Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58758R3
Full Title:	Detecting the Ligand-binding Domain Dimerization Activity of Estrogen Receptor Alpha Using the Mammalian Two-hybrid Assay
Keywords:	Mammalian two-hybrid assay; Estrogen receptor alpha; nuclear receptor; ligand binding domain; Tamoxifen; Species difference; Dimerization; Protein-protein interaction
Corresponding Author:	Yukitomo Arao, Ph.D. National Institutes of Health Research Triangle Park, NC UNITED STATES
Corresponding Author's Institution:	National Institutes of Health
Corresponding Author E-Mail:	araoy@niehs.nih.gov
Order of Authors:	Yukitomo Arao, Ph.D.
	Kenneth S Korach
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Research Triangle Park, North Carolina

TITLE:

Detecting the Ligand-binding Domain Dimerization Activity of Estrogen Receptor Alpha Using the Mammalian Two-hybrid Assay

AUTHORS AND AFFILIATIONS:

Yukitomo Arao[1], Kenneth S. Korach[1]

[1]Reproductive Developmental Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health (NIH), Research Triangle Park, NC, United States of America

Corresponding Author:

Yukitomo Arao (araoy@niehs.nih.gov)

Email Address of Co-author:

Kenneth S. Korach (korach@niehs.nih.gov)

KEYWORDS:

Mammalian two-hybrid assay, estrogen receptor alpha, nuclear receptor, ligand-binding domain, dimerization, tamoxifen, selective estrogen receptor modulator

SUMMARY:

We present a method for analyzing the 4-hydroxy-tamoxifen-dependent estrogen receptor alpha ligand-binding domain dimerization activity using the mammalian two-hybrid assay.

ABSTRACT:

Estrogen receptor alpha (ERα) is an estrogenic ligand-dependent transcription regulator. The sequence of ERα protein is highly conserved among species. It has been thought that the function of human and mouse ERαs is identical. We demonstrate the differential 4-hydroxy-tamoxifen (4OHT) effect on mouse and human ERα ligand-binding domain (LBD) dimerization activity using the mammalian two-hybrid (M2H) assay. The M2H assay can demonstrate the efficiency of LBD homodimerization activity in mammalian cells, utilizing the transfection of two protein expression plasmids (GAL4 DNA-binding domain [DBD] fusion LBD and VP16 transactivation domain [VP16AD] fusion LBD) and a GAL4-responsive element (GAL4RE) fused luciferase reporter plasmid. When the GAL4DBD fusion LBD and the VP16AD fusion LBD make a dimer in the cells, this protein complex binds to the GAL4RE and, then, activates a luciferase gene expression through the VP16AD dependent transcription activity. The 4OHT-mediated luciferase activation is higher in the HepG2 cells that were transfected with the mouse ERα LBD fusion protein expression plasmids than in the human ERα LBD fusion protein expression plasmid transfected cells. This result suggests that the efficacy of the 4OHT-dependent mouse ERα LBD homodimerization activity is higher than human ERα LBD. In general, the utilization of the M2H assay is not ideal for the evaluation of nuclear receptor LBD dimerization activity, because agonistic ligands enhance the basal level of the LBD activity and that impedes the detection of LBD dimerization activity. We found that 4OHT does not enhance ERα LBD basal activity. That is a key factor for being able to determine and detect the 4OHT-dependent LBD dimerization

activity for successfully using the M2H assay. ERα LBD-based M2H assays may be applied to study the partial agonist activity of selective estrogen receptor modulators (*e.g.*, 4OHT) in various mammalian cell types.

INTRODUCTION:

Estrogen receptor alpha (ERα) is an estrogenic ligand-dependent transcription regulator. The amino acid sequence of ERα is highly conserved among species. Because of the higher homology between human and mouse ERα, the function of these receptors is thought of as identical, and the differential activity of estrogenic substances (*e.g.*, tamoxifen) in those species is caused by the species' differences in chemical metabolisms rather than by the structural differences of ERα. ERα has highly conserved domain structures that are common among the nuclear receptor (NR) superfamily, designated A to F domains. The E domain or ligand-binding domain (LBD) includes the ligand-binding pocket and the transcriptional activation function 2, named AF-2. The F domain is localized immediately adjacent to the E domain and is the most variable domain among the NRs. Even between human and mouse ERα, the homology of the F domain is significantly lower than that of the other domains[1]. The ligand-bound LBD of ERα enhances the homodimerization of the ERα protein to bind the specific estrogen-responsive DNA element directly for regulating the ligand-dependent gene transcription (classical action of ERα). Crystallographic studies have revealed the differential positioning of helix 12 (AF-2 core domain) with estradiol (E2)- or 4-hydroxy-tamoxifen (4OHT)-bound LBD dimers[2,3]. The ERα F domain (45 amino acids) connect helix 12 directly. However, there is no information regarding the effect of this extension of 45 amino acids (F domain) from the helix 12 on the ERα LBD dimerization. In this study, we demonstrate the contribution of the F domain to the species-specific 4OHT-dependent ERα LBD homodimerization using a mammalian two-hybrid (M2H) assay.

The M2H assay is a method to demonstrate protein-protein interactions in mammalian cells introducing the three different plasmid DNAs: two protein expression plasmids, which express GAL4 DNA-binding domain (DBD) fusion ERα LBD and VP16AD fusion ERα LBD, and a GAL4RE-fused luciferase expression reporter plasmid. When the GAL4DBD fusion ERα LBD and the VP16AD fusion ERα LBD interact (make a dimer) in the cells, this protein complex binds to the GAL4RE and, then, activates luciferase gene expression through the VP16AD-dependent transactivation function. The level of LBD homodimerization can be evaluated by the luciferase activity.

The yeast two-hybrid (Y2H) assay is an alternative method based on the same principle that uses yeast as the host environment. Previous reports using the Y2H system demonstrated that F-domain-truncated human ERα LBD increases E2-dependent coactivator recruitment, concluding that the F domain prevents E2-mediated transcription[4]. This result is inconsistent with other reports which have demonstrated the attenuated transcriptional activity of F-domain-truncated human ERα in the mammalian cells[5,6]. Recently, our study, using the M2H system, demonstrated that the E2-dependent coactivator recruitment activity of human ERα LBD is decreased by F domain truncation in mammalian cells and is consistent with transcription activity[1]. These observations suggest that the physiological role of protein-protein interaction differs in a cell type-specific manner and context. The M2H assay can demonstrate protein-protein interaction

activity in the same cellular context that is used for determining the transcriptional activity. This provides an advantage of the M2H assay compared to the Y2H or other *in vitro* protein-protein interaction analyses.

There remain questions regarding the molecular mechanisms of the partial agonist activity of selective estrogen receptor modulators (SERMs) (*e.g.*, 4OHT) to regulate ERα-mediated transcription. ERα LBD-based M2H assays may be applied to study the mechanism of the partial agonist activity of SERMs in various mammalian cell types.

PROTOCOL:

1. Preparation of Plasmids for the Mammalian Two-hybrid Assay

1.1. Use the following plasmids: pG5-Luc, pBIND-ERαEF, and pACT-ERαEF.

Note: The plasmids are available from the authors upon request and are sent on paper. pG5-Luc is the reporter plasmid that contains five repeats of GAL4 responsive elements fused with the luciferase expression unit (**Figure 1A**). pBIND-ERαEF is the protein expression plasmid for GAL4DBD-fused ERα LBD proteins. pBIND-ERαEF contains a Renilla luciferase expression unit for normalizing the transfection efficiency (**Figure 1B**). pACT-ERαEF is the protein expression plasmid for the VP16AD-fused ERα LBD proteins (**Figure 1C**). The diagram of the expressed protein structure from the plasmids is shown in **Figure 2**.

