Chapter 4

X-Ray Crystallography of Agonist/Antagonist-Bound Receptors

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Abstract

Crystallographic analysis of the ligand-binding domains of nuclear hormone receptors (NR) has provided a unique insight into the molecular events that underlie the ligand-mediated control of their transcriptional activity. The technique relies on preparing milligram quantities of protein, growing three-dimensional crystals of the desired protein–ligand complex, collecting X-ray diffraction data, and subsequently interpreting the derived electron density maps to reveal the structure of the complex.

Key words: Estrogen receptor, Protein purification, Crystallization, Crystal structure, Carboxymethylation.

1. Introduction

Attempts to crystallize intact nuclear receptors have been hindered by both interdomain flexibility and the unstructured nature of certain regions of the receptor in the absence of interaction partners. Instead a "divide and conquer" approach focussing on isolated ligand-binding domains (LBDs) has yielded a multitude of NR structures in the presence of different classes of ligand (agonists, partial agonists, antagonists, etc.) as well as with a range of coactivator/corepressor peptide fragments *(1, 2)*. Such structural information has provided a better understanding of the relationship between the chemical structure of the ligand and the resultant conformation of the ligand-bound receptor and allowed the design of receptor-specific ligands acting either as agonists, antagonists, or selective receptor modulators *(1)*.

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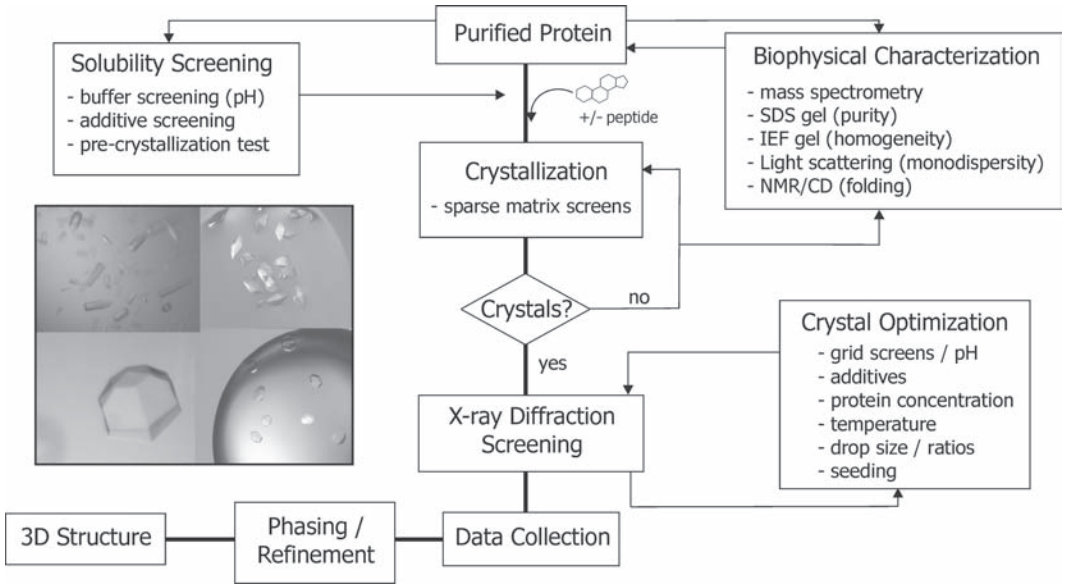

Fig. 1. From protein to 3D structure. A flowchart highlighting the different steps in the crystallization/structure solution process is shown. Inset: Representative examples of various ER-LBD ligand cocrystals.

The structure determination process is not a trivial undertaking, and it should be stressed at the outset that success is by no means guaranteed. The technique relies on obtaining single crystals suitable for diffraction analysis, and the resolution of a specific protein–ligand complex may prove impossible. Particular ligands may be sufficiently destabilizing or result in a population of conformational states that prevent or hinder successful crystallization.

Here, the preparation of the liganded estrogen receptor alpha LBD (ERαLBD) is described along with a protocol for setting up sitting-drop, vapor diffusion crystallization trials. Because of the more generic nature of the subsequent steps in the structure solution process (crystal optimization, data collection and reduction, phasing, refinement), the reader is referred to several excellent handbooks *(3–5)* and detailed protocols *(6)* for further practical information. An overview of the process is shown in **Fig. 1**.

2. Materials

2.1. Expression

1. Luria-Bertani (LB) medium: 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter of distilled water. Autoclave in 1 L baffled shake flasks stoppered with foam bungs at 120°C for 20 min.

