§2. Materials and Methods

2.1 Cells culture

2.1.1 Reagents for cell culture

Chemical and culture medium	Company
4mM L-glutamine	Sigma, USA
Sodium bicarbonate	Sigma, USA
glucose	Sigma, USA
Sodium pyruvate	Sigma, USA
DMEM culture medium	PAN, Germany
Leibovitz's L-15 culture medium	PAN, Germany
Fetal bovine serum	PAN, Germany

2.1.2 Procedures

Ovarian cancer cell lines SKOV-3, OVCAR-3, ES-2 and MDAH-2774 were purchased from American Typical Culture Collection (ATCC) and ovarian cancer cell line OAW-42 and mammary cancer cell line MCF-7 were purchased from European Typical Culture Collection (ETCC), all cell lines were maintained in the Institute of Pathology, Campus Mitte, Charité Universtätmedizin Berlin. The genetic background of the cell lines can be found on the web: www.atcc.org. Ovarian cancer cell lines OVCAR-3, OAW-42, SKOV-3, and breast cancer cell lines MCF-7 were cultured in 90% DMEM medium with 4mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/l glucose, and 1.0 mM sodium pyruvate and supplement with 10% fetal bovine serum. ES-2, MDAH 2774 cell lines were cultured in 90% Leibovitz's L-15 with L-glutamine and supplement with 10% fetal bovine serum, at 37°C in a humidified 5% CO_2 incubator. Every two or three days, the cultured medium was replaced.

2.2 Study Population

A total of 33 ovarian cancer samples and 12 normal unmatched ovarian samples were enrolled in this study. After pathological review, the parts of ovarian surface epithelium (OSE) from the normal ovaries were used as controls. All samples and their related clinical data were from Tumor Bank Ovarian Cancer (T.O.C), Department of Obstetrics and Gynecology, Campus Virchow Klinikum, Charité Universtätsmedizin Berlin, Germany. Samples were collected during 2001-2002. Inform consent was obtained from

each patient. Approval from local ethics board was gained and written. All the samples were confirmed diagnosis by pathological review (Institute of Pathology, Campus Mitte, Charité Universtätmedizin Berlin). The patients enrolled in this study received primary surgery with the attempt of maximal tumor reduction and postoperative systemic chemotherapy with carboplatin and paclitaxel. All ovarian tissues were snap-frozen (shock frozen) in liquid nitrogen as soon as they were separated from body, and stored in -80°C until detection. The clinicopathological characteristics of patients with ovarian cancer were summarized in Table 2-1. The difference of average age were not significant (between groups p=0.350, within groups p=0.563, analyzed by ANOVA, SPSS version 11.0 software). After the primary therapy, all patients were enrolled in a follow-up program. In general, the patients will be visited by telephone call every 3-6 months.

Table 2-1: Clinicopathological characteristics of the study population

	Cases N (%)	**Average age (rang)**
Normal ovaries	12(100%)	42.6(34-48)
Ovarian cancer	33(100%)	54.8(37-71)
Histology		
Serous	20(60.6%)	53.6(44-57)
Mucous	7(21.2%)	57.8(37-71)
Other subtypes	6(18.2%)	52.9(47-59)
Grading		
G1	4(12.1%)	53.7(37-62)
G2	11(33.4%)	54.5(46-57)
G3	18(54.5%)	56.2(44-71)
FIGO clinical stage		
FIGO I	2(6.1%)	52.0(51-53)
FIGO II	3(9.1%)	52.8(37-62)
FIGO III	18(54.5%)	54.2(46-59)
FIGO IV	10(30.3%)	58.9(44-71)
Ascites		
no	8(24.2%)	53.8(44-60)
<500ml	15(45.5%)	54.6(37-71)
≥500ml	10(30.3%)	56.2(46-62)

2.3 Plasmids and plasmid construction

2.3.1 Maps of plasmids

The plasmids encoding full-length cDNA of human ERRα (pSG-hERRα), mouse ERRβ (pSG-mERRβ), mouse ERRγ (pSG-mERRγ), human ERα (pCMV-hERα), mouse ERβ (pCMV-mERβ) were generous gifts from Prof. J.M. Vanacker (Laboratoire de Biologie Moelcuaire de la Cellule, Center National de la Research Scientifique, Lyon, France) and used as standard controls for LightCycler real-time quantitative PCR [31-33]. The maps of plasmids can be seen in Figure 2.1

The green fluorescent protein (GFP) reporter plasmid was a generous gift from Dr. Eckardt Treuter (Karolinska Institute Hospital, Sweden).

The plasmid pSG-HA-tag-hERRγ was a generous gift from Dr. Michel R. Stallcup (University of Southern California, USA) [12]. A nucleotide sequence containing hemagglutinin (HA, amino acids sequence: YPYDVPDYA) epitope tag coding cordon and a new *EcoR*I site was inserted between the original *EcoR*I and *Bam*HI sites of pSG5 basic plasmid.

2.3.2 Plasmid construction

To report the subcellular location of human estrogen receptor-related α in the ovarian cells, the hERRα-GFP reporter plasmid was constructed as follows. By the same method, the pD-GADPH plasmid was also constructed, which was used as an inner standard control of the LightCycler Q-PCR.

2.3.2.1 Reagents and Kist for construction

Reagents and Kist	Company
Qiagen PCR® cloning ^{plus} kit	Qiagen, Germany
Restricted enzyme *EcoR*I	Promega, USA
Restricted enzyme *Bam*HI	Promega, USA
T_4 DNA ligase enzyme	Sigma, USA
QIAquick® gel extraction kit	Qiagen, Germany

2.3.2.2 Excision of the target fragment

The pSG-hERRα or the pCN3-GFP reporter plasmid was digested by the 20 μl double-restricted-enzymes system (the working system can be seen in Table 2-2).

1. The stock solution of hERRα plasmid was thawed on ice and counted the concentration accurately by a DNA-Counter.

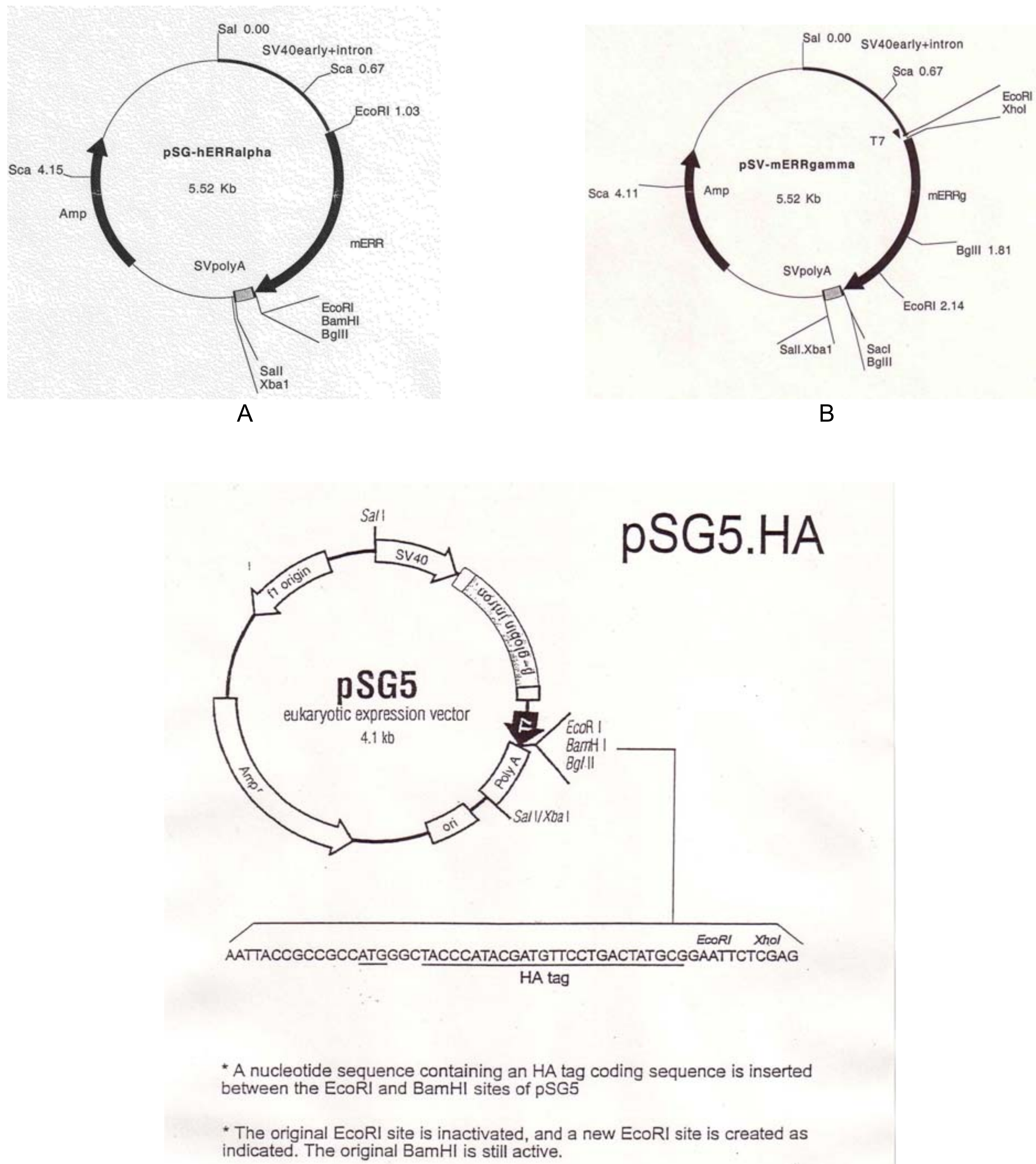


Figure 2.1: Maps of plasmids. (A) pSG-hERRα, containing a full-length cDNA of human ERRα; (B) pSG-mERRγ, containing a full-length cDNA of mouse ERRγ; (C) pSG5-HA-ERRγ, containing a HA encoding sequence and full-length cDNA of hERRγ. A HA tag protein encoding sequence is inserted between the *EcoR*I and *Bam* HI sites of a basic pSG5 plasmid.