1.2. Prepare plasmid DNAs.

1.2.1. Cut out the plasmid-loaded area from the paper using clean scissors and put it into a 1.5 mL tube. Wear gloves and use clean tweezers to pick up the plasmid-loaded paper. Add 100 µL of Tris-EDTA (TE) buffer (pH 8.0) and vortex well.

1.2.2. Add 1 µL of the plasmid DNA solution from step 1.2.1 to competent *Escherichia coli* cells (see **Table of Materials**) and keep the tube on ice for 15 min.

1.2.3. Heat-shock the plasmid-added competent cells; incubate the tube(s) in 42 °C water bath for 30 s and, then, keep them on ice for 2 min.

1.2.4. Add 250 µL of super optimal broth with catabolite repression (SOC) medium (room temperature) to the tube(s) and culture with shaking at 37 °C for 30 min.

1.2.5. Plate 100 µL of the cultured *E. coli* onto a prewarmed Luria broth (LB) plate with 100 µg/mL ampicillin (10 cm-diameter dish) and culture at 37 °C overnight.

1.2.6. Pick one colony from the plate and add it into a 14 mL tube containing 2 mL of terrific broth (TB) with 100 µg/mL ampicillin. Culture with shaking at 37 °C until the medium is faintly clouded.

1.2.7. Add 1 mL of the cultured medium from step 1.2.6 to an autoclaved 250 mL glass flask containing 50 mL of TB with 100 µg/mL ampicillin. Culture with shaking at 37 °C for 20 - 24 h.

1.2.8. Transfer the cultured *E. coli* to a 50 mL conical tube and centrifuge at 3,450 x *g* at room temperature for 30 min using the swing-bucket rotor. Discard the medium. Save the *E. coli* pellet at -80 °C.

1.2.9. Add 3 mL of cell resuspension solution (see **Table of Materials**) to the *E. coli* pellet and suspend it completely. Add 3 mL of cell lysis solution (see **Table of Materials**), mix the tube gently by inverting it 10x, and keep it on ice for 5 min (keep the time punctually). Add 6 mL of neutralization solution (see **Table of Materials**), mix the tube steadily with tapping, and then, keep it on ice for 10 min.

1.2.10. Centrifuge the tube at 3,450 x *g* at 4 ˚C for 30 min using the swing-bucket rotor. Transfer the DNA-containing solution to a new 50 mL conical tube and add 10 mL of DNA purification resin (see **Table of Materials**). Suspend the resin gently.

1.2.11. Centrifuge the tube at 625 x *g* for 1 min using the swing-bucket rotor. Discard the solution and keep the resin at the bottom.

1.2.12. Add 15 mL of column wash solution (80 mM potassium acetate, 8.5 mM Tris-HCl [pH 7.5], 40 µM EDTA, and 55% ethanol) to the 50 mL conical tube and shake it gently to suspend the resin. Repeat step 1.2.11.

Note: The column wash solution must be prepared using diethyl pyrocarbonate (DEPC)-treated water or nuclease-free water.

1.2.13. Add 5 mL of column wash solution to the 50 mL conical tube and suspend the resin with a 5 mL pipet. Apply the resin to the column (see **Table of Materials**) that is set on the vacuum manifold. Vacuum the column until the resin dry. Add 6 mL of column wash solution to the column and vacuum.

1.2.14. Once the resin dries visually, detach the reservoir from the column. Transfer the bottom section of the column (resin) to a 1.5 mL tube. Centrifuge the column at maximum speed for 2 min using a microcentrifuge to dry the resin completely.

1.2.15. Transfer the bottom section of the column (resin) to a new 1.5 mL tube. Add 300 µL of nuclease-free water to the column and wait until the whole resin is soaked.

1.2.16. Centrifuge the column at maximum speed for 1 min to collect the plasmid DNA-containing solution (200 - 250 µL).

1.2.17. Treat the DNA-containing solution with phenol-chloroform (1:1), then with chloroform as follows.

1.2.17.1. Add the same volume of phenol-chloroform (1:1) to the DNA-containing solution (200 - 250 µL), shake well, and centrifuge at maximum speed for 2 min using a microcentrifuge. Collect the upper layer (DNA-containing solution) and add it to a new 1.5 mL tube. Repeat this step 1x.

1.2.17.2. Add the same volume of chloroform to the DNA-containing solution (200 - 250 µL), shake well, and centrifuge at maximum speed for 1 min. Collect the upper layer (DNA-containing solution) and add it to a 1.5 mL tube.

Note: Endotoxin (lipopolysaccharide) induces cellular signaling in some cells. To avoid such an unpredicted effect, step 1.2.17 is recommended, which can reduce endotoxin contamination.

1.2.18. Precipitate the plasmid DNA using the ethanol precipitation technique as follows.

1.2.18.1. Add 0.05x final volume of 3 M sodium acetate (pH 5.5) and 0.5x final volume of 100% ethanol to the DNA-containing solution. For example, add 27.8 µL of 3 M sodium acetate and 278 µL of 100% ethanol to 250 µL of the DNA solution (the final volume is 555.8 µL).

1.2.18.2. Keep the solution at -80 °C for 20 min at least. Centrifuge at maximum speed for 20 min at 4 ˚C. Discard the solution and keep the pellet. Add 200 µL of 75% ethanol and rinse the inside of the tube. Centrifuge for 20 min at 4 °C, discard the solution, and keep the pellet. Dry the DNA pellet using a vacuum concentrator.

1.2.18.3. Add 100 - 250 µL of TE buffer (pH 8.0) to the tube. Dissolve the DNA pellet and check the DNA concentration using a spectrophotometer at a wavelength of 260 nm. Keep the DNA concentration around 0.5 mg/mL.

2. Mammalian Two-hybrid Assay

2.1. Maintain the cells.

2.1.1. Culture human hepatocellular carcinoma HepG2 cells in a 750 mL, 175 cm^2 tissue culture flask with the phenol red-free minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS; heat-inactivated), 2 mM L-glutamine, and 1% penicillin-streptomycin solution (antibiotics) for maintaining the cells.

2.1.2. Culture the cells in a 5% CO_2-supplemented, 37 °C incubator until the cells are approximately 90% confluent.

Note: To reduce estrogenic background activity, phenol red-free medium should be used for all M2H experiments. This step should be performed inside a clean bench/biological safety cabinet. The cells should be maintained following the general mammalian cell culture protocol[7].

2.2. Prepare the cells for transfection.

Note: The following procedural steps should be performed inside the clean bench/biological safety cabinet. The cells are cultured/incubated in a 5% CO_2-supplemented, 37 °C incubator in this step every time.

2.2.1. Replate the 90% confluent cells to the 24-well plates; rinse the cells 1x with phosphate-buffered saline (PBS).

2.2.2. Add 7 mL of 0.05% trypsin-EDTA solution to the culture flask and soak the cells for 1 - 2 min while keeping the culture flask in a clean bench at room temperature. Remove a part of the trypsin-EDTA solution and leave 1 - 2 mL of the solution in the culture flask. Incubate the culture flask in the incubator for 1 - 2 min.

2.2.3. Smack the bottom of the flask to detach the cells and check the cells under the microscope.

Note: If the cells are still attached to the flask, incubate for another 1 - 2 min and, then, repeat step 2.2.3.

2.2.4. Suspend the cells in the phenol red-free MEM supplemented with 10% charcoal-stripped FBS (heat-inactivated), 2 mM L-glutamine, and 1% penicillin-streptomycin solution.

Note: Charcoal-stripped FBS must be used to reduce the effect of serum-derived endogenous estrogen.

2.2.5. Count the cell number and seed cells in a 24-well plate at a density of 1.2 x 10^5 cells/well. Assign three wells for each data point (triplicate).