2. Ampicillin stock (100 mg/mL): 100 mg ampicillin/mL of sterile water. Filter sterilize solution through 0.2 µm filter. Aliquot and store at −20°C.
3. 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) stock. Dissolve IPTG in sterile water. Filter, sterilize, aliquot, and store at −20°C.

2.2. Purification

1. Low/medium pressure chromatographic FPLC system (such as AKTA-FPLC).
   Columns/media: Ni-Sepharose Fast Flow resin; XK-16 column; Superdex S75 16/60 gel filtration column; MonoQ 5/5 or 10/10 ion exchange column (all from GE Healthcare).
2. Sonication buffer (SB): 25 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 10 mM β-mercaptoethanol (β-ME), 10 mM imidazole, 1 COMPLETE™ EDTA-free protease-inhibitor cocktail tablet per 50 mL SB (Roche Diagnostics), 1 mM AEBSF.
3. Ni-NTA running buffer#1 (NTA-RB1): 25 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 10 mM β -ME, 10 mM imidazole, 0.3 M NaCl.
4. Ni-NTA wash buffer (NTA-WB): 25 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 10 mM β -ME, 100 mM imidazole, 0.3 M NaCl.
5. IAA-1 buffer: 10 mM solution of iodoacetic acid (IAA) in 25 mM Tris-HCl pH 8.1. Solutions of IAA are light-sensitive and should be protected accordingly.
6. E2-ligand solution: 25 mM Tris 8.1, 100 µM beta-estradiol (*light sensitive*) (*see* **Note 1**).
7. Ni-NTA elution buffer (NTA-EB): 25 mM Tris 8.1, 0.4 M imidazole.
8. Thrombin stock (0.03 U/µL): Dissolve bovine thrombin (Roche Diagnostics – 30units) in 1 mL water. Store aliquots (50 µL) at −20°C. Thaw on ice and use as required.
9. Ni-NTA running buffer#2 (NTA-RB2): 25 mM Tris-HCl pH 8.0, 5 mM β -ME, 75 mM imidazole, 0.3 M NaCl.
10. Gel filtration running buffer (GF-RB): 25 mM Tris-HCl pH 8.0, 2 mM dithiothreitol (DTT), 0.1 M NaCl. Filter buffer through 0.2 µm filter and degas.
11. IAA-2 solution (10×): 0.2 M solution of iodoacetic acid (IAA) in 250 mM Tris–HCl pH 8.0 (light sensitive). The high Tris concentration is required to buffer the IAA solution (quite acidic) and prevent precipitation of ER-LBD after addition.
12. Mono Q ion exchange buffers: IEX-A, 25 mM Tris-HCl pH 8.0, 2 mM DTT; IEX-B: as IEX-A but including 0.5 M NaCl. Filter buffers through 0.2 µm filter and degas.

13. SDS-PAGE solutions: Resolving gel buffer (1.5 M Tris-HCl pH 8.8, 0.4% (w/v) SDS); Stacking gel buffer (0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS). Running buffer (4×) – 12 g Tris and 57.6 g L-glycine per liter of deionized water. For 1× running buffer, dilute and add SDS to 0.1%. For native-PAGE omit SDS from all solutions. 30% acrylamide (w/v)/0.8% bis-acrylamide stock solution (37.5:1) (National Diagnostics). 10% (w/v) ammonium persulphate solution and TEMED to polymerize.
14. Centriprep/Centricon centrifugal concentrators (10K MWCO; Millipore).

2.3. Crystallization

1. Commercial crystallization screens (e.g. Crystal Screen 1&2; Index; PEG/ion (Hampton Research; http://www.hamptonresearch.com); MDL Nuclear Receptor Ligand binding domain screen; NR-LBD Extension screen (Molecular Dimensions; http://www.moleculardimensions.com) (*see* **Note 2**).
2. 96-well, sitting drop crystallization plates (*see* **Note 3**).
3. Optically clear sealing tape (such as Viewseal (Greiner)).
4. Multichannel pipettes (*optional* but greatly speeds up crystallization setup).

