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Studies of Chaperone-Cochaperone Interactions using Homogenous Bead-Based Assay

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TITLE:

Studies of Chaperone-Cochaperone Interactions using Homogenous Bead-Based Assay

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KEYWORDS:

AlphaScreen, protein-protein interactions, molecular chaperone, Hsp90, FKBP51, FKBP52, TPR proteins, compound screening

SUMMARY:

This protocol presents a technique for probing protein-protein interactions using glutathione-linked donor beads with GST-fused TPR-motif co-chaperones and acceptor beads coupled with an Hsp90-derived peptide. We have used this technique to screen small molecules to disrupt Hsp90-FKBP51 or Hsp90-FKBP52 interactions and identified potent and selective Hsp90-FKBP51 interaction inhibitors.

ABSTRACT:

Targeting the heat shock protein 90 (Hsp90)-cochaperone interactions provides the possibility to specifically regulate Hsp90-dependent intracellular processes. The conserved MEEVD pentapeptide at the C-terminus of Hsp90 is responsible for the interaction with the tetratricopeptide repeat (TPR) motif of co-chaperones. FK506-binding protein (FKBP) 51 and FKBP52 are two similar TPR-motif co-chaperones involved in steroid hormone-dependent diseases with different functions. Therefore, identifying molecules specifically blocking interactions between Hsp90 and FKBP51 or FKBP52 provides a promising therapeutic potential for several human diseases. Here, we describe the protocol for an amplified luminescent proximity homogenous assay to probe interactions between Hsp90 and its partner co-chaperones FKBP51 and FKBP52. First, we have purified the TPR motif-containing proteins FKBP51 and FKBP52 in glutathione S-transferase (GST)-tagged form. Using the glutathione-linked

donor beads with GST-fused TPR-motif proteins and the acceptor beads coupled with a 10-mer C-terminal peptide of Hsp90, we have probed protein-protein interactions in a homogeneous environment. We have used this assay to screen small molecules to disrupt Hsp90-FKBP51 or Hsp90-FKBP52 interactions and identified potent and selective Hsp90-FKBP51 interaction inhibitors.

INTRODUCTION:

Molecular chaperones contribute to protein homeostasis, including protein folding, transport, and degradation. They regulate several cellular processes and are linked to numerous diseases such as cancer and neurodegenerative diseases[1]. Heat shock protein 90 (Hsp90) is one of the most important chaperones whose function is dependent on conformational changes driven by ATP hydrolysis and binding with client proteins mediated by its co-chaperones[2]. Despite an obvious potential of Hsp90 as the therapeutic target, fine-tuning its function represents a big challenge. There are several Hsp90 inhibitors targeting the N-terminal ATP binding region, which have been evaluated in clinical trials, but none of them has been approved for marketing[3]. Due to the lack of a well-defined ligand-binding pocket[4], targeting the C-terminal region of Hsp90 has had limited success[4]. Recently, disruption of Hsp90-cochaperone interactions by small molecules has been investigated as an alternative strategy[5]. Targeting the Hsp90-cochaperone interactions would not elicit general cell stress response and provides the possibility to specifically regulate various intracellular processes. The conserved MEEVD pentapeptide at the C-terminus of Hsp90 is responsible for the interaction with the tetratricopeptide repeat (TPR) motif of co-chaperones[6]. Of the 736 TPR motif-containing proteins annotated in the human protein database, ~20 different proteins interact with Hsp90 via this peptide[7]. Molecules competing for MEEVD peptide-binding would disrupt the interactions between Hsp90 and co-chaperones containing a TPR domain. The peptide binding site has similar tertiary structure but the overall homology between different TPR motif domains is relatively low[7], providing an opportunity to identify molecules specifically capable of blocking interactions between Hsp90 and particular TPR-motif co-chaperones. Among these TPR-motif co-chaperones, FK506-binding protein (FKBP) 51 and FKBP52 are regulators of steroid hormone receptor (SHR) signaling and involved in several steroid hormone-dependent diseases including cancer, stress-related diseases, metabolic diseases, and Alzheimer's disease[8]. Although FKBP51 and FKBP52 share > 80% sequence similarity, their functions differ: FKBP52 is a positive regulator of SHR activity, while FKBP51 is a negative regulator in most cases[8]. Therefore, identifying molecules, specifically blocking interactions between Hsp90 and FKBP51 or FKBP52, provides a promising therapeutic potential for related diseases.

Amplified Luminescent Proximity Homogenous Assay (AlphaScreen) was first developed in 1994 by Ullman EF et al.[9]. Now it is widely used to detect different types of biological interactions, such as peptide[10], protein[11], DNA[12], RNA[13], and sugar[14]. In this technique, there are two kinds of beads (diameter 200 nm), one is the donor bead and the other is the acceptor bead. The biomolecules are immobilized onto these beads; their biological interactions bring donor and acceptor beads into proximity. At 680 nm, a photosensitizer in the donor bead illuminates and converts oxygen to singlet oxygen. Because the singlet oxygen has a short lifetime, it can only diffuse up to 200 nm. If the acceptor bead is in proximity, its thioxene derivative reacts with the singlet oxygen generating chemiluminescence at 370 nm. This energy further activates fluorophores in the same

acceptor bead to emit light at 520-620 nm[15]. If the biological interactions are disrupted, the acceptor bead and donor bead cannot reach proximity, resulting in the singlet oxygen decay and low produced signal.