2.2.6. Culture the cells overnight. Continue to step 2.4.1.

2.3. Prepare a DNA mixture for transfection.

Note: The following plasmid DNAs are transfected in one well: 100 ng of pG5-Luc reporter plasmid, 50 ng of expression plasmids for GAL4DBD fusion proteins (pBIND), and 50 ng of expression plasmids for VP16AD fusion proteins (pACT).

2.3.1. To analyze the ligand-dependent ERα LBD dimerization activity, prepare the following combinations (**Figure 3**). Combination (i): pG5-Luc + pBIND-hERαEF + pACT, and pG5-Luc + pBIND-mERαEF + pACT. Combination (ii): pG5-Luc + pBIND-hERαEF + pACT-hERαEF, and pG5-Luc + pBIND-mERαEF + pACT-mERαEF. Combination (iii): pG5-Luc + pBIND + pACT-hERαEF, and pG5-Luc + pBIND + pACT-mERαEF.

Note: Combination (i) represents the basal experimental level of human ERα LBD and mouse ERα LBD, respectively. If the result shows a remarkably high level with ligand alone, this method is

unfeasible for use with that ligand (*e.g.*, diethylstilbestrol [DES]). Combination (ii) represents the levels of dimerized human ERα LBD and dimerized mouse ERα LBD, respectively. Combination (iii) represents negative controls; use this set only when the experiment is performed for the first time to evaluate the background level of the system.

2.3.2. Before mixing, calculate the total DNA amount required based on the number of wells for transfection. For experiments conducted in triplicates and at five data points, mix the following plasmid DNAs in two 1.5 µL tubes for combinations (i) and (ii): pG5-Luc, 5 (data points) x 3 (wells) x 100 ng = 1,500 ng (15 µL); pBIND-mERαEF, 5 (data points) x 3 (wells) x 50 ng = 750 ng (7.5 µL); pACT (for combination i) or pACT-mERαEF (for combination ii), 5 (data points) x 3 (wells) x 50 ng = 750 ng (7.5 µL).

Note: The concentration of each plasmid DNA solution is 0.1 µg/µL.

2.3.3. Precipitate the DNA mixture using the ethanol precipitation technique. Add 0.05x final volume of 3 M sodium acetate (pH 5.5) and 0.5x final volume of 100% ethanol to the DNA mixture. Then, vortex the tube.

Note: For example, add 3.4 µL of 3 M sodium acetate, and 34 µL of 100% ethanol to the 30 µL of plasmid DNA mixture exemplified in step 2.3.2. This step helps to keep an invariable transfection condition, and it is not necessary to perform this step in a biological safety cabinet.

2.3.4. Keep the mixture overnight at -20 °C. Centrifuge it at maximum speed for 20 min at 4 °C. Discard the solution (the pellet is invisible). Add 120 µL of 75% ethanol to the tube; then, rinse the inside of the tube. Centrifuge for 20 min at 4 °C. Discard the solution and dry the DNA mixture using a vacuum concentrator for 30 min.

2.4. Perform the transfection.

Note: The following steps should be performed in a clean bench or a biological safety cabinet.

2.4.1. The following morning, rinse the cells seeded in a 24-well plate (from step 2.2.6) 2x with 0.5 mL of PBS (room temperature).

2.4.2. Add warm (37 °C) fresh phenol red-free MEM supplemented with 10% charcoal-stripped FBS (heat-inactivated) and 2 mM L-glutamine without antibiotics to each well (0.5 mL medium/well). Keep the cells in a 5% CO_2-supplemented, 37 °C incubator.

2.4.3. Suspend the dried plasmid DNA mixture (from step 2.3.4) in Dulbecco's modified Eagle's medium (DMEM) (no serum, phenol red-free, without glutamine; 13 µL/well). Calculate the amounts of DMEM from the number of wells for transfection.

Note: 15 wells for combination (i) x 13 µL = 195 µL, and 15 wells for combination (ii) x 13 µL = 195 µL.

2.4.4. Suspend the transfection reagent (see **Table of Materials**; 1.5 µL/well) in DMEM (12.5 µL/well) in another 1.5 mL tube. Calculate the amounts of transfection reagent and DMEM from the number of wells for transfection.

Note: 30 [15 wells for combination (i) and 15 wells for combination (ii)] x 1.5 µL of transfection reagent + 30 x 12.5 µL of DMEM = 420 µL.

2.4.5. Add an equal amount of the transfection reagent medium (from step 2.4.4) to each tube (from step 2.4.3). Incubate the tubes for 5 - 10 min at room temperature.

Note: 200 µL of transfection reagent medium + 195 µL of combination (i) DNA mixture = 395 µL, and 200 µL of transfection reagent medium + 195 µL of combination (ii) DNA mixture = 395 µL. It is important to add an equal volume of transfection reagent medium to the tubes of combination (i) and (ii). It is not necessary to use up the medium in this step.

2.4.6. Add the combined mixture (from step 2.4.5) to each well (25 µL/well) of the 24-well plate (from step 2.4.2) and incubate the cells for 6 h in the 5% CO_2-supplemented, 37 °C incubator.

2.4.7. Remove the transfection medium from the 24-well plate. Add warm (37 °C) fresh phenol red-free MEM supplemented with 10% charcoal-stripped FBS (heat-inactivated), 2 mM L-glutamine, 1% penicillin-streptomycin solution, and ligand (suspended in ethanol) (0.5 mL medium/well). Culture the cells for 16 - 18 h in the 5% CO_2-supplemented, 37 °C incubator.

2.5. Harvest the samples.

2.5.1. Rinse the cells with 1 mL of PBS and, then, add 100 µL of 1x passive lysis buffer (see **Table of Materials**).

2.5.2. Vigorously shake the 24-well plates with a vortex mixer to detach the cells.

2.5.3. Freeze the plates (cell lysates) 1x by liquid nitrogen or save them at -80 °C before analysis. Immediately before the assay, vigorously shake the plates on a shaker until they are at room temperature.

Note: This process prevents irregularly appearing anomalous values of luciferase activity.

2.6. Perform a dual-luciferase assay.

2.6.1. Prepare the reagents for the dual-luciferase assay system (see **Table of Materials**).

2.6.1.1. For making the luciferase assay substrate (LAS) buffer, add all luciferase assay buffer into the luciferase assay substrate which is in a glass brown bottle. Save the LAS buffer at -20 °C.

Note: The LAS buffer should be warmed to room temperature before use.

2.6.1.2. For making the Renilla luciferase substrate (RLS) buffer, mix Renilla luciferase substrate and Renilla luciferase buffer in a ratio of 1:50. Make fresh RLS buffer for every assay and use a black tube or a tube shaded by tinfoil. Calculate the amount of RLS buffer making a fresh buffer.

Note: The RLS buffer should be warmed to room temperature before use.

2.6.2. Set the reagents in the microplate reader.

2.6.2.1. Wash the injection lines 2x with 70% ethanol; then, wash the lines 2x with water.

Note: This is important to prevent disturbing the assay results.

2.6.2.2. Set the LAS buffer to the P-injector line (first injection) and set the RLS buffer to the M-injector line (second injection). Do not mix these buffers. RLS buffer inhibits luciferase activity.

2.6.3. Apply 10 µL of harvested samples (from step 2.5.3) to the 96-well white plastic plate.

2.6.4. Apply the following settings to the microplate reader. For the P-injector, use a volume of 50 µL, a delay of 2 s, and a speed of 50 µL/s. For the M-injector, use a volume of 50 µL, a delay of 2 s, and a speed of 50 µL/s. Set an integration time of 15 s. Set the temperature to 25 °C.

2.6.5. Run the microplate reader.

2.6.6. Record the values of the luciferase activity (IPValue) and the values of the Renilla luciferase activity (IMValue) using the data acquisition program (see **Table of Materials**).