2. According to the sequence listed in the Table2-2, the reagents were pipetted into a fresh microcentrifuge tube.
3. The mixture was incubated at 37°C water bath for 4 h.

4. The plasmid pSG-hERRα was excised to two linear DNA fragments, including the pSG (3.3kbp) vector and hERRα full-length cDNA (2.2kbp). By the same method, the plasmid pCN3-GFP was opened to a linear plasmid with an *EcoR*I and *Bam*HI terminal.
5. After reaction, 4 μl 6× lodding buffer was added into the above 20 μl-working system and 12 μl mixture was proceeded to gel analysis.

Table 2-2: *EcoR*I 和 *Bam*HI double-restricted enzymes working system(20 μl)

Reagents	Volume	Finial concentration
Sterile deionizer water	5.8 μl	
BSA(10 μg/μl)	0.2 μl	0.1 μg/μl
10×Multicore® buffer	2 μl	1×
Plasmid DNA	1 μg	0.05 μg/μl
*EcoR*I（10 u/μl）	1 μl	0.5 u/μl
*Bam*HI（10 u/μl）	1 μl	0.5 u/μl
Final volume	20 μl	

2.3.2.3 Recovery of the target fragment

1. After electrophoresis on a 1.5% agar-gel at 100 V for 45 min, the full-length hERRα-1 cDNA (2221bp) and pSG vector (3.3kbp) were separated on the gel. The full-length cDNA was recovered and purified by the QIAquick Gel Extraction Kit.
2. The DNA fragment was excised from agarose gel with a clean, sharp scalpel, Minimizing size of the gel slice by removing extra agarose.
3. Weigh the gel slice in a colorless tube. Add 3× volume buffer QG to 1 volume gel (almost 100 mg gel≈100 μl gel), the gel should be less than 400 mg.
4. Incubate at 50°C until the gel slice has completely dissolved while shaking at 300 rpm.
5. After the gel has completely dissolved, make sure the color of the mixture is yellow (it is essential that the dissolution is pH ≤7.5).
6. Place a QIAquick spin column in 2 ml collection tube provide by the kit.
7. To bind the target DNA, apply the sample to the QIAquick column and centrifuge for 1 min at 13, 000 rpm.
8. Discard the flow-through and place the QIAquick spin column in the same collection

tube, then, add 500 μl QG buffer to the column and centrifuge for 1 min at 13, 000 rpm.

9. Add 750 μl PE buffer to the column and wash the column by centrifuged for 1 min at 13,000 rpm.
10. Place the QIAquick column into a clean 1.5 ml microcentrifuge tube.
11. To elute DNA, add 30 μl EB buffer (10 mM Tris-Hcl, pH 8.5) to the center of the QIAquick column membrane and hold for 1 min, then centrifuge for 2 min at 13,000 rpm.

2.3.2.4 Recombinant of the target fragment and GFP vector

1. Full-length hERRα-1 cDNA fragment was directly inserted into the sites in the pCN3-GFP reporter plasmid by PCR cloning kit and named as hERRα-GFP reporter plasmid.
2. The molar ratio of hERRα cDNA should be 10 times more than pGFP reporter vector. According to the concentration of hERRα cDNA and pGFP vector, calculated out the solution volume need in the reaction.
3. Prepare a ligation-reaction mixture sequentially according to the following Table 2-3.
4. Briefly mix the ligation-reaction mixture and incubate at 16°C for 2 h in a water bath.
5. Proceed to the transformation protocol (chapter ***2.3.3.1***) or store the ligation-reaction mixture at -20°C until use.

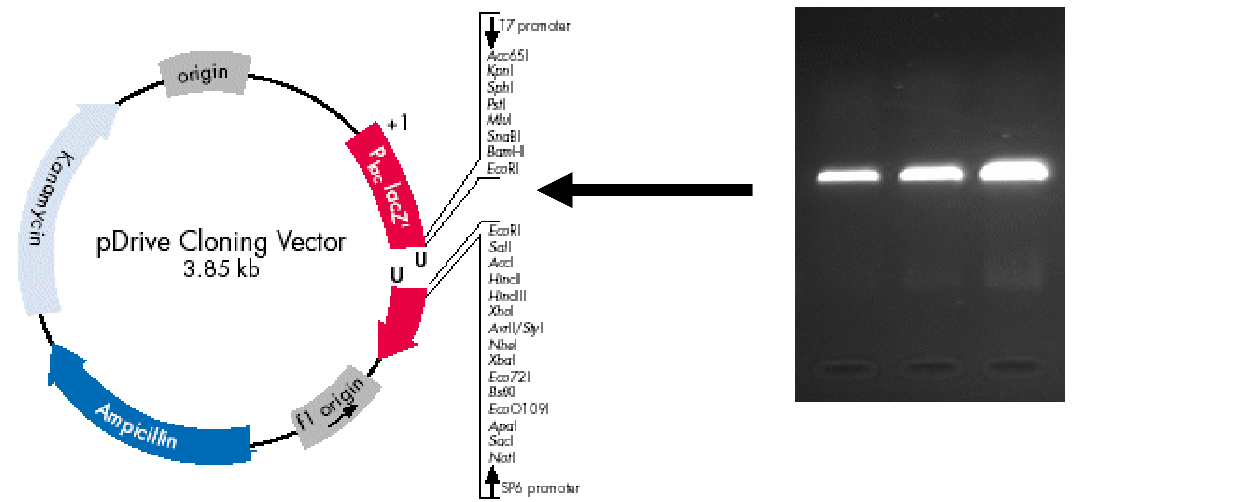
Table 2-3: T_4DNA Ligation-Reaction System

Reagents	Volume
Sterile, deionized water	10.8 μl
pGFP vector (55 ng/μl)	4.2 μl
hERRα cDNA (33 ng/μl)	2 μl
10×buffer	2 μl
T_4DNA ligase (1 u/μl)	1 μl

2.3.2.5 Construction of pD-GADPH plasmid

The pDrive Cloning Vector includes a large number of unique restriction enzyme recognition sites, universal sequencing primer sites, and promoters for *in vitro* transcription. In addition, the vector allows ampicillin or kanamycin selection as well as blue/white screening of recombinant colonies. The pDrive Cloning Vector is supplied in a linear form with a “U” overhang at each 3’ end, which hybridizes high specificity to the “A”

overhang of PCR products generated by Taq and other non-proofreading DNA polymerases.



Linear pDrive Cloning Vector The PCR product of GADPH was recovered

Figure 2.2: The recovered PCR product of GADPH was directly inserted into the linear pD Cloning Vector with a “U” overhang at each 3’ end.

Table 2-4: The universal amount of PCR product to use in the reaction

PCR product size	Amount of PCR product to use in the reaction	
	5-time moral ration	10-time moral ration
100 bp	6.5 ng	13.0 ng
200 bp	13.0 ng	26.0 ng
500 bp	32.5 ng	65.0 ng
1000 bp	65.0 ng	130.0 ng
1500 bp	97.5 ng	195.0 ng
2000 bp	130.0 ng	260.0 ng
3000 bp	195.0 ng	390.0 ng

1. According the following Table 2-4 and formula, calculate out the amount of recovered GADPH PCR product needed in the reaction.

   Calculate for 50ng pDrive Cloning Vector using for the following equation

   $$\text{ng PCR product required} = \frac{50\text{ng} \times \text{PCR product size (bp)} \times \text{molar ration}}{3851\text{bp (pD vector's size)}}$$

2. Thaw 2× Ligation-Master-Mixture. pDrive Cloning Vector DNA and distilled water, place on ice after all solution thawing.