Here we describe a protocol using this technique for screening small molecules inhibiting interactions between Hsp90 and TPR co-chaperones, especially FKBP51 and FKBP52. The 10 amino acid long peptides corresponding to Hsp90 extreme C-terminus are attached to acceptor beads. Purified GST-tagged TPR co-chaperones interact with glutathione-linked donor beads. When the interaction between Hsp90-derived peptides and TPR-motif co-chaperones brings the beads together, an amplified signal is produced (**Figure 1A**). If the screened small molecules can inhibit the interactions between Hsp90 and TPR-motif co-chaperones, this amplified signal will be decreased (**Figure 1B**). Their IC_{50} can be calculated by quantitative measurement. This protocol can be extended to any chaperone - TPR-motif co-chaperone interactions of interest and is of great importance in the development of novel molecules, specifically blocking the interaction between Hsp90 and FKBP51 or FKBP52.

[Place **Figure 1** here]

PROTOCOL:

NOTE: An overview of this protocol is shown in **Figure 2**.

1. Expression and purification of GST-FKBP51 and GST-FKBP52 (Figure 2A)

1.1 Plasmids

NOTE: Obtain cDNA clones for human FKBP51 (clone id: 5723416) and for human FKBP52 (clone id: 7474554) from IMAGE consortium.

1.1.1 Amplify the human FKBP51 DNA by PCR with primers (forward; 5`GGATCCATGACTACTGATGAAGGT-3`, reverse; 5`-CTCGAGCTATGCTTCTGTCTCCAC-3`) containing BamHI and XhoI overhangs and clone into pGEX6-1 vector at BamHI / XhoI restriction sites.

1.1.2 Amplify the human FKBP52 DNA by PCR with primers (forward; 5`-GAATTCATGACAGCCGAGGAGATG-3`, reverse; 5`-CTCGAGCTATGCTTCTGTCTCCAC-3`) containing EcoRI and XhoI overhangs and clone into pGEX6-2 vector at EcoRI / XhoI restriction sites.

NOTE: PCR reaction set up and conditions are shown in **Table 1** and **Table 2**.

1.1.3 Verify the inserted sequence and transform the plasmids into the chemically competent *E. coli* according to the manufacture protocol.

1.2 Protein expression and purification

1.2.1 Add 25 g of Luria broth (LB) base in 1 L of distilled water to make the LB solution. Autoclave it at 121 ˚C for 15 min. After cooling, add 50 µg/mL ampicillin.

1.2.2 Take a colony of bacteria expressing GST-FKBP51 or GST-FKBP52 and mix with 500 µL of LB solution in a 1.5 mL tube. Vortex.

1.2.3 Add the mixture of "1.2.2" into 1 L of LB solution in the Erlenmeyer flask covered with an aluminum foil. Incubate the Erlenmeyer flask in the shaker overnight at 37 ˚C.

1.2.4 Induce protein expression by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) to the Erlenmeyer flask and continue the incubation for a further 2 h.

1.2.5 To get cell pellets, centrifuge at 5,000 x *g* for 15 min. Remove the supernatant.

NOTE: The cell pellets can be stored at -20 ˚C.

1.2.6 Resuspend the cell pellets in 40 mL of PBS and sonicate 3 x 20 s on ice. Add 1 mM PMSF, 1 mM EDTA, and protease inhibitor cocktail (1 tablet) to prevent proteolysis.

1.2.7 Centrifuge the suspension for 30 min 50,000 x *g* to remove cell debris and apply the supernatant onto 5 mL GST-trap column.

1.2.8 After washing the column with 30 mL PBS, elute GST-FKBP51 and GST-FKBP52 with 5 mL of 10 mM glutathione in PBS.

1.2.9 Concentrate proteins on 15 mL 10.000 MWCO centrifugation unit. To remove free glutathione, pass concentrates through PD-10 column equilibrated with 0.5x PBS and again concentrate on the filter centrifuge device.

1.2.10 Collect protein-containing fractions. Verify the proteins in SDS-PAGE and adjust protein concentrations to 1 mg/mL.

NOTE: Typical protein yield is 2-5 mg/L culture. The protein can be stored at -20 ˚C.

2. Coupling of Hsp90 C-terminal peptide to the acceptor beads (Figure 2B)

2.1 Hsp90 peptide preparation

2.1.1 Synthesize ten amino acid peptide NH_2-EDASRMEEVD-COOH corresponding to amino acids 714-724 of human Hsp90 beta isoform (UniProt ID: P08238) by a peptide synthesis service.