2.6.7. Wash the injection lines after the data collection (see step 2.6.2.1).

2.7. Perform data analysis.

2.7.1. Use the normalized values (IPValue/IMValue) for data analysis.

2.7.2. Set the normalized value of combination (i) [pG5-Luc + pBIND-ERαEF + pACT] with vehicle (0 nM ligand) as 1 for the calculation of the basal level and the homodimerized ERα LBD level. The levels are represented as “Relative activity”.

REPRESENTATIVE RESULTS:

Figure 3 displays the scheme of possible responses in the combination (i) and combination (ii) plasmid-transfected cells. The experimental results are shown in **Figure 4**. The activity of combination (i) (pG5-Luc + pBIND-mERαEF + pACT) shows stimulation by 10 nM E2 (**Figure 4A**), because the ERα LBD contains the ligand-dependent transactivation functional domain, AF-2. Agonist (*e.g.*, E2) that binds to the ERα LBD recruits transcription coactivator(s) to the AF-2

domain. This event causes transcriptional activation without interaction with the VP16AD fusion LBD and makes it difficult to evaluate the LBD homodimerization activity because of the nonspecific background (**Figure 3C**). The activity of combination (ii) (pG5-Luc + pBIND-mERαEF + pACT-mERαEF) was observed with 1 nM and 10 nM E2 treatment (**Figure 4A**). It would be possible to determine the agonist-dependent ERα LBD homodimerization activity if the appropriate ligand concentration for detecting the activity of combination (ii) without the activation of combination (i) can be found (*e.g.*, the condition of 1 nM E2 in **Figure 4A**). Thus, it is difficult to detect the agonist-mediated ERα LBD homodimerization activity using the M2H assay. On the other hand, the activity of combination (i) with 4OHT was the same as the vehicle control (0 nM) level (**Figure 4B**). These results suggest that the AF-2 function of ERα is not activated by 4OHT. Thus, the M2H assay is a feasible option for analyzing the homodimerization activity of ERα LBD with 4OHT (**Figure 3B, 3E**).

The activity from the combination of mouse ERα LBD expression plasmids (pBIND-mERαEF + pACT-mERαEF) with 4OHT was significantly higher than the combination of human ERα LBD expression plasmids (pBIND-hERαEF + pACT-hERαEF) (**Figure 5A, 5B**). This result indicates that the 4OHT-dependent homodimerization activity of mouse ERα LBD is more potent than human ERα LBD. Furthermore, we analyzed 4OHT-dependent LBD homodimerization activity using the human-mouse F-domain-swapped ERα LBD expression plasmids, mERαEhF (mouse ERα E domain with human F domain) and hERαEmF (human ERα E domain with mouse F domain). The homodimerization activity of mERαEhF was significantly lower than that of mERαEF. In contrast, the homodimerization activity of hERαEmF was significantly higher than hERαEF (**Figure 5C**). Thus, it seems like the F domain has an influence on species-specific 4OHT-dependent ERα LBD homodimerization activity.

These results suggest that the ERα LBD dimerization activity of 4OHT-like ligands, such as SERMs, can be detected by the M2H assay. Next, we demonstrate a possible way to analyze the ERα LBD homodimerization activity of agonistic chemicals. ERα LBD dimerization activities of E2 and diethylstilbestrol (DES) can be detected by the M2H assay when using a single-amino-acid-mutated ERα LBD (mouse ERα-I362D). This mutation disrupts the E2-dependent coactivator recruitment activity of ERα LBD[8]. Combination (i) (pG5-Luc + pBIND-mERα-I362D + pACT) was not activated by E2 nor DES at any concentration we analyzed (**Figure 6B**), which was different from WT ERα LBD (**Figure 6A**). The ERα-I362D LBD dimerization activity of DES was more potent than E2 (**Figure 6B**). This mutant can be used for a comparative study of agonist-dependent ERα LBD dimerization activity; however, it requires further analysis and the characterization of the biological functionality of this mutant ERα.

FIGURE LEGENDS:

Figure 1: Diagram of plasmids used for the M2H assay. (**A**) This panel shows pG5-Luc, the reporter plasmid that contains a GAL4-responsive element (GAL4 RE) fused with a luciferase coding gene. (**B**) This panel shows pBIND-mERαEF, the protein expression plasmid for GAL4DBD fusion mERαEF; Renilla luciferase works for normalizing the transfection efficiency. (**C**) This panel shows pACT-mERαEF, the protein expression plasmid for the nuclear localization signal (NLS)-fused VP16AD fusion mERαEF.

Figure 2: Diagram of ERα LBD expression plasmids used in this experiment.

Figure 3: Scheme of the M2H assay. This figure shows the representative condition of the cells transfected with combination (i) plasmids (left) and the cells transfected with combination (ii) plasmids (right).

Figure 4: Ligand-dependent dimerization activity of mouse ERα LBD. (**A**) This panel shows the luciferase activity of the cells transfected with combination (i) plasmids (pG5-Luc + pACT + pBIND-mERαEF) or combination (ii) plasmids (pG5-Luc + pACT-mERαEF + pBIND-mERαEF) with or without E2. (**B**) This panel shows the luciferase activity of the cells transfected with combination (i) plasmids or combination (ii) plasmids with or without 4OHT. The activity is represented as the mean ± the standard deviation (SD). The graph of panel **A** has been recreated from previously published data[11].

Figure 5: Differential dimerization activity of mouse and human ERα LBD with 4OHT. (**A**) This panel shows the luciferase activity of the cells transfected with the combination of mouse ERα LBD expression plasmids (pACT-mERαEF + pBIND-mERαEF) or the combination of human ERα LBD expression plasmids (pACT-hERαEF + pBIND-hERαEF) with or without 4OHT. (**B**) The low activity range of panel **A** is magnified to show the dimerization activity of human ERα LBD and experimental basal levels (pACT + pBIND-mERαEF, pACT + pBIND-hERαEF). (**C**) This panel shows the luciferase activity of the cells transfected with mouse-human F-domain-swapped LBD expression plasmids. mERαEhF denotes mouse ERα E domain fused with human F domain. hERαEmF denotes human ERα E domain fused with mouse F domain. The activity is represented as the mean ± the SD. The graphs have been recreated from previously published data[1].

Figure 6: Detection of agonist-mediated ERα LBD dimerization activity using an AF-2 mutated ERα LBD. (**A**) This panel shows the luciferase activity of the cells transfected with combination (i) plasmids (pG5-Luc + pACT + pBIND-mERαEF) or combination (ii) plasmids (pG5-Luc + pACT-mERαEF + pBIND-mERαEF) with or without ligands; E2 (red), DES (purple). (**B**) This panel shows the luciferase activity of the cells transfected with combination (i) plasmids (pG5-Luc + pACT + pBIND-mERα-I362D) or combination (ii) plasmids (pG5-Luc + pACT-mERα-I362D + pBIND-mERα-I362D) with or without ligands; E2 (red), DES (purple). The activity is represented as the mean ± the SD.

DISCUSSION:

Herein, we described the protocol for the M2H assay, focusing on the assay conditions for detecting the homodimerization activity of ERα LBD as an example. In general, the M2H assay is not popular for the assessment of ligand-dependent ERα LBD dimerization activity. This is due to the ERα LBD possessing a transcriptional activation function; the activity of which disturbs, in some cases, the results of the M2H assay. However, as we demonstrate here, the M2H assay can be used for analyzing the LBD dimerization activity of certain substances that do not activate the AF-2 transactivation function of LBD (*e.g.*, 4OHT, SERMs). To reduce the intrinsic activity of LBD (background activity), we selected the lowest E2-containing charcoal-stripped FBS from the

production lots and used heat-inactivated charcoal-stripped FBS. These factors are important to the success of the M2H assay using the WT ERα LBD and to detect weak interaction activity. Additionally, we showed the ERα LBD dimerization activity of agonists (E2 and DES) using the mERα-I362D mutant, which disrupts the agonist-dependent coactivator recruitment activity to the AF-2. These results suggest that the M2H assay could be more useful for the assessment of ligand-dependent ERα LBD dimerization activity.