3. Prepare a ligation-reaction mixture sequentially according to the Table 2-5.

Table 2-5: The Ligation-Reaction System

Reagents	Volume
Sterile, deionized water	1.8 μl
pDrive Cloning Vector (50 ng/μl)	1 μl
PCR product of GADPH(230 bp)	2.2 μl*
Ligation Master Mix, 2× buffer	5 μl
Total Volume	10 μl

* The concentration of recovered GADPH PCR product is 14.5ng/μl

4. Briefly mix the ligation-reaction mixture and incubate at 16°C for 2 h in a water bath.
5. Proceed to the transformation protocol (chapter ***2.3.3.1***) or store the ligation-reaction mixture at -20°C until use.

2.3.3 Plasmids Cloning

2.3.3.1 Kits

Kits	Company
Top-10® One shot Kit	Invirtrogen, USA
QIAGEN® PCR Cloning ^{plus} Kit	Qiagen, Germany

2.3.3.2 Procedures of transformed

1. Thaw the appropriate number of tubes of TOP-10 one shot cells or QIAGEN EZ competent cells on ice. Thaw SOC medium and warm to 37°C (Note: the TOP-10 cells and EZ competent cells should only be thawed on ice and do not allow the unused cells to thaw.) After thawing, immediately proceed to the transformation step.
2. Add 1-2 μl ligation-reaction mixture (from chapter ***2.3.2.3*** or ***2.3.2.5***) per tube of the Top-10 shot cells (all the plasmids listed in the Chapter **2.3.1** and the hERRα-GFP recombinant plasmid) or EZ competent cells (for pD-GADPH plasmid), mix gently by flicking the transformation mixture several times and incubate on ice for 5 min.
3. Heat the tubes at 42°C in water bath for 45 s without shaking.
4. Incubate the tubes on ice for 2 min.
5. After warmed to room temperature, add 250 μl SOC medium into per tube cells, incubate the cells at 37°C while shaking for 1 h.
6. Pipet 100 μl transformation mixture onto the LB-x-gal-ampicillin screening plate.

Preparation for the LB-x-gal-ampicillin screening plate

Reagents for the LB-x-gal-ampicillin screening plate

Reagents	Amount
tryptone	10 g
yeast extraction	5 g
NaCl	10 g
Agar	15 g
X-gal stock solution(40 mg/ml)	2 ml
IPTG stock solution(100 mM)	0.5 ml

Adjust pH to 7.0 by adding deionized water to a final volume of 1,000 ml. Autoclave the solution for 20 min, allow the autoclaved solution to cool to 55°C then add 1 ml filter-sterilized (100 mg/ml) ammpicillin stock solution.

7. Incubate the plate at room temperature until the transformation mixture has been absorbed by the agar. Invert the plate and incubate at 37°C overnight (for 16 h)

2.3.3.3 Cloning

1 After 24 h incubation, the cells seeded on the plate will form many clones. The successfully transformed cells achieve the ability to resist the ampicillin and lose the ability to decompose the x-gal, and then the clone will show a white color.

2 Prepare for the LB liquid cloning medium.

LB liquid cloning medium

Reagents	Amount
tryptone	10 g
yeast extraction	5 g
NaCl	10 g
Agar	15 g

Adjust pH to 7.0 by adding deionized water to a final volume of 1,000 ml. Autoclave the solution for 20 min, allow the autoclaved solution to cool to 55°C then add 1 ml filer-sterilized (100 mg/ml) ammpicillin stock solution.

3 Choose a white single clone (positive) and transfer it to a starter culture of 5 ml LB medium containing the appropriative selective antibiotic

4 Incubate the starter culture medium that contains the positive clone for 8 h at 37°C while shaking at 180 rpm.

2.4 Plasmid purification

2.4.1 Kits and equipment for purification

Kits and equipments	Company
QIAprep Spin Minipre Kit	Qiagen, Germany
QIAGEN® Plasmid Maxi Kit	Qiagen, Germany
Centrifuge 5417 R	Eppendof, Germany
Centrifuge Avanti^{TM}-J25	Beckman, USA

2.4.2 Small scale plasmid purification

Perform according to the protocol of QIAprep Spin Minipre Kit (250)

1. Transfer 1.5 ml starter culture (result from chapter ***2.3.3.3***) to a fresh microcentrifuge tube.
2. Harvest the bacterial cells by centrifuge at ≥11,000× g for 2 min at 4°C.
3. Resuspend the bacterial pellet in 250 µl P1 buffer.

   Before use the P1 buffer, add the RNAse provided by the kit to the P1 buffer. Use one vial of RNAse A (spin down briefly) per bottle of P1 buffer, to achieve a final concentration of 100 µg/ml.
4. Add 250 µl P2 buffer, mix thoroughly by gently inverting 4-6 times at room temperature (less than 5 min).
5. Add 250 µl chilled N3 buffer and mix immediately by gently inverting 4-6 times (less than 5 min).
6. Centrifuge at ≥18,000× g for 10 min at 4°C, during the centrifugation, place the QIA pre-spin column into 2 ml collection tube.
7. Remove the supernatant containing plasmid DNA promptly and pipet into the QIA pre-spin columns, centrifuge at ≥18,000× g for 1 min at room temperature.
8. Remove the liquid in the collection tube.
9. Wash the column with 500 µl PB buffer, centrifuge at ≥18,000× g for 1 min at room temperature.
10. Apply 750 µl PE buffer to the spin column, centrifuge at ≥18,000× g for 1 min at room temperature.
11. Remove the flowthrough in the collection tube, centrifuge at ≥18,000× g again for 1 min at room temperature.
12. Place the spin column containing the DNA in a new microcentrifuge tube.
13. Apply 50 µl EB buffer (or TE buffer containing RNAse inhibitor) to the spin column,

elute the DNA for 5 min.

14. Centrifuge at ≥18,000× g for 1 min at room temperature. The solution in the microcentrifuge tube contains the plasmid DNA. Store at -20°C.

2.4.3 Large scale plasmid purification

Perform according to the protocol of QIAGEN® Plasmid Maxi Kit (10).

1. Dilute 2 ml starter culture (result from chapter ***2.3.3.3***) into a 100 ml selective LB medium, grows at 37°C for 16 h with vigorous shaking (about 300 rpm).
2. Harvest the bacterial cells by centrifuge at 6,000× g for 15 min at 4°C.

Before use the P1 buffer, add the RNAse provided by the kit to the P1 buffer. Use one vial of RNAse A (spin down briefly) per bottle of P1 buffer, to achieve a final concentration of 100 μg/ml

3. Resuspend the bacterial pellet in 10 ml P1 buffer.
4. Add 10 ml P2 buffer, mix thoroughly by gently inverting 4-6 times at room temperature (less than 5 min).
5. Add 10 ml chilled N3 buffer and mix immediately but gently by inverting 4-6 times (less than 5 min) and incubate on ice for 30 min.
6. Centrifuge at ≥18,000× g for 30 min at 4°C.
7. Remove the supernatant containing plasmid DNA promptly.
8. Equilibrate a QIAGEN-Tip-500 by applying 10 ml buffer QBT, and allow the column to empty by gravity flow.
9. Apply the supernatant from step 8 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
10. Wash the QIAGEN-tip with 2× 30 ml QC buffer.
11. Elute DNA with 15 ml QF buffer.
12. Precipitate DNA by adding 10.5 ml (0.7× volume) room temperature isopropanol to elute the DNA.
13. Mix and centrifuge immediately at 15,000× g for 30 min at 4°C, carefully decant the supernatant.
14. Wash the DNA pellet with 5 ml of room temperature 70% ethanol and centrifuge at 15,000× g for 10 min at 4°C, carefully decant the supernatant.
15. Air-dry the pellet for 10 min and redissolve the DNA in a suitable volume of TE buffer (pH 8.0).

2.4.4 Quantification of the plasmid concentration

After small-scale purification or large-scale purification of the plasmid, 2 μl product of the purification was diluted in 98 μl sterile, deionized water, and counted by the DNA-counter

accurately. Only the sample with an OD 260/280 ≥ 1.6 was used in the followed steps.

2.5 FuGENE 6 mediated transfection

According to the protocol provided by the manufacture, the 6:1, 3:1 or 3:2 ratios of volume FuGENE 6 [μl] and mass DNA [μg] was performed, which provided the excellent transfection levels.

2.5.1 Kits and equipment

Kits and equipments	Company
4-chamber slide	Nunc, USA
35-mm culture dish	Nunc, USA
FuGENE 6	Roche, Germany

2.5.2 Preparation fro the transfection

2.5.2.1 Preparation of cell culture

To minimize both the intra- and extra- experimental variance in conditions of transfection efficiency, the cells are harvested from log-growth phase and plated at a consistent density.

1. One day before transfection, the cells are trypsinized in log –growth phase.
2. Adjust the cell concentration and seed the cells in the chosen cell culture vessel: plate $3×10^5$ cells in 35-mm culture dish with 2 ml medium, seed $1×10^4$ cells per chamber (about 2 cm^2 culture surfaces) in a 4-chamber slide. Cultured overnight, it will achieve the desired density of 50%-80% confluence.
3. According to the different cultured size, the starting volume of FuGENE 6 reagent and the starting mass of DNA were adjusted in proportion to the relative surface area (see the refs showed in Table 2-6)..