2.4. Inspection/ Optimization

1. Good quality stereomicroscope (40×) preferably with a cold light source (*see* **Note 4**).

3. Methods

Despite the caveats mentioned in the Introduction section, successful structure determination can be greatly enhanced by careful protein preparation. The sample should be of high purity, homogeneous, and monodisperse. A full biophysical characterization of the purified protein is recommended before embarking on crystallization (**Fig. 1**). The sample should typically be at least 90–95% pure as adjudged from a Coomassie stained SDS-polyacrylamide gel. Samples of lower purity may produce crystals but impurities can often affect their diffraction quality. The reader should remember that behind every structure lies good quality crystals and attempts to crystallize a poor quality sample is often destined to failure. It is also vital to keep a history of each batch as it is not uncommon to find that one batch of protein will crystallize whereas the next will not.

The following describes the preparation of human ERαLBD liganded to beta-estradiol as a generic example, although other ER ligands may be substituted. Because of tendency of the apo-form of ERαLBD to aggregate at low/moderate protein

concentrations, it is not possible to prepare unliganded ERαLBD to which ligands could be added prior to crystallization. Consequently, each individual ligand complex should be prepared separately. The purification scheme is unusual in that the exposed free cysteines in the protein are alkylated by iodoacetic acid treatment. This introduces a single negative charge per cysteine and improves solubility and, in our hands, greatly enhances crystallizability of the sample. Most other NR-LBDs will not require such a treatment to obtain crystals.

Hexa-histidine (his_6)-tagged ERαLBD is initially affinity purified using nickel Sepharose, and the free cysteines are alkylated by treatment with iodoacetic acid (IAA) while still bound to the column. Immobilized, alkylated LBD is then incubated with ligand prior to elution followed by removal of the his-tag by thrombin cleavage. Size-exclusion chromatography allows removal of aggregated species. Finally, ion-exchange chromatography is used as a final polishing step. The purification protocol can be comfortably completed within 4–5 days. The final yield will vary but a conservative estimate is 10–15 mgs purified, liganded ERαLBD per liter of cells.

A generic protocol for obtaining diffraction quality crystals is impossible to provide because of the unique properties of each protein–ligand complex. As the nature of the successful combination of crystallising agents cannot be predicted at the outset, the target LBD/ligand complex is instead screened against a range of preformulated mixtures of chemicals. This approach affords the highest probability of success and should allow the experimenter to identify conditions required for crystal growth. Temperature and protein concentration are important variables, and it is suggested that experiments are carried out in parallel at 4 and 20°C (preferably in incubators). In most structural biology laboratories, the setup and inspection of crystallization experiments is often semiautomated. Nanoliter-sized drops (150–300 nl) comprising protein and reservoir are mixed and equilibrated against the reservoir solution using a liquid handling robot. Small volumes allow many more trials to be carried out from a fixed volume of sample compared with conventional methods. Such volumes cannot be accurately pipetted manually, and the protocol below assumes that the experimenter does not have access to such specialized equipment. The screening strategy outlined below can be equally applied to other NR-LBD complexes.

3.1. Expression

1. His_6-tagged ERαLBD (residues 304–554) can be expressed in soluble form in *Escherichia coli* from a pET15b derived expression plasmid *(7)*. This vector encodes an N-terminal polyhistidine tag that can be cleaved by thrombin (*see* **Note 5**).
2. Dilute (1/50) an overnight culture of *E. coli* strain C41 DE3 *(8)* transformed with pET15b-ERαLBD *(7)* into fresh LB supplemented with 100 µg/mL ampicillin (*see* **Note 6**).

3. Grow cells at 37°C in a shaker until $OD_{600\,nm}$ is between 0.6–0.8. Induce expression by addition of 1 mM IPTG (1/1,000 IPTG stock) and grow cells overnight at 25°C. Add additional antibiotic (1/1,000 ampicillin stock) at induction to maintain selection.
4. Harvest cells by centrifugation and store pellets at –70°C until required.
5. Samples can be taken preinduction and after overnight incubation for SDS-PAGE analysis according to the method of Laemmli *(9)* to check the expression level (*see* **Note 7**).

3.2. Purification

1. All procedures should be carried at 4°C unless stated. Resuspend frozen cell pellet in SB (25–50 mL SB per liter cells) and sonicate on full power for 10 × 15 s burst (15 s pulse followed by 15 s rest on ice. Repeat ten times).
2. Sonicate is centrifuged at 27,000 × *g* in SS34 rotor for 30 min and supernatant is retained.
3. Supernatant (soluble extract) is filtered through 0.45 µm syringe filter (e.g. Acrodisc/Minisart) to remove large particulates. Add NaCl to a final concentration of 0.3 M. Ensure solution is mixed quickly after NaCl addition to avoid excessive local concentration. Addition of NaCl prior to sonication is not encouraged as this reduces the amount of the hydrophobic LBD recovered from the whole cell extract.