2.1.2 Dilute the Hsp90 peptide in PBS to 1 mg/mL concentration.

2.2 Acceptor beads preparation

2.2.1 Dilute the unconjugated acceptor beads in PBS to 1 mg/mL concentration and transfer to a 1.5 mL tube.

2.2.2 Perform the washing by centrifugation at 16,000 x *g* for 15 min. Carefully remove the supernatant.

2.3 Conjugation

2.3.1 Set the ratio between beads and peptide as 10:1. In the 1.5 mL tube containing 1 mg of acceptor bead pellet (prepared as described above), add 1 mL of PBS (pH 7.4), 0.1 mg of diluted peptide, 1.25 µL of Tween-20, 10 µL of a 400 mM solution of sodium cyanoborohydride ($NaBH_3CN$) in water.

CAUTION: NaBH3CN is toxic; use a fume hood and gloves. $NaBH_3CN$ solution should be freshly prepared.

2.3.2 Incubate for 24 h at 37 ˚C with end-over-end agitation (10-20 rpm) on a rotary shaker.

2.4 Reaction quenching and beads washing

2.4.1 Add 20 µL of 1 M Tris-HCl (pH 8.0) solution to the reaction to block unreacted sites. Incubate for 1 h at 37 ˚C.

2.4.2 Centrifuge at 16,000 x *g* (or maximum speed) for 15 min at 4 ˚C. Remove the supernatant and resuspend the bead pellet in 1 mL of Tris-HCl solution (100 mM, pH 8.0).

2.4.3 Repeat the washing step three times.

2.4.4 After the last centrifugation, resuspend the beads at 1 mg/mL in storage buffer (1 mL of 0.5 × PBS with 0.01% sodium azide as a preservative). Store the conjugated acceptor bead solution at 4 ˚C light protected.

CAUTION: Sodium azide is toxic; use a fume hood and gloves.

3. The assay probing the interaction between GST-FKBP51 or GST-FKBP52 and Hsp90 C-terminal peptide, and inhibition with small molecular mass compounds (Figure 2C)

3.1 GST-tagged proteins interacting with glutathione donor beads

3.1.1 Set up the reactions in 384-well plates.

3.1.2 Prepare the solution containing 10 µg/mL of the glutathione donor beads in 0.5x PBS, pH

7.4.

NOTE: After prolonged storage, beads settle down and need to be vortexed.

3.1.3 Add GST-FKBP51 or GST-FKBP52 to a final concentration of 10 µg/mL.

3.1.4 Incubate in the dark at 25 ˚C for 10 min.

NOTE: At this step, GST-tagged proteins will interact with glutathione attached to the beads. For each well, 22.5 µL of this mixture will be used. The concentration of the binding partners must be determined empirically. Titrate GST-FKBP51 and GST-FKBP52 and choose the concentration that gives the best signal.

3.2 Compound addition

3.2.1 Make serial dilutions of test compounds in DMSO.

NOTE: The concentrations used are typically 10, 30, 100, 300, 1,000, and 3,000 µM.

3.2.2 Add 0.25 µL of DMSO (negative control) or Hsp90 C-terminal peptide (positive control, 30 µM) or compounds in DMSO to the corner of each well of the plate. Use triplicates for every compound concentration.

3.2.3 Add 22.5 µL of the solution containing glutathione donor beads with GST-tagged proteins to each well.

3.2.4 Shake the plate gently with hand but thoroughly. Incubate in the dark at 25 ˚C for 15 min.

NOTE: During this time, compounds will interact with the TPR domain at the Hsp90 C-terminal peptide binding site.

3.3 Acceptor beads addition

3.3.1 Dilute the acceptor beads with attached Hsp90 C-terminal peptide to 100 µg/mL in 0.5x PBS.

3.3.2 Add 2.25 µL of diluted acceptor beads to each well.

3.3.3 Mix gently but thoroughly. Incubate in the dark at 25 ˚C for 15 min.

NOTE: At this step, donor and acceptor beads are brought into proximity by the protein-peptide interactions. The final volume of the reaction mixture is 25 µL. Therefore, the final concentrations of compounds are ranging from 0.1 to 30 µM.

3.4 Plate reading

NOTE: Read the plate using a plate reader set in the relevant mode.

3.4.1 Turn on the instrument and open the software

3.4.2 Choose the relevant protocol.

3.4.3 Click **Edit plate map** and select the well being used in the plate for measurement.

3.4.4 Click **Next** to continue and **Run** the selected protocol.

3.4.5 After measurement, click **Show Results** to view results.

3.4.6 Export the data.

4. Data analysis

4.1 Z' factor and signal-to-background (S/B) ratio

4.1.1. Calculate the Z' factor and S/B ratio for the assay using the following equation:

$Z'=1-(3\sigma pos+3\sigma neg)/|\mu pos-\mu neg|$ [16]

$S/B=\mu neg/\mu pos$

where, σ and μ represent the standard deviations and means of the positive (Hsp90 C-terminal peptide, 30 μM) and negative (DMSO) controls, respectively. A Z' factor > 0.5 will ensure that the assay is robust enough for screening. To monitor the assay sensitivity, the S/B ratio has also been calculated.