2.5.2.2 Preparation of DNA

It is critical to determine accurately the plasmid DNA concentration using 260 nm absorption. Determine the DNA purity using a O.D 260nm/280nm ration, the ration should be ≥1.6. The concentration of plasmid DNA in sterile TE buffer or water should be between 0.02 μg/μl and 2.0 μg/μl.

2.5.2.3 Preparation of FuGENE 6 reagent: DNA mixture

Using Fugene 6 reagent: DNA mass on ratios of 3:1, 3:2 and 6:1 (μl: μg, μl for FuGENE 6 reagent and μg for DNA, respectively). These ratios will function very well for commonly used adherent cells. The FuGENE 6 reagent: DNA complex must be prepared in the

serum-free medium.

1. Before the transfection, the FuGENE 6 reagent should be warmed to the room temperature for more than 30 min.
2. The serum-free medium must first be pipetted to a freshly microcentrifuge tube. Dilute the FuGENE 6 reagent with serum-free medium (without any antibiotics or fungicides). The order and manner of accession is critical.
3. The FuGENE 6 reagent was pipet directly into medium without allowing contact with the walls of the plastic tube.
4. Vortex for 1 s or flick the tube to mix, incubate for 5 min at room temperature.
5. According to the Table 2-6, add proportionally amount of the plasmid DNA to the FuGENE 6 reagent diluted in the serum-free medium, which were prepared in step 1.
6. Mix and incubate the complex: tap the tube or vortex for 1 s to mix the contents. Incubate the transfection reagent-DNA complex for minimum of 15 minutes at room temperature. (Continued incubation for up to 45 min, for some cell lines up to 2 h, will not affect the transfection efficiency.)

Table 2-6: Refers to the setting up of transfection reactions system

Cultured Container	Surface area cm^2	Medium volume ml	FUgene6 Reagent (µl)		Mass of DNA (µg)		Working complex (µl/well)
			Amount	Range	Amount	Range	
96well MTP	0.3	0.1-0.2	0.15	0.1-0.3	0.05	0.03-0.06	5
24well MTP	1.9	0.5-2.0	0.6	0.6-1.8	0.2	0.2-0.4	20
12well MTP	3.8	0.5-1.0	1.5	1.2-3.6	0.5	0.4-0.8	50
35mm dish	8	2.0	3.0	3.0-9.0	1.0	1.0-2.0	100
6well MTP	9.4	2.0	3.0	3.0-9.0	1.0	1.0-2.0	100
60mm dish	21	4.0-6.0	6.0	6.0-20.0	2.0	2.0-4.5	200
10mm dish	55	10.0	18.0	17.0-51.0	6.0	5.6-11.0	600
T-25 bottle	25	5.0-7.0	7.5	7.5-24.0	2.5	2.5-5.3	250
T-75 bottle	75	15.0-30.0	24.0	24.0-72.0	8.0	8.0-16.0	800

2.5.3 Transfection

2.5.3.1 Procedures

1. Remove culture vessel from the incubator. Removal of growth medium is not necessary.

2. Add the transfection reagent: DNA complex to the cells in a drop-wise manner. Swirl the wells or flasks to ensure distribution over the entire plate surface.
3. Return the cells to the incubator until the assay for gene expression is to be performed.
4. Incubate the cells for 48-72 h. The length of incubation depends upon the transfected vector construct, the cell type being transfected and the type of protein being expressed.

2.5.3.2 Note

•For stable transfection experiments, the complex-containing medium should be left unchanged until the cells need to be passaged and treated with selection antibiotics.

•To prepare transfection complexes for larger experiments or parallel experiments, proportionally increase the quantity of all components according to the total surface area of the cell-culture vessel being used (Table 2-6).

•For ease-of-use when transfecting small volumes, as in 96 wells with 0.1 ml, prepare 100 μl of complex and add 5 μl to each well.

•Test different media and optimize the level of each sera medium component for these effects. It is not usually necessary to remove the transfection reagent:DNA complex, but it is necessary to feed cells with fresh media for extended growth periods.

2.6 RNA Reverse Transcription

2.6.1 Kits and equipment for RNA reverse transcription

Kits and equipment	Company
RNeasy Mini Kit	Qiagen, Germany
Reverse transcription kit	Promega, Germany
DNA counter	Bio-RAD, USA
dNTP	Invirtrogen, USA
centrifuge	Heraeuse, Germany

2.6.2 Extraction of total RNA

Total RNA of cell lines and samples were isolated according to the procedure provided by the kits of MiniRNA (Qiagen, Germany). Cells were grown to 70%-80% confluence in 10-cm plates. The concentration of RNA was measured by the ***DNA Counter*** (Bio-RAD, USA).

2.6.2.1 Before use

1. β-Mercaptoethanol (β-ME) must be added into RLT buffer before use. Add 10 μl β-ME per 1 ml RLT buffer. RLT Buffer is stable for 1 month after addition of β-ME. *Notice: β-ME is toxic.*
2. RPE buffer is supplied as concentrate. Before using for the first time, add 4 volumes of ethanol (100%), as indicated on the bottle, to obtain a working solution.

2.6.2.2 Extraction from cultured cells

1. Harvest cells, when the cell cultured on a T-25 bottle achieved an 80% confluence (about 1~2×10^6 cells), discard the culture medium and wash by sterile pre-chilled PBS.
2. Discard the PBS totally, add 350 μl buffer RLT to the bottle, and incubate on ice for 3~5 min.
3. Disrupt cells on ice by RLT buffer. Collect cell lysate with a rubber scrape. Pipet lysate into a freshly microcentrifuge tube. Pipet the lysate several times to mix, and ensure that no cell clumps are visible.
4. Homogenize the sample. Pass the lysate at least 20 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.
5. Add 350 μl (1× volume) of 70% ethanol to homongenize lysate and mix well by pipetting gently.
6. Apply up to 700 μl of the sample, including any precipitate that may have formed to an RNeasy Mini column placed in a 2 ml collection tube (supplied by the kit). Close the tube gently and centrifuge for 30 s at ≥11,000 g. Discard the flow-through in the collection tube.
7. Add 700 μl RW1 buffer to the RNeasy column. Incubate for 2 min, close the tube gently and centrifuge for 30 s at ≥11,000 g. Discard the flow-through in the collection tube.
8. Transfer the RNeasy column into a new collection tube (supplied by the kit). Pipet 500 μl RPE buffer onto the RNeasy column. Close the tube gently and centrifuge for 30 s at ≥11,000 g to wash the column. Discard the flow-through in the collection tube.
9. Add another 500 μl RPE buffer onto the RNeasy column. Close the tube gently and centrifuge for 2 min at ≥18,000 g to dry the RNeasy column silica-gel member. Discard the flow-through.
10. Centrifuge for 1 min at ≥18,000 g, discard the flow-through and collection tube.
11. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 30 μl RNase-free, DNase-free waster directly onto the RNeasy column silica-gel member.

Close the tube gently and centrifuge for 1 min at ≥11,000 g (if the RNA expected more than 30 μg, repeat this step with second 30 μl water).

2.6.2.3 Extraction from sample tissues

1. Excise 20 mg sample from -70°C frozen ovarian tissues (about 2~5×10^6 cells) and place into a 1.5 ml tube in liquid nitrogen.
2. Grind the tissues thoroughly with a liquid-nitrogen-cooled 2 ml tube. Do not allow the tissue to thaw.
3. Add 350 μl buffer RLT to the bottle, and homogenize the samples by passing the lysate at least 20 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.
4. Centrifuge the tissues at 22,000 g for 1 min at 4°C. Carefully transfer the supernatant to a new 1.5 ml tube by pipetting. Only use this supernatant in subsequent steps.
5. Add 350 μl (1× volume) of 70% ethanol to the cleared lysate and mix well by pipetting gently.
6. Apply up to 700 μl of the sample, including any precipitate that may have formed to an RNeasy Mini column placed in a 2 ml collection tube (supplied by the kit). Close the tube gently and centrifuge for 30 s at ≥11,000 g. Discard the flow-through in the collection tube.
7. Add 700 μl RW1 buffer to the RNeasy column. Incubate for 2 min, close the tube gently and centrifuge for 30 s at ≥11,000 g. Discard the flow-through in the collection tube.
8. Transfer the RNeasy column into a new collection tube (supplied by the kit). Pipet 500 μl RPE buffer onto the RNeasy column. Close the tube gently and centrifuge for 30 s at ≥11,000 g to wash the column. Discard the flow-through in the collection tube.
9. Add another 500 μl RPE buffer onto the RNeasy column. Close the tube gently and centrifuge for 2 min at ≥18,000 g to dry the RNeasy column silica-gel member. Discard the flow-through.
10. Centrifuge for 1 min at ≥18,000 g, discard the flow-through and collection tube.
11. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 30 μl RNase-free, DNase-free waster directly onto the RNeasy column silica-gel member. Close the tube gently and centrifuge for 1 min at ≥11,000 g (if the RNA expected more than 30 μg, repeat this step with second 30 μl water).