Ni-chelated Sepharose (8–10 mL bed volume) is prepared as manufacturer's instructions and packed into a suitable column (e.g. XK-16 column (*see* **Note 8**)). Resin is equilibrated with NTA-RB1 (10 column volumes (10 CV)), soluble extract is loaded at 3 mL/min and then washed with NTA-RB1 (10 CV or until A280 absorbance baselines). Resin washed with NTA-WB until baseline (9 CV), then with 25 mM Tris pH 8.1 to remove β-ME (5 CV). Resin washed with 2–3 CV IAA-1 buffer. Column is capped, wrapped in foil, and stored overnight.

4. Column washed with buffer (25 mM Tris-HCl pH 8.1) to remove alkylation solution (5 CV).
5. Column washed with E2 ligand solution (4 CV) and then capped and incubated for 30 min (*see* **Note 9**). E2-liganded ERαLBD complex is eluted with NTA-EB in 5 mL fractions. Assess which fractions to pool by SDS-PAGE.
6. Beta-mercaptoethanol (β-ME) is added to pooled fractions to a final concentration of 5 mM and pooled material is dialysed against 2 L of 25 mM Tris pH 8.0/5 mM β-ME in dialysis tubing (12–14 kDa cutoff) for a minimum of 2 h. Buffer is replaced and dialysed for further 1 h. It is not necessary to add additional ligand to the dialysis buffer. Dialysate collected and protein concentration estimated by recording absorbance at 280 m (*see* **Note 10**).

7. The his-tag can be removed with thrombin. NaCl (5 M stock) and $CaCl_2$ (1 M stock) are added to the dialysate at concentrations of 100 mM and 1.5 mM, respectively. Thrombin stock solution (0.05 units thrombin/mg ER) added and incubated without mixing at 22°C overnight (*see* **Note 11**).
8. SDS-PAGE analysis can be used to check that thrombin cleavage has proceeded to completion. Thrombin activity is inhibited by addition of AEBSF to a final concentration of 1 mM.
9. Imidazole and NaCl are added to thrombin-treated LBD at final concentrations of 75 and 300 mM, respectively. Addition of imidazole is important as it prevents nonspecific binding of his(-)ERαLBD to Ni-NTA resin while allowing uncleaved material (his(+)ERαLBD) to be retained. ERαLBD is unusual in that it has a high intrinsic affinity for nickel resin and higher concentrations of imidazole are required to prevent nonspecific binding compared with other proteins.
10. Thrombin-cleaved material is passed down Ni-Sepharose column equilibrated with NTA-RB2 to remove uncleaved material. This is a negative step and column flow-through containing cleaved his(-)ERαLBD should be retained. Check purification progress by SDS-PAGE analysis.
11. Superdex S75 16/60 gel filtration (GF) column is connected to FPLC system and equilibrated with GF-RB overnight.
12. His(-)ERαLBD-E2 complex is transferred to a rinsed 10K Centriprep™ centrifugal concentrator (Millipore) and spun at 4°C to reduce volume to ca. 4 mL. Concentrate is spun at 20,000 × *g* to remove particulates prior to loading onto the GF column via a 5 ml superloop. The column should be run at 1 mL/min. Aggregated material will elute around 42 mL while the majority of the protein elutes around 65 mL (corresponding to ERαLBD dimer). Collect 1–2 mL fractions over the dimer peak. Fractions containing ER are pooled and the concentration estimated.
13. A second alkylation step of the liganded LBD has been found to be necessary for successful crystallization. IAA-2 solution (1×) is added to the pooled fractions and incubated at 22°C for 2–3 h. The reaction is quenched by addition of DTT to a final concentration of 20 mM.
14. The final polishing step uses ion exchange chromatography to partially separate the various charge states introduced by carboxymethylation (*see* **Note 12**).
15. MonoQ 10/10 column (8 mL bed volume/35–40 mgs capacity) is connected to FPLC system and equilibrated with IEX-A buffer (5 CV). This column can be run at 3–4 mL/min depending on the back pressure.