4.2 Dose-response curve and IC_{50}

NOTE: Use nonlinear regression analysis to fit the data of Hsp90-cochaperones PPI inhibitors by software.

4.2.1 Create an XY data table in the **Welcome** dialog and select X **Numbers,** and Y **Enter 3** (if triplicates) **replicate values in side-by-side columns**.

4.2.2 Normalize the signal data of samples to the negative control group. Import concentration values to the X column and the signal values to the Y column.

4.2.3 Click **Analyze** and choose **Transform concentration (X)** under **Transform | Normalize**. Choose **Transform to Logarithms**.

NOTE: This will transform the concentration to a log scale. If your starting concentration is zero, set it to a very small number that is effectively zero (e.g., 0.1 nM) not to lose those values since the logarithm of zero is not defined.

4.2.4 Click **Analyze** and choose **Nonlinear regression (curve fit)** under **XY analyses**, open the **Dose-Response-Inhibition** and choose **Log(inhibitor) vs. response -- Variable slope**.

4.2.5 Click **OK** to view the **Results** (containing IC_{50} value) and **Graphs**.

[Place **Figure 2** here]

REPRESENTATIVE RESULTS:

In our assay, Z' factor and S/B ratio are 0.82 and 13.35, respectively (**Figure 3A**), demonstrating that our assay is robust and reliable for high-throughput screening. We then used it to screen small molecular mass compounds. **Figure 3B** presents dose-dependent inhibition of chaperone-cochaperone interactions with a selected small molecule (D10). The dose-response curves for D10 are generated by nonlinear regression analysis, based on which the values of IC_{50} are calculated. D10 shows dose-dependent inhibition both on Hsp90 - GST-FKBP51 and Hsp90 - GST-FKBP52 PPIs. But the values of IC_{50} are different: its IC_{50} for Hsp90 - GST-FKBP51 interactions is 65 nM, whereas, for Hsp90 - GST-FKBP52 interactions, complete inhibition was not achieved with the highest compound concentration (100 µM), its IC_{50} is estimated to be > 30 µM. These results provide evidence that selective inhibition of Hsp90-HKBP51 or Hsp90-FKBP52 PPIs with small molecules can be achieved (TPR domains of FKBP51 and FKBP52 have 60% sequence identity and > 80% sequence similarity), and this assay can be applied for this screening.

[Place **Figure 3** here]

FIGURE AND TABLE LEGENDS:

Table 1: PCR reaction set up for human FKBP51 and FKBP52 DNA amplification.

Table 2: Thermocyler conditions for human FKBP51 and FKBP52 DNA amplification.

Figure 1: The basic principle of this assay. (**A**) Purified GST-FKBP51 interacts with glutathione-linked donor beads. The 10 amino acid long peptides corresponding to the extreme C-terminus of Hsp90 are attached to acceptor beads. The interaction between Hsp90-derived peptides and TPR domain of FKBP51 brings the donor and acceptor beads into proximity. At 680 nm, a photosensitizer in the donor bead illuminates and converts oxygen to singlet oxygen. The thioxene derivative on the acceptor bead reacts with the singlet oxygen and generates chemiluminescence at 370 nm. This energy further activates fluorophores in the same acceptor bead to emit light at 520-620 nm. (**B**) When small molecules inhibit the interactions between Hsp90 and FKBP51, the donor and acceptor beads cannot reach proximity. Then the singlet oxygen with short lifetime decays, and no detectable signal is produced.

Figure 2: Schematic of this protocol. (**A**) Expression and purification of GST-FKBP51 and GST-FKBP52. (**B**) Coupling of Hsp90 C-terminal peptide to the acceptor beads. (**C**) The assay probing the interaction between GST-FKBP51 or GST-FKBP52 and Hsp90 C-terminal peptide. Inhibition with small molecular mass compounds. Created with BioRender.com

Figure 3: Assay analysis and results. (**A**) Z' factor and signal-to-background (S/B) ratio of this assay. Data represent signals of (○) positive control (Hsp90 C-terminal peptide, 30 µM) and (●) negative control (DMSO) from 48 wells. A Z' value of 0.82 and an S/B ratio of 13.35 were calculated from these two populations of data. (**B**) The inhibition of selected compound (D10) on interactions of Hsp90 with FKBP51 (●) or FKBP52 (○) in this assay. D10 inhibits Hsp90-FKBP51 or Hsp90-FKBP52 dose-dependently. Its IC_{50} is 65 nM for Hsp90-FKBP51 interaction but above 30 µM for Hsp90-FKBP52 interaction. Data are normalized to the control group and expressed as means ± SEM.

