be analyzed quantitatively comparing to standard ligands, i.e., endogenous hormones.

2-3. Test method

The procedures of receptor binding assay and reporter gene assay were described in 1-1 and 1-2, respectively.

2-4. Results and discussion

We have been demonstrated receptor binding and reporter gene assay of following twelve substances to medaka estrogen receptors α and β. The relative binding affinities and reporter gene transactivational activities of test chemicals were summarized in Tables 1 and 2, respectively. When compared receptor binding assay to reporter gene assay, former was more sensitive and quantitative than latter assay. Tributyltin chloride and triphenyltin chloride seemed to bind to both receptors strongly, however, they have strong protein denaturation property. Then, we examined GST activity of fusion protein (ER α) using 1-chloro-2,4-dinitrobenzene as a substrate. The GST activity was decreased in a dosedependent manner and it was suggested that the apparent binding affinity of each organo-tin compound for both receptors was caused by their denaturative characteristics.

Table 1 Relative binding affinities of chemicals to Medaka ERs α and β

Chemical	Relative binding affinity (%)	
	ER α	ER β
17β-estradiol	100	100
dibutyl phthalate	0.023	0.0063
dicyclohexyl phthalate	0.045	0.016
di-2-ethylhexyl phthalate	0.79	0.80
4-*t*-octylphenol	16	0.83
nonylphenol	8.1	0.83
benzophenone	0.02	not determined
octachlorostyrene	0.023	0.021
tributyltin chloride	0.10	0.19
triphenyltin chloride	0.24	0.29
butylbenzyl phthalate	0.23	0.057
diethyl phthalate	0.012	0.0024
di-2-ethykhexyl adipate	0.014	0.0040

Table 2 Gene transactivational activities of chemicals mediated by Medaka ERs α and β

Chemical	Relative potency (%)	
	ER α	ER β
17β-estradiol	100	100
dibutyl phthalate	not determined	negative
dicyclohexyl phthalate	negative	negative
di-2-ethylhexyl phthalate	negative	negative
4-*t*-octylphenol	1.3	negative
nonylphenol	0.35	negative
benzophenone	negative	negative
octachlorostyrene	negative	negative
tributyltin chloride	negative	negative
triphenyltin chloride	negative	negative
butylbenzyl phthalate	not determined	not determined
diethyl phthalate	negative	negative
di-2-ethykhexyl adipate	negative	negative

3 Medaka androgen receptor (meAR) reporter gene assay

Similarly, reporter gene assay using medaka AR was carried out (Table 3). All chemical substances tested in this study had no gene transcriptional activity mediated by binding to medaka AR.

Table 3 Gene transactivational activities of Chemicals mediated by Medaka AR

Chemical	Relative potemcy(%)
5α-dihydrotestosterone	100
dibutyl phthalate	negative
dicyclohexyl phthalate	negative
di-2-ethylhexyl phthalate	negative
4-*t*-octylphenol	negative
nonylphenol	negative
benzophenone	negative
octachlorostyrene	negative
tributyltin chloride	negative
triphenyltin chloride	negative
butylbenzyl phthalate	negative
diethyl phthalate	negative
di-2-ethykhexyl adipate	negative

4. Future work

As shown in Table 1, 4-*t*-octylphenol and nonylphenol bound to medaka ER α at 1/6 and 1/12 of E2, respectively. On the other hand, they bound to human ER α at approximately 1/3,000 of E_2. These results suggest there are species differences in receptor binding characteristics and show importance of the differences in sensitivities to chemicals among diverse species to assess the endocrine disrupting effects of chemicals to ecosystem. Consequently, we should clarify these issues by receptor binding assay using various fish estrogen receptors.