16. IAA-treated material is diluted 50:50 with 25 mM Tris–HCl pH 8.0 to reduce NaCl concentration to 50 mM. Filter through 0.2 µm acrodisc and load onto column in batches from a 10 mL superloop at 3–4 mL/min. Multiple loading will be necessary given volume of diluted material but do not load more than 35–40 mgs. Column is first washed with 5 CV IEX-A then the gradient should be stepped to 30% IEX-B and column washed for a further 5 CV. Bound material is eluted using a gradient from 30–65% IEX-B over a minimum of 10 CV. Finally gradient stepped to 100% IEX-B. Three milliliter fractions are collected. The major peak of ERαLBD should elute between 45 and 55% IEX-B. Fractions should be checked on 15% SDS-PAGE and native 7.5% PAGE before deciding which fractions should be pooled (*see* **Note 12**).
17. Most homogeneous fractions are pooled and dialysed thoroughly in 20 mM Tris-HCl 8.0, 150 mM NaCl, 2 mM DTT prior to concentration (*see* **Note 13**). his(-)ERαLBD-E2 complex can be concentrated in a Millipore 10K MWCO centrifugal concentrator to 10–12 mg/mL (*see* **Note 14**).
18. Concentrated material can be stored in small aliquots (50–100 µL) by flash-freezing in liquid nitrogen prior to storage at –80°C. Thin-walled PCR tubes are recommended for storage. Frozen material should be rapidly thawed by rubbing between the fingers rather than slowly on ice as this will minimize damage. Repeated freeze-thaw cycles should definitely be avoided as this can lead to serious sample heterogeneity. Once thawed, protein should be stored on ice and used up as quickly as possible (i.e., thaw a fresh sample rather than use material that has been thawed and stored for more than 3–4 days).

3.3. Crystallization

1. Pipette 40 µl of each of the 96 crystallization screen solutions into each reservoir well of the Wilden plate (*see* **Note 15**). Repeat for each screen/temperature to be tested using a new plate.
2. Freshly prepared or rapidly-thawed, frozen liganded LBD is centrifuged at 20,000 × *g* in a benchtop centrifuge at 4°C to remove particulates.
3. One microliter of concentrated protein–ligand solution is pipetted into subwell A of the crystallization plate (**Fig. 2**). An equal volume of the corresponding reservoir solution is pipetted into subwell A on top of the concentrated protein without mixing (*see* **Note 16**). If a multisubwell plate is used, additional samples can be set up in other subwells as required (*see* **Note 17**).
4. Seal plate with tape (*see* **Note 18**).
5. Place at desired temperature (*see* **Note 19**).

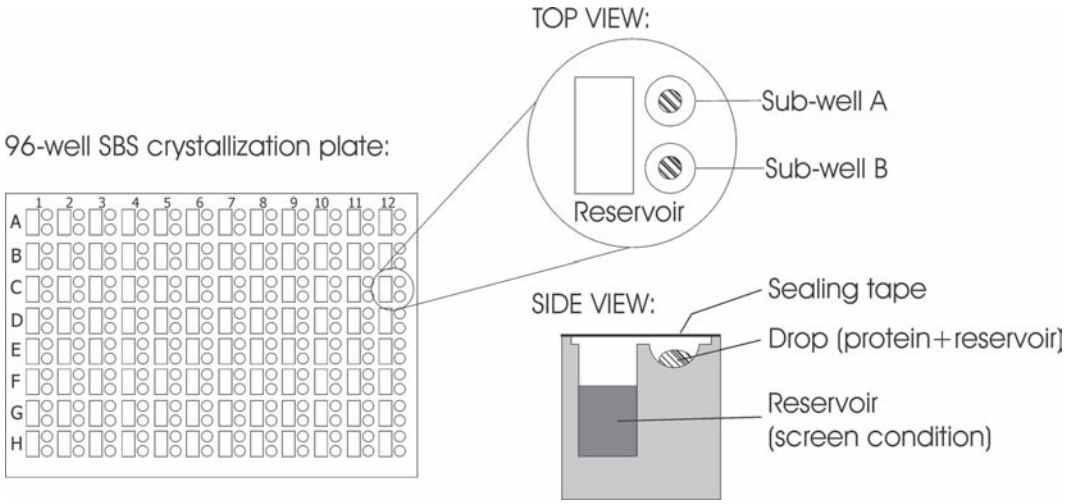

Fig. 2. A 96-well sitting-drop crystallization plate. The layout of one of the 96 individual crystallization chambers is shown in detail on the *right*.

3.4. Inspection and Optimization

1. Progress of crystallization experiments should be monitored after 24 h and then on day 2, day 3, day 7 and then at weekly intervals. Inspect each drop under the microscope and record hits (*see* **Note 20**). Optimize crystallisation conditions as necessary to obtain diffraction quality crystals (*see* **Note 21**).