DISCUSSION:

Here we describe a protocol using the assay for screening small molecules inhibiting interactions between Hsp90 and TPR-motif co-chaperones, especially FKBP51 and FKBP52. Its high Z' score (>0.8) demonstrates the robustness and reliability for a high-throughput format. Results can be obtained within one hour, and small amounts of beads, protein and compounds are required. Moreover, this protocol could easily be extended to any Hsp90/Hsp70 - TPR-motif co-chaperone interactions of interest. Several TPR-motif co-chaperones of Hsp90 have been implicated in various human disorders ranging from Alzheimer's disease to autoimmune diseases, cancer, etc. The protocol described here provides an *in vitro* robust and inexpensive assay for high-throughput screening of small molecules inhibiting chaperone-cochaperone interactions of high medical importance.

In addition, drugs targeting TPR domains and inhibiting interaction with Hsp90 must be assessed not only for their affinity towards the target but also for their selectivity towards other TPR-motif proteins. The human genome encodes > 20 TPR motif proteins capable of interacting with the Hsp90 C-terminal peptide. This assay using glutathione beads and GST-tagged proteins allows assessment of the drug effect on multiple binding TPR partners. Our laboratory possesses a library of 20 human TPR proteins in their GST-tagged form and can obtain affinity and selectivity profiles for every small molecule tested.

It has been previously shown that PPI between Hsp90 and TPR co-chaperones is mediated by the C-terminal peptide of Hsp90; the deletion of Hsp90 C-terminus completely abolishes the binding of TPR-motif co-chaperones[6]. We have found that compounds efficient in our assay where the Hsp90 C-terminal peptide was used also were able to inhibit the interaction of full-length Hsp90 with GST-FKBP51/52 in a pull-down assay using glutathione sepharose beads (data not shown). Some of the selected compounds also show the binding affinity with FKBP51 by surface plasmon resonance technology, and their specific binding sites determined by co-crystallization are currently under investigation.

A critical step in our protocol is the order of addition; we first form a complex between a small

molecule and its TPR target. If complexes between FKBP51 and Hsp90 C-terminal peptide have already been formed, longer incubation times and higher drug compound concentrations are required to break the PPIs. This is mostly due to the steric hindrance of bead limiting access of molecules to the site of interaction.

It is possible to covalently couple TPR proteins and Hsp90 C-terminal peptides to donor and acceptor beads, respectively, and directly probe PPIs. However, it is not recommended to covalently attach both binding partners to the beads due to the reduced movement of molecules in solution affecting the kinetics of the interactions. This also increases the risk of steric hindrance due to the bead size. Therefore, we choose acceptor beads coupled with Hsp90 C-terminal peptide and glutathione donor beads that are interacting with GST-tagged proteins in our assay, where multiple TPR partners can be assessed.

In this assay, it is probable that some identified compounds can be false-positive because of their interference with the assay technology, such as quenching singlet oxygen, quenching light, and scattering light. False-positive results can also be induced by disrupting the binding of GST tag with glutathione donor beads. These false-positive results can be avoided when screening molecules both on Hsp90-FKBP51 and Hsp90-FKBP52 PPIs to identify selective inhibitors. Other methods detecting PPIs should also be applied to verify the selected inhibitors.

The limitation of our assay is that it cannot be used to detect the interaction between Hsp90 and TPR-motif co-chaperones in crude biological samples, such as cell lysates. After the high-throughput screening, the inhibitions of selected compounds on Hsp90 - TPR-motif co-chaperones PPI in biological samples need to be verified by other methods, such as co-immunoprecipitation and proximity ligation assay.

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DISCLOSURES:

Authors report no conflicts of interest.