In the NR research field, the M2H assay has been used for the assessment of ligand-dependent coactivator or corepressor recruitment activity of NR LBDs[9,10]. The plasmid of pBIND fused with the NR interacting region of the cofactor is used for this type of experiment instead of using the pBIND-ERαEF. This experiment uses the short protein element containing the NR interacting motif (LxxLL) rather than a larger structural domain of the coactivator. One possibility is that the larger coactivator element may activate transcription without the recruitment of the VP16AD fusion LBD; that could disturb the results from the M2H assay. Alternatively, this protocol could be used for analyzing the binding activity of a variety of substances to the ERα. For instance, the cells were transfected with pG5-Luc, pBIND-fused NR interacting motif, and pACT-ERαEF plasmids and, then, treated with various chemicals, such as endocrine-disrupting chemicals or potential hormonal therapeutics. If the luciferase activity is increased by a chemical in this assay, then it is predicted that the chemical should be interacting with the ERα LBD to recruit the NR interacting motif[8].

The M2H assay demonstrates protein-protein interactions in mammalian cells. As we mentioned in the **Introduction**, the physiological role of protein-protein interactions may be different in a cell-type-specific context. M2H assays can provide the results of protein-protein interaction activity in the same cellular context that is used for other experiments, such as the analysis of transcriptional activity. Additionally, it can analyze the effect of cell-signaling modulators (*e.g.*, inhibitors or activators of kinases) involved with protein-protein interactions in the cells. This is an advantage of the M2H assay compared to other *in vitro* protein-protein interaction studies.

However, it needs to be considered that the protein-protein interactions detected *via* the M2H assay might not represent a direct interaction between two proteins. In other words, there is the possibility that cellular factor(s) exist that are allowing a connection between two proteins. If such a consideration arises, then the evaluation of *in vitro* protein-protein interaction studies should be required, such as the GST-fusion protein pulldown assay. In addition, it is better to check the expression level of pBIND- and pACT-plasmid-derived proteins in the cells to evaluate the performance of the assay system. Especially when the interaction activity cannot be detected, the information of the protein expression levels helps to determine the cause of the result. Anti-Gal4DBD antibody and anti-VP16AD antibody can be used for a western blot analysis.

If the laboratory has the experimental set-up for luciferase reporter assays, which can be used for analyzing the transcription activity of regulatory elements or regulating factors, it is quite simple to perform the M2H assay. The M2H assay is an advantageous additive method for transcriptional regulation studies.

ACKNOWLEDGMENTS:

The authors thank Drs. Sueyoshi and Wang at the National Institute of Environmental Health Sciences (NIEHS) for their critical reading of the manuscript. This work was supported by the National Institutes of Health Grant 1ZIAES070065 (to K.S.K.) from the Division of Intramural Research of the NIEHS.

DISCLOSURES:

The authors have nothing to declare.

REFERENCES

1. Arao, Y., Korach, K. S. The F domain of estrogen receptor α is involved in species-specific, tamoxifen-mediated transactivation. *Journal of Biological Chemistry.* **293** (22), 8495-8507, doi:10.1074/jbc.RA117.001212 (2018).

2. Brzozowski, A. M. *et al.* Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature.* **389** (6652), 753-758, doi:10.1038/39645 (1997).

3. Shiau, A. K. *et al.* The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell.* **95** (7), 927-937 (1998).

4. Yang, J., Singleton, D. W., Shaughnessy, E. A., Khan, S. A. The F-domain of estrogen receptor-alpha inhibits ligand induced receptor dimerization. *Molecular and Cellular Endocrinology.* **295** (1-2), 94-100, doi:10.1016/j.mce.2008.08.001 (2008).

5. Montano, M. M., Müller, V., Trobaugh, A., Katzenellenbogen, B. S. The carboxy-terminal F domain of the human estrogen receptor: role in the transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists. *Molecular Endocrinology* (Baltimore, MD). **9** (7), 814-825, doi:10.1210/mend.9.7.7476965 (1995).

6. Koide, A. *et al.* Identification of Regions within the F Domain of the Human Estrogen Receptor α that Are Important for Modulating Transactivation and Protein-Protein Interactions. *Molecular Endocrinology*. **21** (4), 829-842, doi:10.1210/me.2006-0203 (2011).

7. Mitry, R. R., Hughes, R. D. *Human Cell Culture Protocols*. Humana Press, (2011).

8. Arao, Y., Coons, L. A., Zuercher, W. J., Korach, K. S. Transactivation Function-2 of Estrogen Receptor α Contains Transactivation Function-1-regulating Element. *Journal of Biological Chemistry.* **290** (28), 17611-17627, doi:10.1074/jbc.M115.638650 (2015).

9. Huang, H.-J., Norris, J. D., McDonnell, D. P. Identification of a negative regulatory surface within estrogen receptor alpha provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. *Molecular Endocrinology* (Baltimore, MD). **16** (8), 1778-1792, doi:10.1210/me.2002-0089 (2002).

10. Chang, C. Y. *et al.* Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. *Molecular and Cellular Biology.* **19** (12), 8226-8239 (1999).

11. Arao, Y., Hamilton, K. J., Coons, L. A., Korach, K. S. Estrogen receptor α L543A, L544A mutation changes antagonists to agonists, correlating with the ligand binding domain dimerization associated with DNA binding activity. *The Journal of Biological Chemistry.* **288** (29), 21105-21116, doi:10.1074/jbc.M113.463455 (2013).