4. Notes

1. The majority of ER ligands are hydrophobic and poorly soluble in water. Ligand stocks should be prepared in 100% dimethylformamide (DMF) or 100% dimethylsulphoxide (DMSO) at appropriate concentrations (5–50 mM). Dilution of the concentrated ligand stock in buffer can result in significant precipitation of ligand and the working solution may appear quite cloudy. Nevertheless, sufficient ligand should remain in solution to saturate the LBD binding sites. Up to 10% DMF can be included in the ligand solution to enhance solubility without affecting Ni-Sepharose column performance. Diluted ligand solutions should be freshly prepared and not reused.
2. A variety of crystallization screens are commercially available. They comprise a set of solutions (referred to as conditions) that can be mixed with the concentrated protein sample and used to induce crystallization. Each solution typically comprises a precipitant (a salt or polyethylene glycol (PEG)) along with

various additive salts at a specific pH. As a whole, each screen has a broad coverage of different precipitants, salts, and pH range (4.5–9.0). Different screens have some unique conditions but there can be considerable overlap between the conditions. The screens listed here have been successfully used in the initial screening of ER–ligand complexes and provide comprehensive set of starting conditions. The Hampton Crystal Screens 1&2 can be combined in a 96-well plate as can the MDL NR-screens. The Hampton Index screen takes up an entire 96-well plate while the PEG/ion screen comprises 48 conditions.

3. A variety of 96-well SBS (Society for Biomolecular Screening) format, sitting-drop crystallization plates are commercially available. 2-drop MRC/Innovaplate SD-2 plates (Wilden/Innovadyne) are recommended as they have large circular wells with excellent optical properties. The advantage of multiple drop plates is that several samples (different ligand complexes) can be setup against the same reservoir solution (**Fig. 2**).
4. If possible the microscope should have a large, flat viewing platform with a cold (fiber optic) light source illumination in the base. Protein crystals (and solutions) are highly sensitive to changes in temperature. If a cold light source is unavailable, drop inspection should be carried out in stages allowing the viewing stage/bulb to cool down before resuming.
5. The pET15b expression vector is described here as it consistently (and reproducibly) produces the highest quality protein in our hands. Nevertheless, thrombin cleavage is not particularly efficient/specific/cost-effective and has to be carried out at room temperature to achieve cleavage in a sensible timeframe. Other N-terminally tagged expression vectors may be used (C-terminal tags are best avoided for NR-LBDs due to possible interference with the carboxy-terminal AF2 helix). However, the author has also tested a variety of ERαLBD expression constructs that are not tagged or contain a 3C protease (pET28a based) cleavage site rather than a thrombin site. Despite the inherent advantages of these constructs, expression of soluble LBD is, in all cases, both significantly reduced and much less reproducible.
6. The *E. coli* strain C41 (DE3) *(8)* was found to give the best percentage of soluble expression under the conditions tested. The expression plasmid is stably retained and transformed C41 cells can be stored at –80°C.
7. His-tagged (his(+)) ERαLBD has a molecular weight of 31,019 Da (reducing to 29,137 after removal of tag (his(–)). A standard 12 or 15% SDS-PAGE gel can be used to resolve these species.

8. An FPLC system is not required for all steps. A column attached to a simple peristaltic pump will suffice for Ni-NTA affinity chromatography. The column volumes (CV) of each wash step are indicated so that the whole procedure can essentially be carried out "blind". Although self-packed columns are the most flexible for this step, prepacked 1 or 5 mL Ni-Sepharose HiTRAP columns (GE Healthcare) can also be used. The advantage of the 5 mL columns is that they tolerate high flow rates. Alternatively columns can be left to flow by gravity or the whole procedure can be carried out "in batch" in 50 mL tubes. In cases where working with a precious or limiting quantity of ligand, the amount of resin can be optimized on the basis of the amount of complex being prepared so that the minimum amount of ligand solution is required.
9. It is not necessary to include ligand in all the purification/dialysis buffers after the immobilized LBD has initially been exposed to ligand on the Ni-Sepharose. However, addition of ligand is recommended during the final centrifugal concentration step as it ensures that the LBD if fully saturated with ligand prior to crystallization.
10. Protein concentration can be estimated assuming that a 1 mg/mL solution of his(+)ERαLBD has an absorbance of 0.757 at 280 nm. Use dialysis buffer as a reference and dilute sample to get an accurate measurement (depending on dynamic range of equipment used). After removal of the tag, a 1 mg/mL solution has an absorbance of 0.81. Alternatively, protein concentration can be estimated using standard colorimetric methods such as the Bradford assay.
11. Thrombin cleavage is notoriously inefficient and can be seriously impacted by contaminants. In this case, the tag is specifically cleaved after overnight incubation if the dialysis step is followed prior to addition of enzyme. If SDS analysis suggests incomplete cleavage then additional thrombin solution can be added and incubated further.
12. ERαLBD contains 4 free cysteines of which 3 are accessible to the iodoacetic acid treatment. As ERαLBD dimerizes in the presence of ligand (agonist or antagonist), incomplete alkylation results in a range of charge species. SDS-PAGE analysis will not reveal this heterogeneity, and it is worthwhile to run samples on a native PAGE. For native gel analysis, prepare a 7.5% cross-linked acrylamide/bis-acrylamide gel using resolving/stacking gel buffers that do not contain SDS. Run at 100 V at 4°C in Tris/glycine running buffer (no SDS) and stain with Coomassie.
13. The final material is dialysed against a relatively low concentration of buffer so that the pH can be readily modulated by