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Figure

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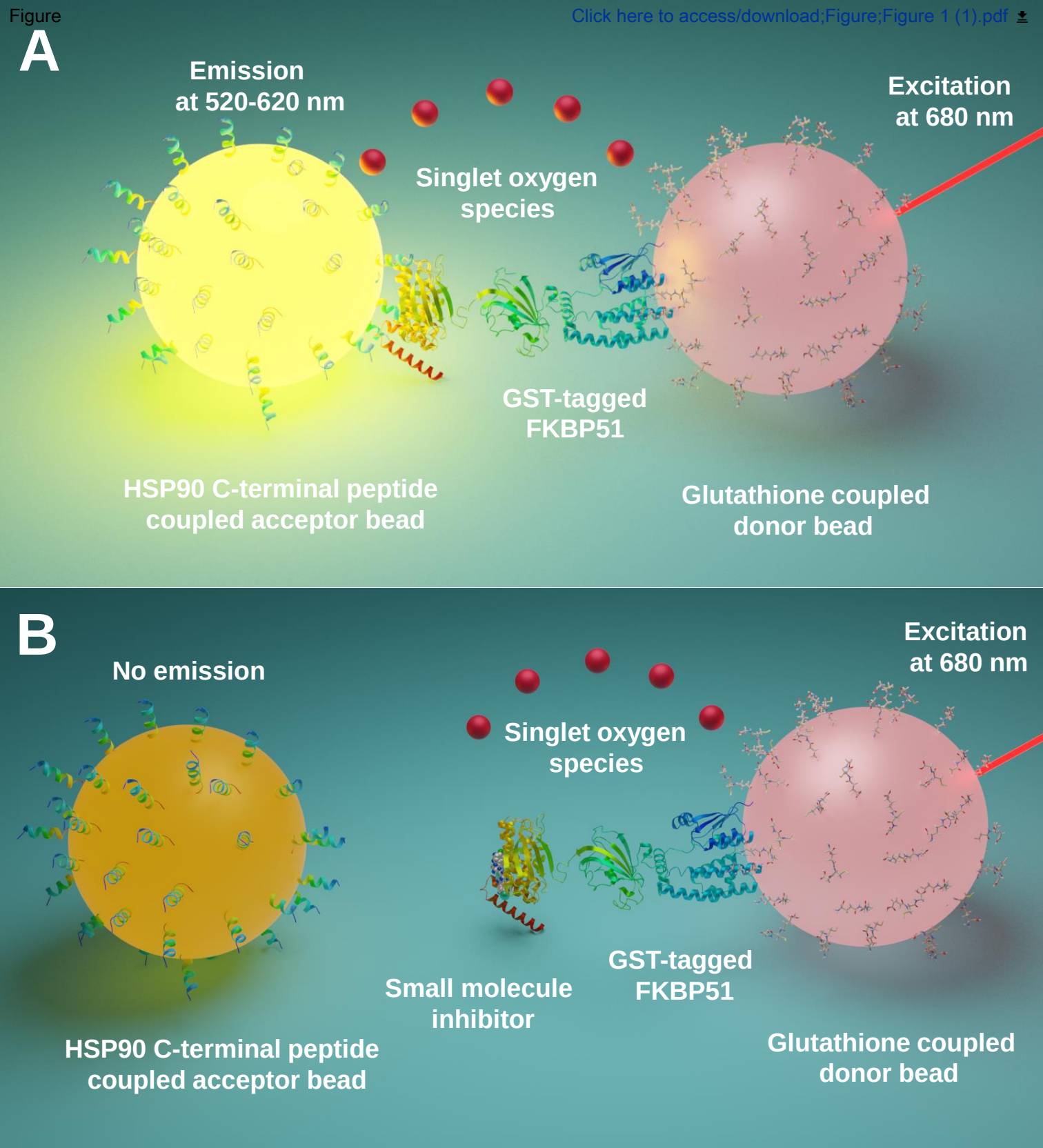

Figure

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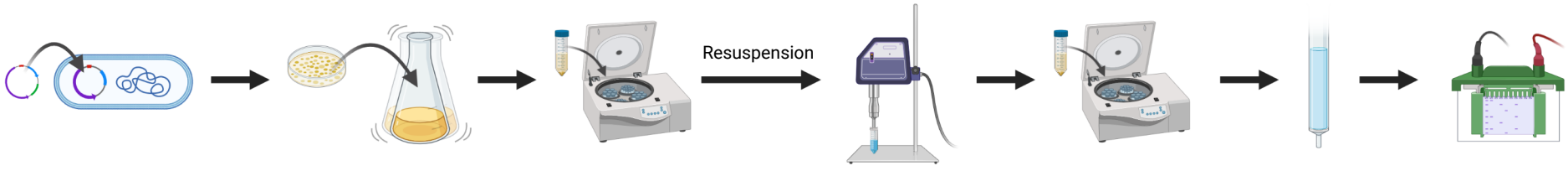
A. Expression and purification of GST-FKBP51 or GST-FKBP52