2.6.3 The first stand cDNA synthesis

According to protocols provided by the manufacture (Reverse transcription, Promega), 2 μg of RNA was used on the 40 μl reverse transcription system

1. The reagents were pipetted in a 250 μl PCR-reaction tube sequentially following the Table 2-7.
2. Pipet the mixture gently.
3. Pre-incubate at 22°C 10 min, reverse transcription at 48°C for 45 min, then inactivation the reaction at 95°C for 2 min.

Table 2-7: The reverse transcription system

Reagents	Volume/Reaction
Sterile, deionized water	Adjust to a final volume of 40 μl
10× RT buffer	4 μl
10 mM dNTP	4 μl
Hexamere	4 μl
RNasin	1 μl
Template RNA	2 μg
M-Mlv	2 μl

2.7 LightCycler real-time quantitative RT-PCR

2.7.1 Kits and equipment for LighCycler PCR

Kits and equipment	Company
LightCycler instrument	Roche, Germany
LightCycler capillaries	Roche, Germany
LightCycler Control Kit DNA	Roche, Germany
LightCycler Standard bench	Roche, Germany
LightCycler benchtop microcentrifuge	Roche, Germany
A rotor for 20.0 μl capillaries	Roche, Germany

Brief theory of LightCycler PCR system

The “ROCHE LightCycler FastStart DNA Master SYBR Green I” kit is a ready-to-use “Hot Start” reaction mixture for quantitative PCR applications, which contain Faststart Taq DNA polymerase and DNA double-stand specific SYBR Green I dye for detection. The FastStart Taq DNA polymerase is a modified form of thermostable recombinant

Taq DNA polymerase. It is inactive at room temperature and it is "activated" by removing the blocking groups at a high temperature (for example an incubation at 95 for a maximum of 10 min). SYBR Green I dye binds to the amplified PCR products and the amplification is detected by its fluorescence. Combining amplification with melting curve analyzed by the Roche LightCycler Run 5.0 Software can enhance specificity and sensitivity of amplification reactions.

2.7.3 Primers design and synthesis

The primer sets were design by Oliogo 6.0 software (The software was downloaded from the official website) and confirmed by BLAST homologous analysis (National Center of Biology Information, NCBI). All primers were synthesized by the TIB Molecular Lab (Berlin, Germany). The sequence of special primer sets and their location in the nucleotide were listed in Table 2-8.

Table 2-8: Special primer sets of ERRs and ER family and GADPH

GENE	Primers sequence		Position	Length
ERRα	UP	5'-Tgg TCC AgC TCC CAC TCg CT-3'	(471-490)	483 bp
	DOWN	5'-TgA gAC ACC AgT gCA TTC ACT g-3'	(932-953)	
ERRα-1	UP	5'-AAA gTg CTg gCC CAT TTC TAT-3'	(1537-1557)	100 bp
	DOWN	5'-CCT TgC CTC AgT CCA TCA T-3'	(1618-1636)	
ERRβ	UP	5'-TCA AgT gCg AgT ACA TgC TC-3'	(601-620)	340 bp
	DOWN	5'-gAA ATT TgT AAg CTC Agg TA-3'	(921-940)	
ERRβ-1	UP	5'-CAT TCC Acg gAg gCA TCC TC-3'	(168-187)	537 bp
	DOWN	5'-TgC Aag CCT CgC Agg Agg CC-3'	(686-705)	
ERR_{γ}	UP	5'-CTC gCC ACC TCT CTA CCC TT-3'	(389-408)	395 bp
	DOWN	5'-gCT TgT ACT TCT gCC gAC CTC-3'	(763-781)	
ERα	UP	5'-AGATAATCGACGCCAGGGTG-3'	(822-841)	115 bp
	DOWN	5'-AGCATAGTCATTGCACACTGCA-3'	(914-936)	
ERβ	UP	5'-TCCTATGTAGACAGCCACCATG-3'	(521-542)	103 bp
	DOWN	5'-GCCCACCTTCCAAGTTAGTGAC-3'	(602-623)	
GADPH	UP	5'-ACg CAT TTg gTC gTA TTg gg-3'	(68-83)	230 bp
	DOWN	5'-TgA TTT Tgg Agg gAT CTC gC-3'	(278-298)	

2.7.4 Preparation of Q-PCR

2.7.4.1 Primers

Use the primers at a final concentration of 0.3-1 M each. A recommended starting concentration is 0.5 M each. Refer to the LightCycler operator's manual for recommendations concerning the primer design; usually the amplification product should be no longer than 700 bp.

2.7.4.2 MgCl

For specific and efficient amplification, it is essential to optimize the target-specific $MgCl_2$ concentration. The Kit contains a basic $MgCl_2$ concentration of 1mM (final concentration). The optimal concentration for PCR with the LightCycler instrument may vary from 1 to 5 mM. The Table 2-9 below gives the volumes of the $MgCl_2$ stock solution (vial 2, blue cap), which give the designated $MgCl_2$ concentration by different using in a 20-μl final Q-PCR system.

Table 2-9: The refs of Mg^{2+} concentration used in the Q-PCR system

To a final Mg2+ concentration	Volumes of 25 mM MgCl2 stock Solution
1(mM)	0.0(μl)
2(mM)	0.8(μl)
3(mM)	1.6(μl)
4(mM)	2.4(μl)
5(mM)	3.2(μl)

2.7.4.3 Negative control

Always run a negative control (set the negative control by replacing the template DNA with PCR-grade water) with the samples.

2.7.4.4 Series of standard control

The plasmids pSG-hERRα, pSG-mERRβ, pSG-mERRγ, pD-GADPH were used to set the serial dilution of standard control. For an X μg/ml target gene (Y bp size) stock solution, the copies number of target gene can be calculated out as follows:

$$\text{The target gene copies/}\mu l = \frac{X\ \mu g/ml \times 10^{-9} \times 6 \times 10^{23}}{660D \times Y\ (bp)}$$

Then, use the TE buffer to serially dilute the target gene stock solution to final concentration of $1\times10^7, 1\times10^6, 1\times10^5, 1\times10^4, 1\times10^3, 1\times10^2, 1\times10^1$ copies/μl working solutions and using as standard control.

2.7.4.5 Dilution of sample

The cDNA samples reverse transcripted from 1µg total RNA of ovarian cancer cell lines or ovarian tissues, were diluted in a ratio of 1:10 by TE buffer.

2.7.5 Setting the experimental protocol

The experimental protocol consists of four programs. The parameters of these four programs were listed in followed table. The analysis software was ***LightCycler*** version 5.02. The display model was set up as follow: Channel 1(F1). A real-time running program for amplification of hERRα could be seen in Figure 2.3. In this figure, the marker “A” pointed out the display model.