Figure 1

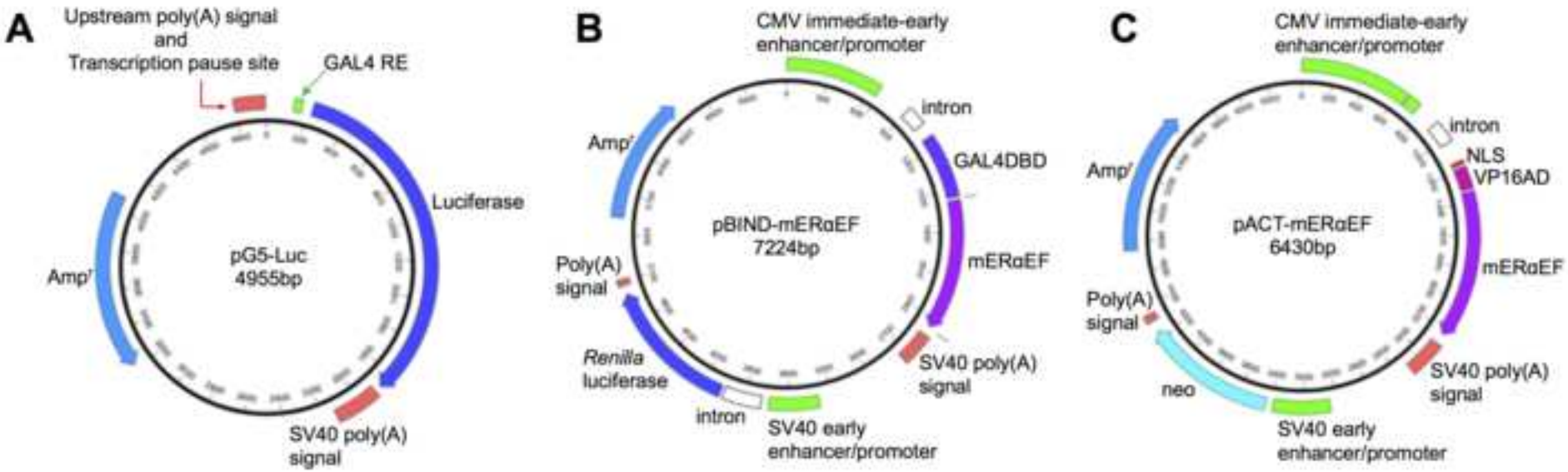

Fig.1 Arao & Korach

Figure 2

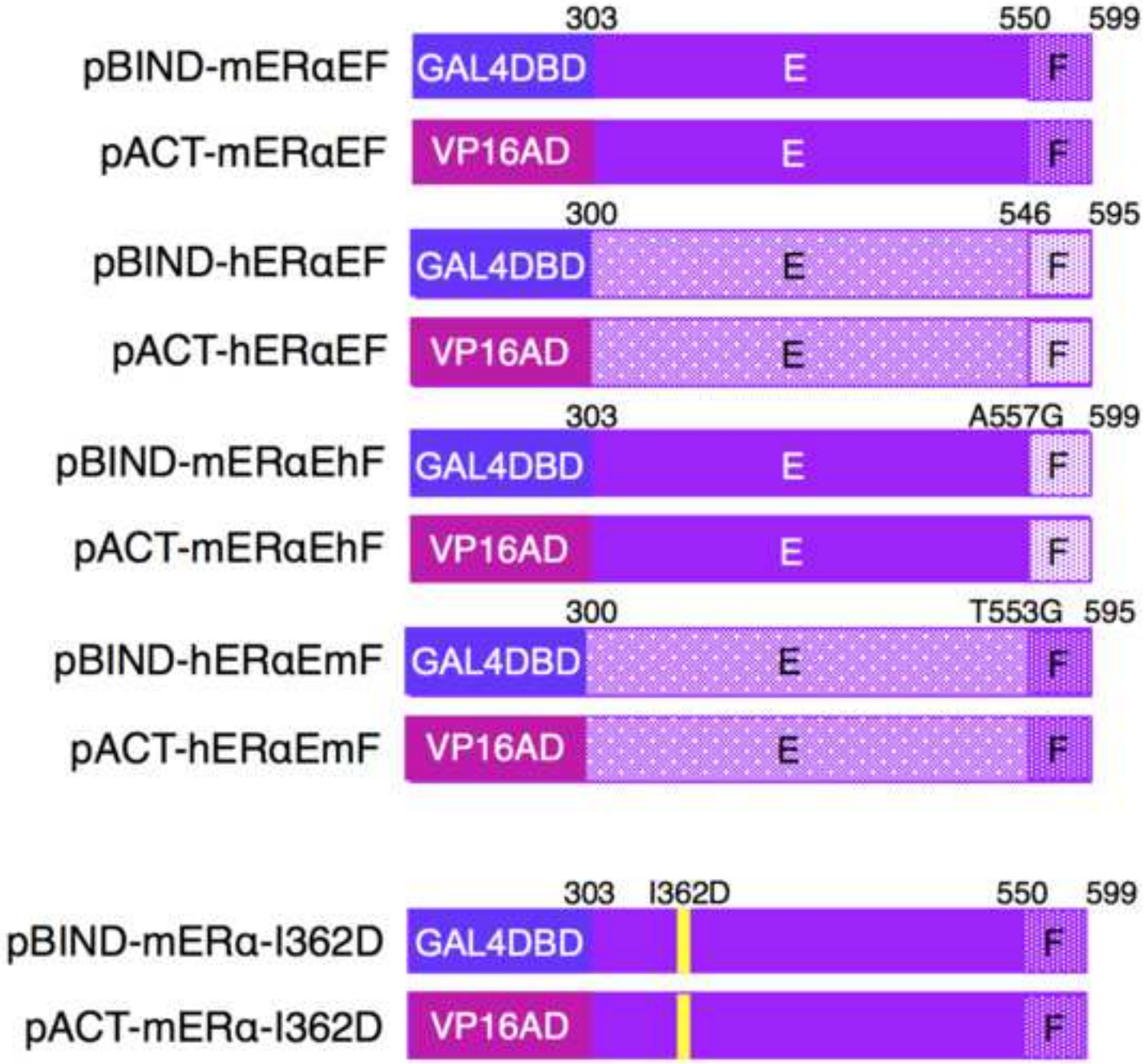

Fig.2 Arao & Korach

Figure 3

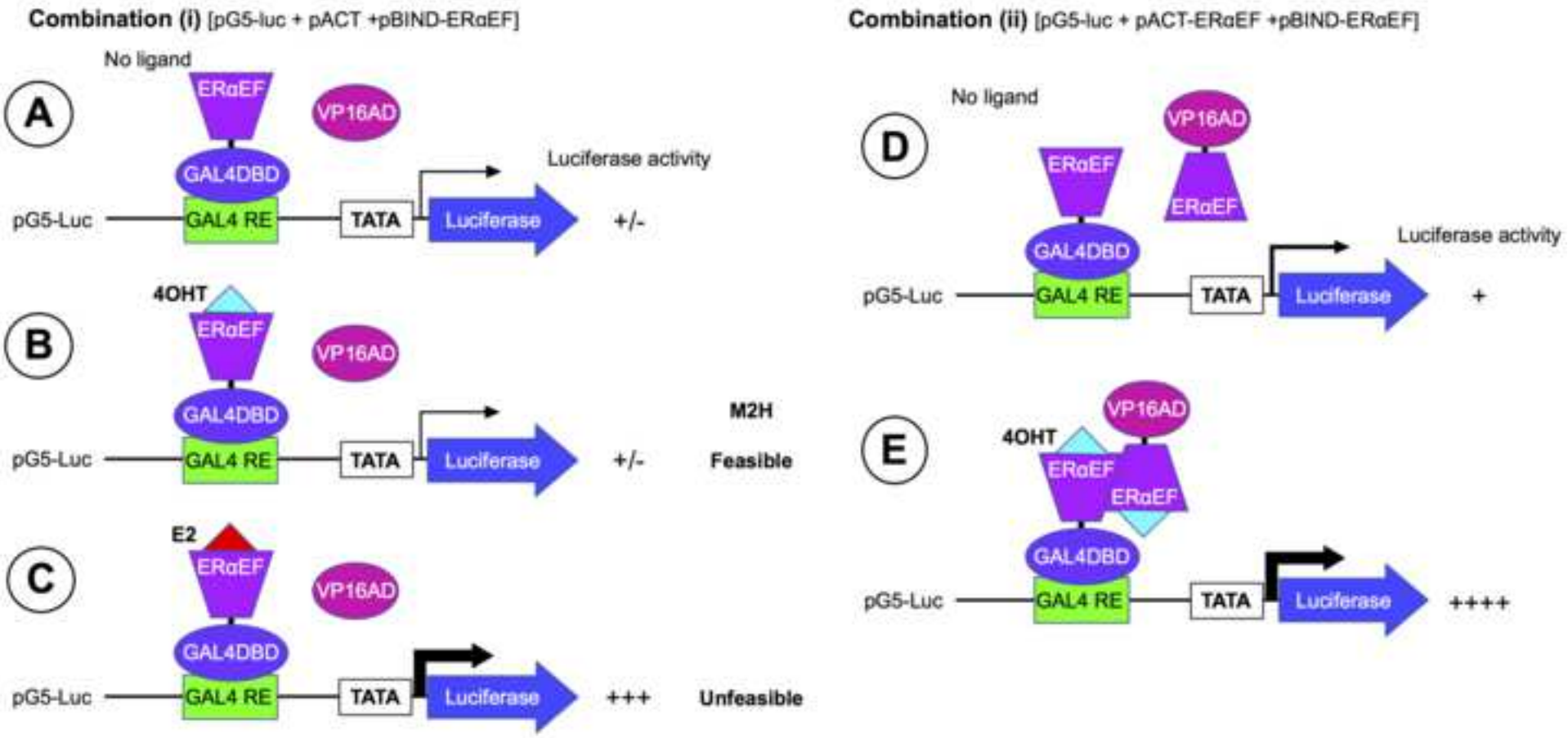

Fig.3 Arao & Korach

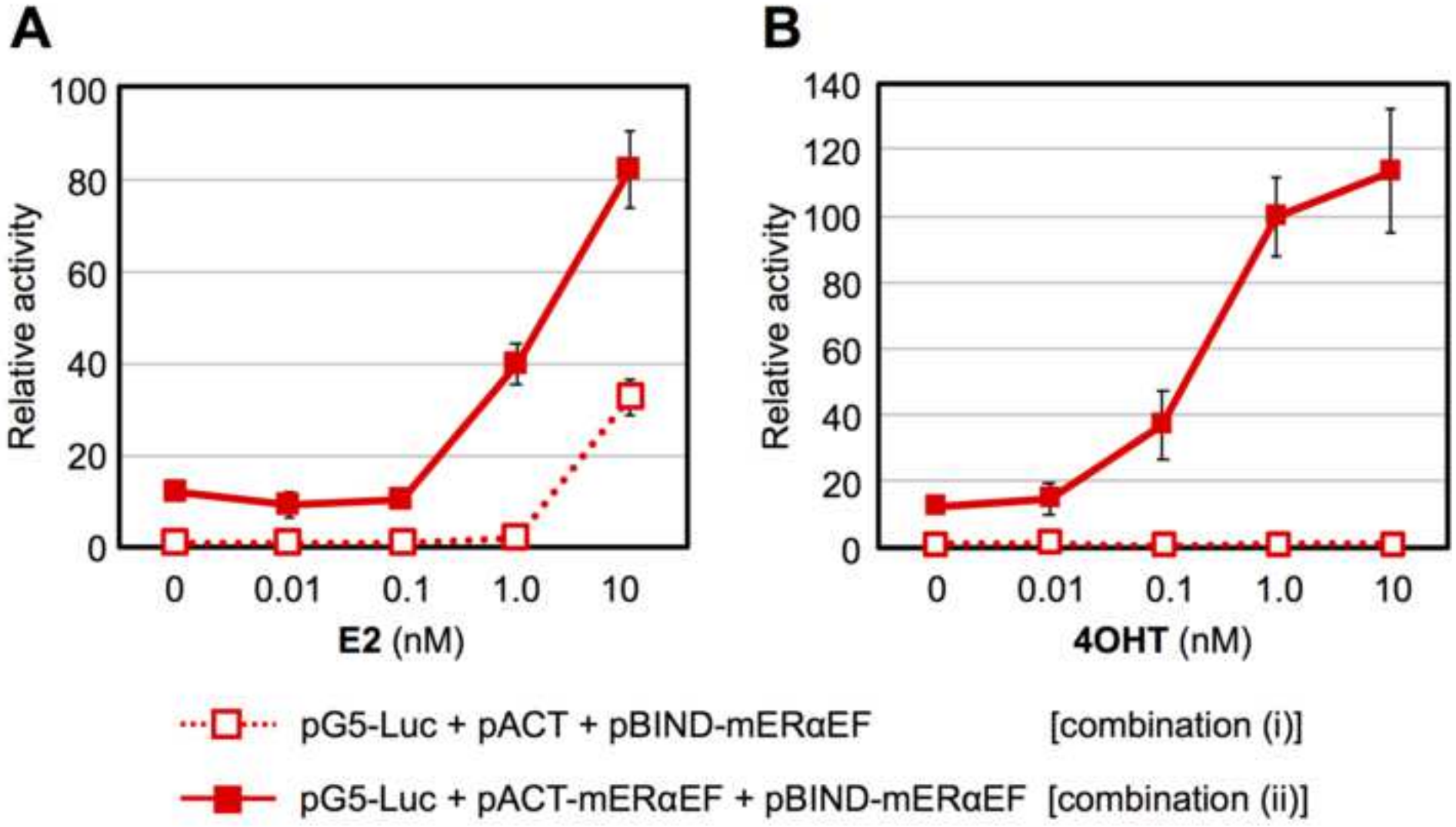

Fig.4 Arao & Korach

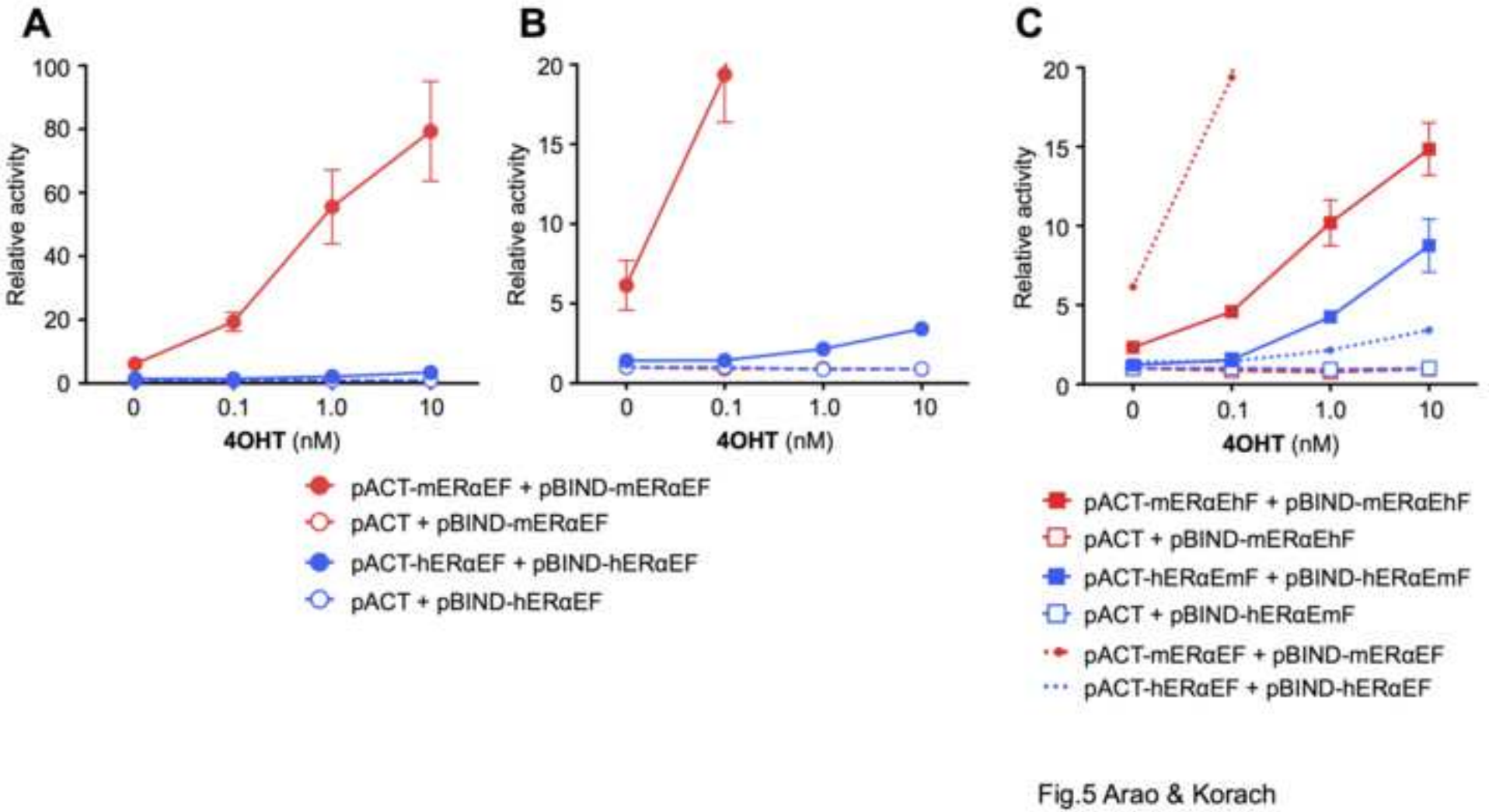

Fig.5 Arao & Korach

Figure 6

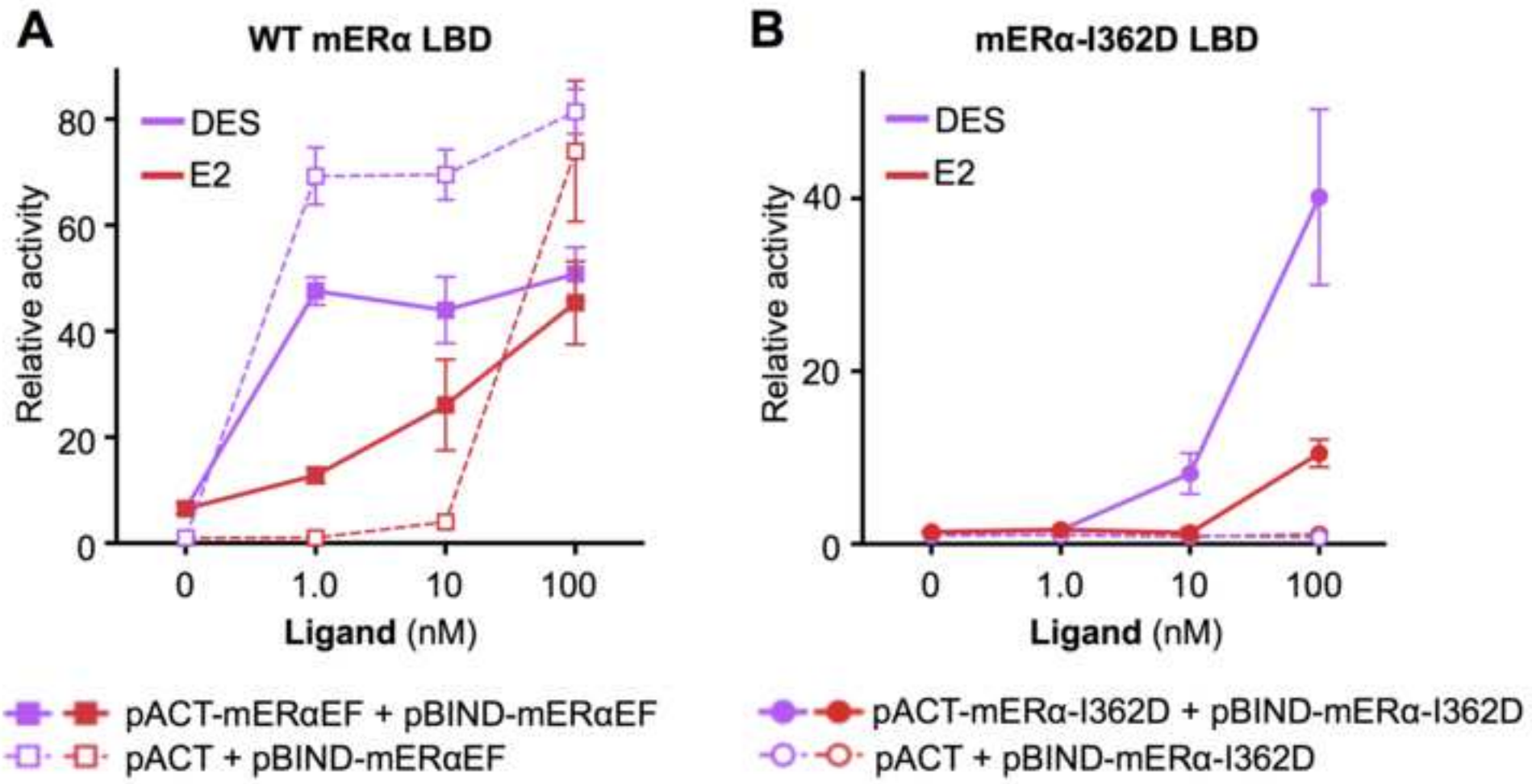

Fig.6 Arao & Korach

Name of Material/ Equipment	Company	Catalog Number
pG5-Luc	Promega	E249A
pBIND	Promega	E245A
pACT	Promega	E246A
One Shot TOP10 Chemically Competent *E. coli*	Invitrogen	C404010
Centrifuge Rotor	SORVALL	75006445
Swing Buckets	SORVALL	75006441
Cell Resuspension Solution (CRA)	Promega	A7112
Cell Lysis Solution (CLA)	Promega	A7122
Neutralization Solution (NSA)	Promega	A7131
Column Wash Solution (CWB)	Promega	A8102
Wizard Midipreps DNA Purification Resin	Promega	A7701
Wizard Midicolumns	Promega	A7651
MEM, no glutamine, no phenol red	Gibco	51200038
L-glutamine (200 mM)	Gibco	A2916801
0.5% Trypsin-EDTA (10x)	Gibco	15400054
Penicillin-Streptomycin (100x)	Sigma-Aldrich	P0781
BenchMark fetal bovine serum (FBS)	Gemini-Bio	100-106
Charcoal:dextran stripping fetal bovine serum	Gemini-Bio	100-119
DMEM, high glucose, no glutamine, no phenol re	Gibco	31053028
Lipofectamine 2000	Invitrogen	11668027