Transform the plasmid into BL21 E.coli strain

Cluture bacteria in LB solution

Centrifuge to get cell pellets

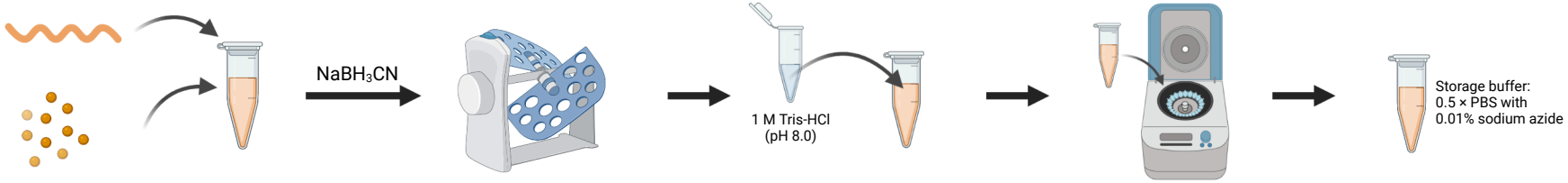
Sonicate cells to release proteins

Centrifuge to remove cell debris

Purify GST-FKBP51/52 by GST-trap column

Concentrate proteins and verify by SDS-PAGE

B. Coupling of Hsp90 C-terminal peptide to AlphaScreen acceptor beads



Prepare Hsp90 peptide and acceptor beads

Dilute and combine peptides and acceptor beads

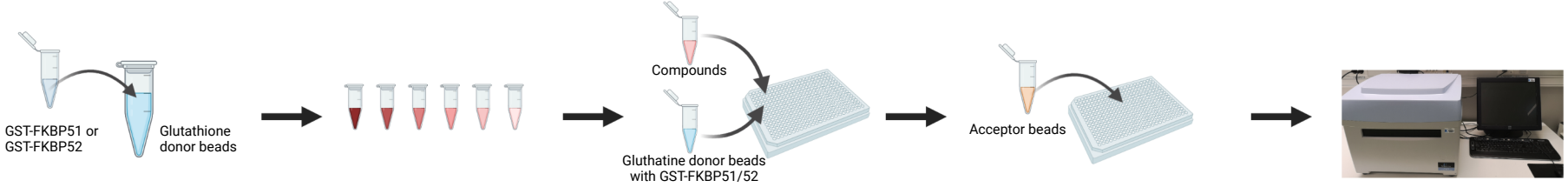
Incubate for 24h at 37 °C with end-over-end agitation for conjugation

Block unreacted sites

Wash acceptor beads three times

Resuspend acceptor beads Store and protect from light

C. AlphaScreen assay probing interaction between GST-FKBP51 or GST-FKBP52 and Hsp90 C-terminal peptide Inhibition with small molecular mass compounds



Combine GST-FKBP51/52 with glutathione donor beads and incubate in darkness at 25 °C, 10 min

Make sterial dilutions of compounds

Add them into a 384-well alpha plate and incubate in darkness at 25 °C, 15 min

Add acceptor beads into the plate and incubate in darkness at 25 °C, 15 min

Read plate and analyze data

Figure

Click here to access/download;Figure;Figure 3.pdf

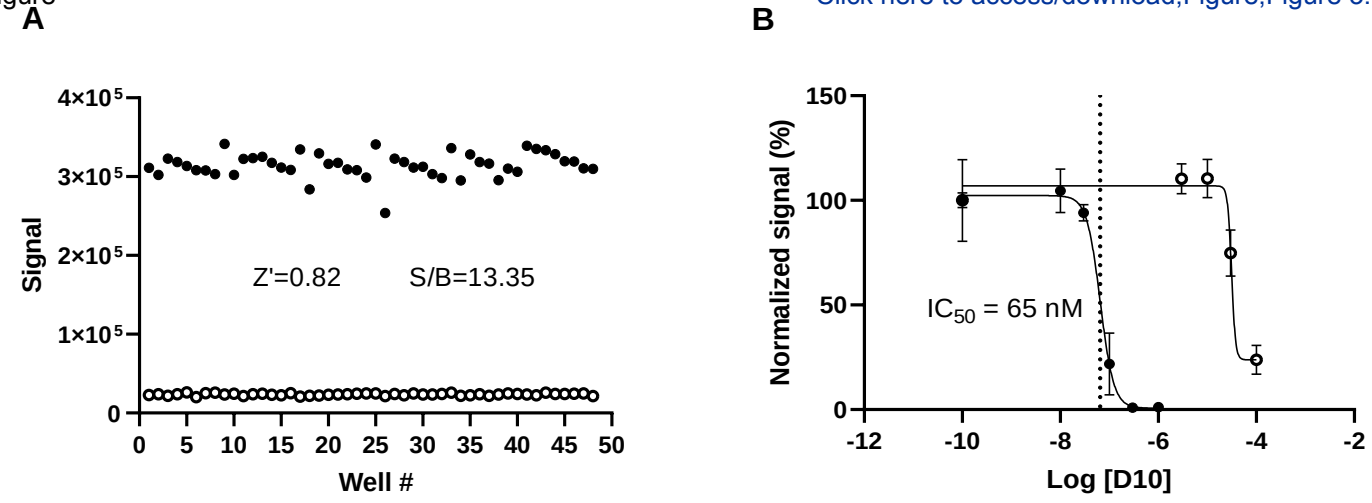


Table 1. PCR reaction set up for human FKBP51 and FKBP52 DNA amplification.

Reaction component	Volume (µL)
PCR buffer (5 x concentrate)	4
Forward primer	1
Reverse primer	1
Plasmid	0.5
dNTP mix (10 mM each)	0.5
Phusion DNA polymerase	0.5
Water (DNA grade)	12.5
Total	20

Table 2. PCR conditions for human FKBP51 and FKBP52 DNA amplification.

Stage	Temperature (℃)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	
Annealing	56	30 sec	35
Extension	72	1 min	
Final extension	72	5 min	1

Note: Lid temperature is 105 ℃.

LETTER TO EDITOR

Dear Editor,

Thank you for providing us the opportunity to submit the revised version of our manuscript to Journal of Visualized Experiments. Now we have revised the manuscript and provide the point-by-point response to editorial and reviewer's comments.