the crystallization screen conditions. NaCl can also be added up to 0.5 M to enhance solubility of certain ER–ligand complexes. The amount of NaCl required should be determined on a case-by-case basis.

14. Other ER-LBD ligand complexes, especially those with antagonists may exhibit reduced solubility. It is extremely important that the protein solution is not over-concentrated as this can affect the ability of the sample to crystallize. Concentration should be stopped at the first signs of precipitation – often when thread-like filaments appear in solution or when the rate of ultrafiltration gets very slow (indicative of material precipitating on membrane). The protein solution should also be mixed at regular intervals to avoid over-concentration at the membrane surface of the concentrator. If moderate-to-heavy precipitation occurs it may be reversed by addition of NaCl (up to 0.5 M) but concentration should be halted and any remaining precipitate should be removed by centrifugation. Achieving a "high" protein concentration for all complexes is not necessarily essential as we have successfully crystallized ER complexes as low as 3.5 mg/mL. A precrystallization test (kit available from Hampton Research) can be used to assess whether the protein concentration is suitable for crystallization screening.
15. Each crystallization plate comprises 96 individual compartments. Each compartment comprises a central reservoir for the crystallization solution and a raised platform containing 1–3 wells (subwell) where the protein-reservoir "sitting-drops" are set up (**Fig. 2**). Once the plate is sealed each set of subwells are able to equilibrate with the parent reservoir. Initially the concentration of components will be halved as the protein and crystallization solution are mixed in a 1:1 ratio. However, the drop will rapidly equilibrate with the reservoir by vapor diffusion (effectively water will be drawn from the drop to the reservoir) so that the drop is slowly dehydrated and the concentration of components will reach or exceed their individual starting concentrations. The minimum amount of reservoir solution (crystallization reagent) required varies depending on plate type (40–100 µL).
16. A single channel pipette can be used to dispense protein in columns. A multichannel (8-channel) pipette can then be used to add reservoir solution on a column-by-column basis. Protein solution should always be dispensed first and then reservoir solution added second. Exhaustive mixing is not necessary and solutions can be left to diffuse together. If the protein sample is limited, the drop size may be reduced to 0.5 µL protein + 0.5 µL reservoir if these volumes can be accurately pipetted.