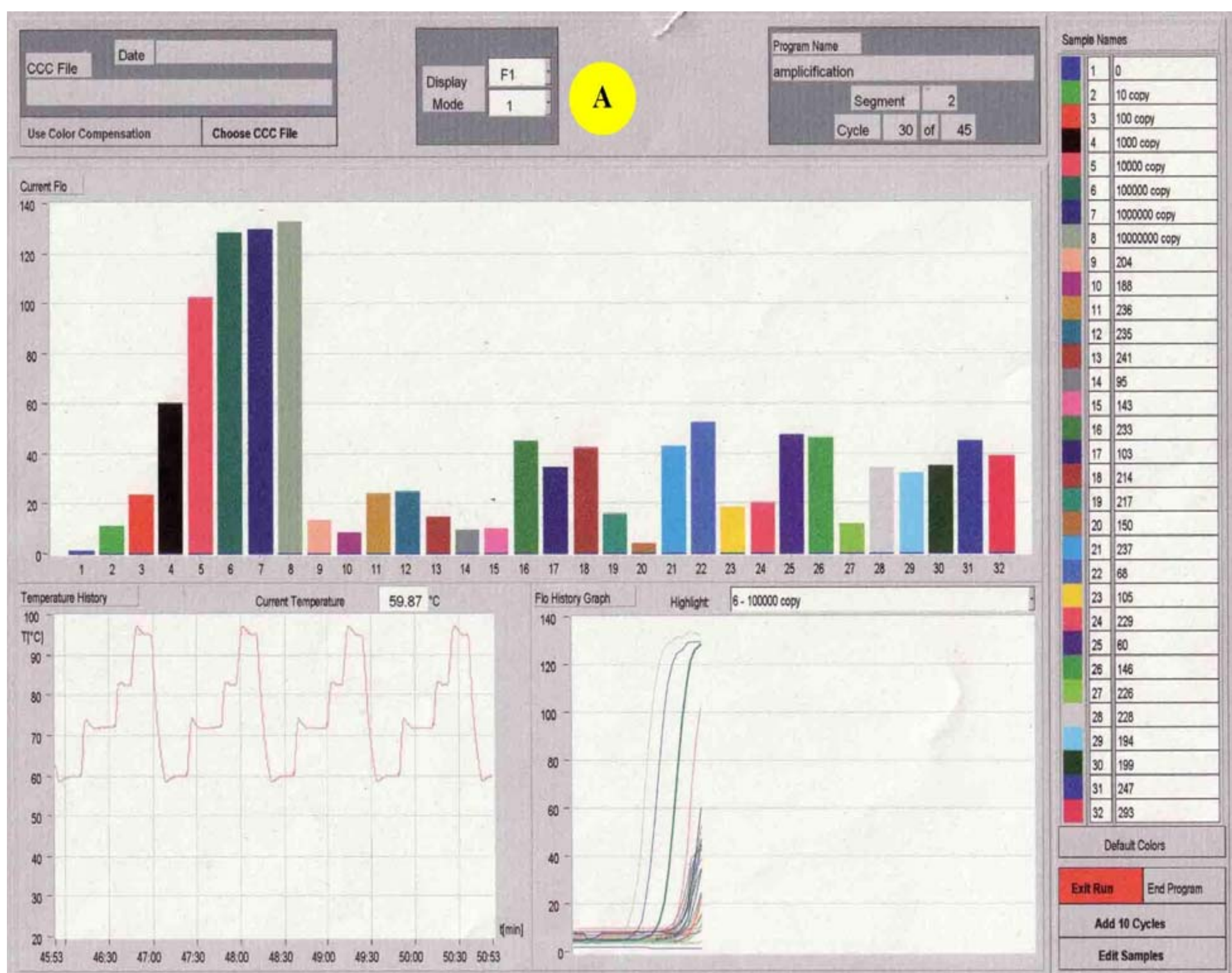


Figure 2.3: A real-time running program of LightCycler Q-PCR. Marker A (yellow) point out the display model in the program: F1, 1.The lane 1 stands for the negative control and the lane 2 to lane 8 stand for the series of positive control (from 10 copies/µl to 10^7 copies/µl). The other lanes stand for the respectively samples. The down-left column showed the real-time temperature achieved during the running program.

According to the protocol provided by the LighCycler Master SYBR Green I kit, the parameters were set up as followed programs:

2.7.5.1 Program 1: pre-incubation and denaturation of the cDNA

Program Parameters	Cycle Program Data
Cycles	1
Analysis Mode	None
Temperature Target	*Segment 1*
Target Temperature (°C)	95
Incubation time(h:min:s)	15:00 min
Temperature Transition Rate(°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size(°C)	0.0
Step Delay (Cycles)	0
Acquisition Model	None

2.7.5.2 Program 2: Amplification of the target sequence

Program Parameters	Cycle Program Data			
Cycles	1			
Analysis Mode	None			Acquisition
Temperature Targets	*Segment 1*	*Segment 2*	*Segment 3*	*Segment 4*
Target Temperature (°C)	95	60*	72	82
Incubation time(h:min:s)	10 s	10 s**	15 s***	0 s
Temperature Transition Rate (°C/s)	20.0	20.0	20.0	20.0
Secondary Target Temperature(°C)	0	0	0	0
Step Size(°C)	0.0	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0	0
Acquisition Model	None	None	None	Single.

* Annealing temperature (°C) dependent on the primer of special gene, for GADPH and hERRβ were 58°C and hERβ was 62°C;

** Annealing time (s) 0–10 dependent on the primer sets; for hERRβ was 15 s;

*** Elongation temperature (°C) 72 and elongation time (s) length of product can calculate as: [bp]/25, for hERRβ was 10s

2.7.5.3 Program 3: Melting curve analysis for product identification

Program Parameters	Cycle Program Data		
Cycles	1		
Analysis Mode	Melting Curves		
Temperature Targets	*Segment 1*	*Segment 2*	*Segment 3*
Target Temperature(°C)	95	65	95
Incubation time(h:min:s)	0 s	15 s	0 s
Temperature Transition Rate(°C/s)	20.0	20.0	0.1
Secondary Target Temperature (°C)	0	0	0
Step Size(°C)	0.0	0.0	0.0
Step Delay (Cycles)	0	0	0
Acquisition Model	None	None	Cont.

2.7.5.4 Program 4: Cooling the rotor and thermal chamber

Program Parameters	Cycle Program Data
Cycles	1
Analysis Mode	None
Temperature Target	*Segment 1*
Target Temperature (°C)	40
Incubation time(h:min:s)	30 s
Temperature Transition Rate(°C/s)	20.0
Secondary Target Temperature (°C)	0
Step Size(°C)	0.0
Step Delay (Cycles)	0
Acquisition Model	None

2.7.6 Procedures of Q-PCR

1. Thawing the reagents, mix gently, centrifuge brief and keep on ice.
2. In a 1.5 ml microcentrifuge tube chilled on ice, add the following components in the order mentioned below and mix gently.
3. Pipet 18 μl Master Mix into the pre-cooled LightCycler capillary, add 2 μl of the plasmid DNA template or 2 μl cDNA reverse transcription from 1 μg total RNA in a final volume of 20 μl per reaction.

Table 2-10: The LightCycler PCR work system

Reagents	Volume	Final Concentration
RNAse, DNAse free water	13.2 μl*	
25mM Mg2+	0.8 μ*	2 mM
10mM sense primer	1 μl	0.5 mM
10mM antisense primer	1 μl	0.5 mM
Master SYBR Green I	2 μl	1×
Sample cDNA (0.1μg RNA/20μl)	2 μl	5 ng/μl RNA

*4 mM Mg2+ was need in the Q-PCR system for hERRβ-1

4. Seal each capillary with a stopper and place the adapter, containing the capillary, into a standard benchtop microcentrifuge. Centrifuge at 700× g for 5 s (3000 rpm in a standard benchtop microcntrifuge). Note: place the centrifuge adapters in a balanced arrangement within the centrifuge.
5. Place the capillaries in the rotor of the LightCycler instrument. Cycle the samples as described in section 3.3.

2.8 Confocal Scan Microscope

FuGENE6 (0.6 μl) and hERRα-GFP reporter plasmid (0.1 μg) were mixed in 19 μl serum-free medium for 15 min. 5 μl mixture per well were added into the normal cultured medium in a 4-chamber-cultured slide (Nunc, USA). 20 μl serum-free DMEM medium was used as a negative control and added into the control group. After 48 h of culture, the medium was discarded. Ovarian cancer cell lines OVCAR-3 and SKOV-3 were washed 2 times with PBS for 5 min and fixed by methanol at -20°C for 10 min. After fixation, the cells were washed by 2 times PBS for 5 min each time. Cell nuclei were stained with 4',6-diamidino-2 phemylindole (DAPI, 1:1000 dilution). The cells were analyzed using a confocal scanning microscope (Leica, Solms, German) according the setting listed in the Table 2-11.

Table 2-11: The setting for the confocal scanning microscope

Target	Staining	Excited Illumination	Color
hERRα-GFP	**No**	**480 nm(FITC)**	**Green**
Nuclear	**DAPI(1:1000)**	**330 nm(UV)**	**Blue**

2.9 Protein extraction and quantification assay

2.9.1 Kits and equipment for protein assay

Kits and equipment	Company
CellLytic™ NuCLEAR™ extraction kit	Sigma, USA
BCA protein assay kit	Pierce, USA
ELISA reader	Dynal Biotech & Nordic

2.9.2 Whole-cell protein extraction

Perform all the steps on ice.

1. Plated cell lines at a density of 3×10^5 cells/well in 35-mm cultured dish.
2. Discard the medium after the cells achieved a density of 80% confluence. Wash the cells by 2 times PBS.
3. Discarded the PBS, pipet 100 μl of 62.5 mM Tris-HCl (pH6.8) containing 2% sodium dodecy sulfate, 10% glycerol and 50 mM DTT into the cells.
4. Incubate on ice for 3-5 min, and then harvest the cells into a new and fresh micocentrifuge tube by a rubber scraper.
5. lyses the cells by repeated crushing through a 20-gauge needle (0.9 mm diameter)
6. Centrifuge at 14,000 rpm, at 4°C for 15 min.
7. Transfer the supernate to a fresh microcentrifuge tube and go on the Protein Quantification Assay, or store at -20°C.