Passive Lysis 5X Buffer	Promega	E1941
Dual-Luciferase Reporter Assay System	Promega	E1980
SpectraMax L microplate reader	Molecular Devices	
SoftMax Pro Software	Molecular Devices	

Comments/Description
Component of CheckMate Mammalian Two-Hybrid System
Component of CheckMate Mammalian Two-Hybrid System
Component of CheckMate Mammalian Two-Hybrid System
Heat inactivated
Heat inactivated
for transfection

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58758_R2_RE2

Thank you for your editing. I edited some to clarify the process. I hope it is more understandable.

Page 5: Commented [A1] **Author** **8/30/18 2:25:00 PM**

Centrifugation parameters?

at maximum speed for 2 min using a microcentrifuge

Page 5: Commented [A2] **Author** **8/30/18 3:33:00 PM**

Centrifugal force?

at maximum speed

Page 5: Commented [A3] **Author** **8/31/18 8:44:00 AM**

Please note that I highlighted these steps for filming, because we cannot start filming step 2.2.5 without showing the cells first.

I agree with you.

Page 6 [A4]

I moved this Note to 2.2.4 where FBS is first mentioned.

I agree with you.

Page 6 [A5]

Please note that I have heavily edited 2.3 and sub-steps so they comply with JoVE guidelines. For instance: The Protocol should be made up almost entirely of discrete steps **without large paragraphs** of text between sections and contain only **action items** that direct the reader to do something. Please read carefully to ensure that the intended meaning has remained.

Thank you for your edits. I added some modifications.

Page 7 [A6]

As I noticed that these are the only differences between combination (i) and (ii), so I combined the original two paragraphs here.

Thank you for your edits. I added some modifications.

Page 7 [A7]

Please note that calculations are not appropriate for filming. Therefore I unhighlighted calculation steps.

I understand.

Page 8 [A8]

It is unclear to me why the volume of transfection reagent in DMEM should be 195 uL. Based on lines 317-318, it should be 210 uL. Please check.

I edited this part.

Page 8 [A9]

It is unclear to me why the volume of transfection reagent in DMEM should be 195 uL. Based on lines 317-318, it should be 210 uL. Please check.

I edited this part.

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Please specify the antibiotics used in this step.

I added 1% penicillin-streptomycin solution.

Page 9: Commented [A11] **Author** **8/31/18 9:14:00 AM**

Do you mean warm to room temperature before use?

Yes. Your edit is right.

Page 9 [A12]
Do you mean warm to room temperature before use?

Yes. Your edit is right.