17. Appropriate coregulator peptides can be added to the protein–ligand complex to stabilize the LBD conformation and increase the chances of success. Alternatively, several initial concentrations of protein–ligand complex can be evaluated (100, 75, 50%). Protein can be diluted with GF-buffer. Coactivator peptides derived from the LxxLL-containing regions will stabilize the agonist-bound state. Antagonist complexes may be stabilized by corepressor peptides or peptides derived from phage-display studies. Peptides should be of high purity (>95%) and cocrystallization will require relatively large amounts (1–5 mg). Peptide stocks (mM) can be prepared in water/buffer and added at two to fivefold molar excess with respect to the LBD concentration followed by brief incubation prior to setting up crystallization drops. Alternatively, peptide can be added to dilute LBD solution to improve solubility and behavior during the final concentration step. Peptide stocks can be stored in aliquots at –20°C. Additional ligand may also be added prior to crystallization from concentrated stocks to ensure an excess of ligand is present so that all LBDs contain bound ligand. This is particularly important for lower affinity ligands that may have leached out during the purification. Ensure that organic component (DMSO/DMF – *see* **Note 1**) is around 1–2% (v/v). Higher concentrations may cause precipitation.
18. Drops should not be allowed to dry out/evaporate during setup. This will be an issue if setting up more than one protein–ligand complex per plate. Depending on the relative humidity, it may be necessary to complete a 96-well plate in several stages (e.g., 3 columns at a time), temporarily sealing with clear tape prior to final sealing with optically clear tape.
19. Incubators should be used if available to maintain experiments at a constant temperature. Otherwise plates should be isolated from temperature fluctuations and vibrations, which both have a negative effect on the crystallization process. Plates can be placed in polystyrene boxes, which afford some protection, prior to storage in a fridge/cold room or at room temperature. Temperature fluctuations should also be minimized when inspecting the progress of crystallization experiments and 4°C experiments should ideally be examined in a cold room.
20. The drops will take time to equilibrate and so crystals are unlikely to appear immediately. Because of the wide range of chemical/pH space that the listed crystallization screens cover, it is normal that anywhere from 1/3 to a 1/2 of all drops will produce precipitate. Precipitation is a positive outcome as it suggests that the drop has reached a supersaturated

state – a prerequisite for crystallization. If all the drops are clear then the protein solution is too dilute and should be concentrated further if possible. Conversely, if greater than 75% of drops produce precipitate then consider diluting the sample before repeating the crystallization trials. As mentioned in **Note 17,** it is worthwhile setting up initial experiments at several different protein concentrations if sample is not limiting. Alternatively, a precrystallization test (*see* **Note 14**) can be performed. In certain cases it may be necessary to further optimize the sample's solubility prior to successful crystallization using buffer and additive screening (*see (6) for protocols detailing these techniques*). If you are lucky some drops will yield crystals of some description. Almost certainly these will not be large or perfectly shaped but rather could be "sea-urchins" of thin needles, stacked plates or showers of tiny crystals so careful observation is necessary. See http://www.hamptonresearch.com/stuff/Gallery.aspx for examples of the myriad of protein crystal morphologies.

21. In the majority of cases, initial crystals will be unsuitable for diffraction analysis and will require some degree of improvement/optimization. Because of the sparse-matrix (random) design of the crystallization solutions, a scoring system greatly simplifies the process of identifying conditions (pH, precipitant, additives) that favor crystallization (*see (6) for example*). Briefly, a simple 1–10 scale can be used to score denatured precipitate (will appear brown under the microscope), clear drops, precipitates, and crystals of various sizes. Analysis of the initial screens should provide clues to the pH dependence of the complex's solubility as well as its behavior in the presence of different anions, cations, and metals. Optimization parameters include concentration of both the protein–ligand complex and components of the crystallization solution. The pH of the crystallization solution may also have a large impact on resultant crystals. A strategy for optimization can include a simple dilution screen of the original hit condition that gives crystals (100, 95, 90%, etc, using ultrapure water as diluent) as well as exploring conditions 1.5 pH units either side of condition (using 0.5 pH unit steps). In the case of ER-LBD, the majority of crystals are obtained from polyethylene glycol solutions at pHs greater than 6.5.

Diffraction quality of putative complex crystals should be assessed at the earliest possible stage to avoid optimising salt crystals (common with phosphate containing conditions and when calcium and zinc salts are present). ER-LBD crystals typically diffract X-rays weakly and the author's experience is that the majority of datasets will need to be collected at high brilliance synchrotron sources such as the ESRF (European Synchrotron Radiation Facility; http://www.esrf.fr) to obtain sufficiently high resolution data.

Nevertheless, screening crystals on a laboratory rotating anode X-ray source can provide useful information about the quality and diffraction potential of any crystals obtained. In-house diffraction to around 3–4 Å is generally quite encouraging.

Finally, if no crystals are obtained from initial screening then the following approaches could be considered. If sample homogeneity is not a concern (see Biophysical Characterization in **Fig. 1**), then more crystallization conditions can be setup at different temperatures. Otherwise try and purify the sample further with additional chromatographic steps such as ion exchange. Different ligands can have widely differing (de-)stabilizing effects on the protein that can impact crystallization, and it is advisable to try as many ligands as possible. Leaving the his-tag intact (i.e.. omitting the protease cleavage step) can sometimes result in dramatic changes in crystallization behaviour. Other approaches include protein methylation or partial in-drop proteolysis. Nonetheless, be prepared for some LBD-ligand complexes to resist all attempts at crystallization.

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