2.9.3 Nuclear and Cytoplasmic protein extraction

2.9.3.1 Reagents

- 10× Lysis Buffer, hypotonic, 7 ml (100 mM HEPES, pH 7.9, with 15 mM $MgCl_2$ and 100 mM KCl)
- 5× Lysis Buffer, isotonic, 14 ml (50 mM Tris-HCl, pH 7.5, with 10 mM $MgCl_2$, 15 mM CaCl2, and 1.5 M Sucrose)
- Extraction Buffer, 10 ml (20 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, and 25% (v/v) Glycerol)
- 3× Dilution and Equilibration Buffer, 90 ml (60 mM HEPES, pH 7.9, with 4.5 mM MgCl2, 0.6 mM EDTA, 30 mM KCl, and 75% (v/v) Glycerol)
- Dithiothreitol (DTT), 0.4 ml (1 M DTT in deionized water)
- Protease Inhibitor Cocktail, 1 ml (contains 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), Pepstatin A, Bestatin, Leupeptin, Aprotinin, and

trans-Epoxysuccinyl- L-leucyl-amido (4-guanidino)-butane (E-64))

• IGEPAL® CA-630 10% Solution, 4 ml (10% IGEPAL CA-630 in deionized water)

2.9.3.2 Procedures

Perform all steps at 2–8°C. Use precooled buffers and equipment. Ensure all the solutions are defrosted and homogeneous. All centrifugations are performed at 4°C with precooled rotors. The final concentration of DTT in the solutions should be 1 mM. The protease inhibitor cocktail should be diluted 100-fold in the final solutions. Nuclear Protein Extraction from 100 μl of packed cell using a detergent (IGEPAL CA-630) Calculate accordingly for different packed cell volumes.

1. Dilute the 1 M DTT solution with deionized, sterile water to a concentration of 0.1 M. For small-scale preparations (below 100 µl total) the 1 M DTT stock solutions should be diluted to 0.01 M.
2. Prepare 1× Lysis Buffer, hypotonic, from the 10× Lysis Buffer, hypotonic, by diluting 10-fold with sterile, deionized water. For fragile cells use 1× Lysis Buffer, isotonic, prepared from the 5× Lysis Buffer, isotonic, to replace the 1× Lysis Buffer, hypotonic. To 500 μl of 1× Lysis Buffer either hypotonic or isotonic), add 5 μl of the prepared 0.1 M DTT solution and 5 µl of the protease inhibitor cocktail.
3. Collect cells: adherent cells form 70-90% confluent monolayer culture.
   - *Remove the growth medium from the cells.*
   - *Rinse the cells twice with PBS, being careful not to dislodge any of the cells.*
   - *Discard the PBS.*
   - *Scrape the cells using fresh PBS into an appropriate conical centrifuge tube.*
   - *Centrifuge for 5 minutes at 450 x g.*
   - *Decant and discard the supernatant.*
4. Estimate the packed cell volume (PCV).
5. Add 500 μl (5× PCV) of 1× Lysis Buffer (including DTT and protease inhibitors) to 100 µl of PCV. Resuspend the cell pellet gently. Avoid foam formation. If working with small volumes, the suspended cells may be transferred to a microcentrifuge tube.
6. Incubate the packed cells in the selected lysis buffer on ice for 15 minutes, allowing cells to swell. Take several microliters of the cells in the lysis buffer and view them under the microscope. If massive cell lysis is detected under the microscope or a gelatinous mass is observed, the cells may be fragile. In this case, use the Lysis Buffer, isotonic, for cell lysis and consider eliminating the incubation step.

7. To the swollen cells in lysis buffer, add the 10% IGEPAL CA-630 solution to a final concentration of 0.6% (6 μl per 100 μl of mixture). Vortex vigorously for 10 s.
8. Centrifuge immediately for 30 seconds at 10,000-11,000x g.

   • *In order to assess the degree of lysis, before centrifugation, take a sample of the suspended cells and view the nuclei under the microscope. Lysis can be observed by the addition of the Trypan Blue solution to an aliquot of cells. The dye is excluded from the intact cells, but stains the nuclei of lysed cells. If lysis of nuclei is observed under the microscope or if a gelatinous mass is observed, lyses the cells with a lower final concentration of IGEPAL CA-630.*

   • *If cells are not lysed, increase the final percentage of IGEPAL CA-630 in the resuspended cells (step 7).*

   •*For fragile cells use lower concentrations of IGEPAL CA-630. Avoid vortexing the cells and centrifuge at a slower speed.*
9. Transfer the supernatant to a fresh tube. This fraction is the cytoplasmic fraction.
10. Add 1 μl of the prepared 0.1 M DTT solution and 1 μl of the protease inhibitor cocktail to 98 μl of the Extraction Buffer. If it is necessary to extract the proteins of interest at a lower salt concentration, dilute the Extraction Buffer with 1× Dilution and Equilibration Buffer.

    Note: The salt concentration in the Extraction Buffer is 0.42 M, a commonly used extraction condition. In rare cases, a lower or a higher salt concentration may be needed for a better extraction of a particular protein. In that case, dilute the Extraction Buffer with the 1× Dilution and Equilibration Buffer or add NaCl to the Extraction Buffer to reach the desired salt concentration.
11. Resuspend the crude nuclei pellet in ~70 μl (2/3× PCV) of Extraction Buffer containing the DTT and protease inhibitor cocktail.
12. Mount the tube on a vortex mixer and agitate at medium to high speed for 15-30 min. Avoid foam formation.
13. Centrifuge for 5 min at 20,000-21,000× g.
14. Transfer the supernatant to a clean, chilled tube.
15. Snap-freeze the supernatant in aliquots with liquid nitrogen and store at -70°C.

2.9.4 BCA protein quantification assay

2.9.4.1 Preparation of diluted BSA serial standards

The BSA standards were prepared by diluting 1 ml of 2.0 mg/ml BSA stock solution with

different volume of 0.9% sodium chloride solution.

1. 1 ml of 2.0 mg/ml BSA stock solution was mixed with 1 ml 0.9% sodium chloride solution to get a final concentration of 1.0 mg/ml BSA solution.
2. A list of standard dilutions with a working range from 0μg/ml to 500 μg/ml is shown bellow:

Table 2-12: BSA standard working solutions

Volume of 1.0 mg/ml BSA	Volume of diluents	Final BSA concentrations
0 μl	1000 μl	0 μg/ml (A)
5 μl	995 μl	5 μg/ml (B)
10 μl	990 μl	10 μg/ml (C)
25 μl	975 μl	25 μg/ml (D)
50 μl	950 μl	50 μg/ml (E)
100 μl	900 μl	100 μg/ml (F)
250 μl	750 μl	250 μg/ml (G)
500μl	500μl	500μg/ml (H)

2.9.4.2 Protein quantification assay

1. 2 μl samples or 2 μl standard BSA solutions were added with 0.9% sodium chloride solution to a final volume of 200 μl working solution.
2. 30 μl working solution per well were pipetted into 96-well plate, each sample or each concentration BSA standard was added into 2 wells to get an average value.
3. The BSA color developed reagent was prepared by mixing 50 ml BCA protein assay reagent A (contains BCA) with 1 ml reagent B (contains $CuSO_4$).
4. 200 μl of BSA color developed reagent per well were pipetted into the wells of a microwell-plate sequentially.
5. The plate was the covered and incubated at 37°C for 6 min. After incubation, the plate was cooled to room temperature before final measurement.
6. The protein concentration was measured by colorimetric assay at λ=570 nm with an ELISA reader. The program “Revelation Version 2.0” provided by the manufacturer was used for the data record and analysis.

2.10 Western-Blot

2.10.1 Kits and antibody

Kits and antibody	Company
Nitrocellulose membrane	Pinzette, Germany
Filter paper	Whatman, USA
Chemiluminesence Luminol	Dynal Biotech & Nordic, Germany
Anti-HA mouse monoclonal antibody	Roche, Germany

2.10.2 Preparation of the protein

1. Adjust the concentration of nuclear protein, cytoplasmic protein and whole-cell protein to 50μg/20μl, 100μg/20μl and 100μg/20μl, respectively, by 1× lysis buffer.
2. Add 4 μl 6× SDS loading buffer to the 20 μl sample proteins, protein marker and positive control protein, mix gently.
3. Denature the protein at 95°C for 10 min, centrifuge brief and proceed to the protocol listed in chapter **2.10.4**.

2.10.3 Preparation of the SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

1. Mix ingredients of resolving gel gently. In the order showed in Table 2-13, ensure no air bubbles formed in the gels. Pour resolving gel into the glass plate assembly.
2. Overlay gel with isopropanol to ensure a flat surface and to exclude air bubbles. Wash off isopropanol with water after gel was set (about 15 min).
3. Prepare the stack gel as before (The reagents were listed in Table 2-14), then pour stack gel on to the top of set resolving gel, insert comb, allow setting, removing comb, and fill with electrophoresis buffer.
4. Assemble top tank onto glass plate. Fill with electrophoresis buffer.

Table 2-13: **8% Resolve gel (20 ml)**

Reagents	Volume
Deionized water	11.6 ml
30% Acrylamide stock solution	3.0 ml
1.5 mol/L Tris (pH8.8)	5.0 ml
10% SDS	0.2 ml
10% APS	0.1 ml
TEMED	0.010 ml

Table 2-14: 4% Stack gel (10ml)

Reagents	Volume
Deionized water	6.4 ml
30% Acrylamide stock solution	1.0 ml
0.5 mol/L Tris (pH6.8)	2.5 ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.010 ml

2.10.4 Immunoblot

2.10.4.1 Electrophoresis

1. Load the prepared sample proteins into the wells in the stack gel carefully.
2. Run an electrophoresis to separate the proteins. In stack gel, 8V/cm, in resolving gel 15 V/cm, 100 mA per gel for 120 min.

2.10.4.2 Transfer to the membrane

1. During the electrophoresis, prepare 1× transfer buffer by diluting 50 ml 10× transfer buffer stock solution with 450 ml distilled water and mix thoroughly.
2. Draw a line 0.5 cm from the top edge of an 8 × 10 cm nitrocellulose sheet and soak it in 1× transfer buffer for about 5 min.
   ③ Nitrocellulose is both fragile and flammable and easily contaminated during handling.
   ③ When soaking the nicrocellulose, wet first one side and then turn the sheet over and wet the other, to prevent trapping air within the filter.
3. After electrophoresis, wash the gel by 1× fresh PBS and carefully cut off the stack gel.
4. In a clean tray, prepare the blot-system in the following order.
   A. Place 200 ml of blot buffer into a tray and add 3 piece of sponge pat and 3 pieces of filter paper slightly larger than the electrophoretic gel.
   B. Remove the gel from the electrophoresis chamber after the proteins have been separated. Place the gel into the tray containing the filter paper.
   C. Gently slide the gel onto the top of the filter paper. Keep the stacking gel off the paper until the last moment, since it tends to stick and make repositioning difficult.

D. Holding the gel and the filter paper together, carefully remove them from the tray of blot buffer and transfer the paper and gel to a pad of the blot cell with the gel facing up.
E. Transfer the nitrocellulose sheet (ink side down) onto the top of the gel and line up the line drawn on the sheet with the top of the stacking gel.
F. Once the gel and nitrocellulose touch they can not be separates.

Negative Pole (-)
3 pieces of sponge pat
3 pieces of filter paper
Gel
Nitrocellulose membrane (marker side down)
3 pieces of filter paper
3 pieces of sponge pat
Positive Pole (+)

5. Roll a glass stick across the surface of the nitrocellulose to remove any air bubbles and insure good contact between the gel and nitrocellulose.
6. Lay another 3 pieces of wet filter paper on top of the nitrocellulose creating a sandwich of paper-gel- nitrocellulose-paper, all lying on the pad of the blot cell, and roll a glass stick again.
7. Add a second 3 piece of sponge pad to the top of the sandwich and place the entire group inside of the support frame of the blot cell, and assemble the blot cell so that the nitrocellulose side of the sandwich is toward the positive terminal.
8. Fill the tank with transfer buffer and give 100 mA electrify per membrane blotting for 1.5 h.

2.10.4.3 Immune hybridization

1. After the blot, wash the nitrocellulose membrane in 1× fresh PBS and then incubate in the 1×blocking buffer for 2 h at room temperature.
2. In a clean tray with lid, dilute the primary antibodies (mouse anti HA) with 1× blocking buffer in a ratio of 1:1,000-1,500, then transfer the nitrocellulose to the tray. Close the lid and incubate at 4°C overnight while shaking at 45 rpm.
3. Wash the nitrocellulose membrane for 3 times (15min per time) by washing buffer.

4. Incubate the nitrocellulose membrane with second antibodies (rabbit anti mouse) diluted in the block buffer (1:5,000) at room temperature while shaking at 300 rpm.
5. Wash the nitrocellulose membrane 3 times (15 min per time) by washing buffer.
6. Incubate with 1× assay buffer, 2 times, 2 min per time.
7. Put the nitrocellulose membrane in a piece of clean film and wipe off the assay buffer.
8. Incubate with the Chemiluminescence Luminol Working Solution (mixed with 2 ml CSPD® ready to use solution and 100 μl Nitro block) for 5 min, protect from light.
9. Wipe off the excess working solution and develop the image of nitrocellulose membrane with a Koda film in a dark room.

2.11 Immunocytochemistry and immunohistology

2.11.1 Reagents and antibodies

To classify different ER isoforms expression pattern, ERα (F-10) monoclonal antibody, ERβ (N-19) polyclonal antibody and ABC Detection system were purchased from Santa Cruz Bitotechnology (Canada). To detect the human ERRα, ERRβ ERRγ protein, anti-hERRα, anti-hERRβ, anti-hERRγ mouse monoclonal antibody were purchased from Perseus (Japan). To our knownledge, these anti ERRs antibodies are the first commercial antibodies.

Reagents and Antibodies	Company
Monoclonal anti-ERRα	Perseus, Japan
Monoclonal anti-ERRβ	Perseus, Japan
Monoclonal anti-ERRγ	Perseus, Japan
Monoclonal anti-ERα	Santa Cruz, Canada
polyclonal anti-ERβ	Santa Cruz, Canada
ABC staining kit	Santa Cruz, Canada
Fast-red stain kit	Dako, Germany

2.11.2 Immunocytochemistry

2.11.2.1 Prepare for the working buffers

1. Blocking serum: in mixing bottle 1, combine 75 μl normal blocking serum stocks with 5 ml PBS.
2. Biotinylated secondary antibody: in mixing bottle 2, combine 75 μl normal blocking serum stock, 5 ml PBS and 25 μl biotinylated secondary antibody stock.

3. AB enzyme reagent: in AB mixing bottle, combine 50 μl reagent A (avidin), 50 μl reagent B (biotinylated HRP) and 2.5 ml PBS. Mix and let stand for approximately 30 min.
4. Peroxidase substrates: in substrate mixing bottle, combine 1.6 ml distilled H_2O, 5 drops 10× substrates buffer, and 1drop 50× DAB chromogen and 1 drop 50× peroxidase substrate. Sufficient for 15-20 slides.

2.11.2.2 Preparation of cell slides

1. When the cells cultured in a T-25 bottle achieved about 70%-80% confluence (about 1~2×10^6 cells), discard the culture medium.
2. Incubated cells with 0.25% typrinse for about 5 min.
3. Stop the typrinse working by adding fresh medium supplement with 10% serum.
4. Centrifuge at 1,000 g for 5 min and then discard the supernatant carefully.
5. Resuspend the cells and seed the cells into the chambers in the 4-chamber-slides.
6. After incubation in the incubator for 48 h, discard the medium and wash cells with 2× PBS. Fix the cells by 100% methanol at -20°C for 20 min. Then proceed to staining (see the protocol in chapter ***2.11.3.2***).

2.11.3 Immunohistology

2.11.3.1 Preparation of paraffin-embedded tissue sections

1. Cut paraffin-embedded tissue into 4-6 μm thick sections using the microtome and apply to pre-treated slides.
2. Deparaffinize as follows: 3× xylenes for 5 min, 2× 100% ethanol for 10 min, 2× 95% ethanol for 10 min. Wash in deionized water for 5 min on a stir plate.

2.11.3.2 Staining

1. Incubate slide with 0.1% hydrogen peroxide diluted in PBS for 10 min, washed by 2 times PBS for 5 min per time.
2. Incubate sections with 1.5% blocking buffer for 1 h.
3. Incubate with primary antibodies diluted in 1.5% blocking buffer (1:200~400) at 4°C overnight, wash the cells with changes of PBS for 3 times, 5 min per time.
4. Incubate with AB enzyme reagent provided by the ABC detection kit for 30 min, wash with 3 changes of PBS for 5 min per time.
5. Incubate with 2~3 drops peroxidase substrate for 3 min or until desired stain intensity developed. The section should be checked for staining by rinsing with water and viewing under a microscope. If necessary, an additional staining can be continued.

6. Dehydrate as follows: 2× 95% ethanol for 1 min each, 2× 100% ethanol for 1 min each, 3× xylenes for 30 s each. Wipe off excess exylens.
7. Immediately add 1~2 drops of permanent mounting medium and cover with a glass cover slip. Observe under the microscope.

2.12. Serum CA-125 assay by ELISA

The patient sera were stored at –20°C until analysis. The commercially available ELISA-Kit (MEDAC, Hamburg, Germany) was used. Serum CA-125 level of samples was determined using an ELISA reader according to the instructions of the manufacturer. An independent technician, who did not have any clinical information about the patients, performed the serum CA-125 ELISA assay.

2.13 Statistical Analysis

The statistical analysis was performed via SPSS, version 11.0 software (SPSS, Inc, Chicago, USA). ANOVA and the independent T-sample test were used to compare the parametric data; Chi-square test was used to analyze non-parametric data. The correlations between expression of ERRs and clinicopathological parameters were analyzed by the bivariate correlation analysis. To analyze the prognosis of ovarian cancer patients according to the different expression of ERRs, the primary outcome measure of this non-randomized study was overall survival; secondary outcome was progression-free survival. Overall survival was defined as the time from first surgery to death from any cause. Progression-free survival was defined as the time from first surgery to first clinical or pathological evidence of recurrence. The Kaplan-Meier method was used to calculate overall survival time or progression-free survival time and survival curves were compared by the log-rank test. Multivariate survival analysis was performed using the Cox regression model. Generally, p value less than 0.05 was considered as significant.