Response to editorial comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- ✓ We have checked the manuscript. There are no spelling or grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

- ✓ We have changed the format of our manuscript according to your suggestions.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: AlphaScreen, One Shot BL21 Star, Eppendorf, Peptide 2.0 inc, Perkin Elmer plate, EnSpire Manager, Excel, etc.

- ✓ We have removed all commercial language from our manuscript and referenced them in the Table of Materials and Reagents.

4. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "AlphaScreen" within your text. The term may be introduced once in the introduction where directly relevant. Otherwise, please refer to the term using generic language.

- ✓ We have replaced nearly all the "AlphaScreen" with general words, except the first appearance in the introduction. We also use "AlphaScreen" at the title. If it is not suitable, we can change it to other general words such as "Studies of chaperone-cochaperone interactions using homogenous bead-based assay".

5. Please revise the following lines to avoid overlap with previously published work: 32-36, 170-171.

- ✓ We have revised these lines.

6. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents…"

- ✓ We have rewritten our Summary (48 words) as your suggestions.

7. Please ensure that the Abstract is between 150-300 words and clearly states the goal of the protocol.

- ✓ We have rewritten our Abstract (172 words) and clearly stated the goal of the protocol.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

- ✓ We have described all the actions in the imperative tense and put the text that cannot be written in the imperative tense as the “NOTE”.

9. Please ensure you answer the “how” question, i.e., how is the step performed? There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

- ✓ We have described all detailed actions in the revised protocol that can be replicated by the viewers.

10. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

- ✓ We have described each step with maximum 3 actions in the protocol.

11. 1.1: Please include PCR conditions and reaction set up as a table and upload it separately as .xlsx file to your editorial manager account.

- ✓ We have included PCR conditions and reaction set up in Table 1 and 2.

12. 2.1.1: How do you decide on the sequence?

- ✓ MEEVD pentapeptide at Hsp90 extreme C-terminus is the binding determinant for TPR proteins. The peptide NH2-EDASRMEEVD-COOH represents last ten amino acids of Hsp90 extreme C-terminus, which includes MEEVD. Therefore, attaching this ten amino acid peptide to acceptor beads can be used to screen compounds that disrupt Hsp90-TPR cochaperones interactions.

13. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- ✓ We have highlighted 3 pages of the protocol for video recording.

14. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. However, for figures showing the experimental set-up, please reference them in the Protocol.

- ✓ In the revised manuscript, we have included the Z’ factor and S/B ratio of our assay to demonstrate it is reliable for high-throughput screening. We also modified the expression of

results from a selected compound (D10) to show that our assay can be applied to screen small molecules selectively disrupting Hsp90-HKBP51 or Hsp90-FKBP52 interactions.

- ✓ We have referenced the Figure 2 (Schematic of the protocol) under the title of Protocol.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

- ✓ All figures in our manuscript are original.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

- ✓ We have discussed all above-mentioned points in the revised discussion section.

17. Please do not abbreviate the journal titles in the references section.

- ✓ We have cited the full name of journals in the references section.

18. Please sort the materials table in alphabetical order.

- ✓ We have sorted the materials in alphabetical order.

Response to reviewer´s comments

Reviewer #1:

Manuscript Summary: Wang et. al developed a Alphascreen protocol for probing interactions of Hsp90 and its co-chaperones, FKBP51 and FKBP52. They used purified GST-tagged FKBP51 or FKBP52 coupled to the glutathione-linked donor beads. Hsp90-derived peptide was attached to the activated acceptor beads via N-terminal amino group. When donor and acceptor beads are brought into proximity by the interaction between TPR proteins and Hsp90-derived peptide, bioluminescence signals indicate the resulting interactions. Based this protocol, they screened the small molecule library to identify inhibitors of Hsp90-FKBP interaction and identified potent and selective inhibitors of Hsp90-FKBP51 interaction. In general, this protocol is well designed and showed success in practice.

Major Concerns: No major concerns.

Minor Concerns:

Please clarify (1) compound concentration dilution ratio from 0.1 to 30uM and (2) positive control well (page 195-197). I am not clear about the reproducibility of screen (Z' score).

- ✓ We have specified concentrations used in 3.2.1 section and positive control in 3.2.2 section.
- ✓ We have added data regarding assay Z´score in Figure 3A, and relevant text in 4.1 section.

Reviewer #2:

Manuscript Summary: Pavlov P. and et al. presented the work titled with Studies of chaperone-cochaperone interactions using AlphaScreen assay. This study is interesting but not suitable for publication in current status with the following comments:

Major Concerns:

1. AlphaScreen assay is good for PPI. However, it's not popular or not a golden standard (co-PI) for PPI. This reviewer thinks the as-prepared chaperone-cochaperone interactions should be verified by co-Immunoprecipitation (co-PI) or surface plamon resonace technology (SPR), as well.

- ✓ Yes, the chaperone-cochaperone interactions should be verified by co-IP or SPR in the development of inhibitors. We have performed the co-IP and SPR for the hits and briefly discussed them in the discussion section. Because this protocol is mainly focused on AlphaScreen, so we did not include detailed information of co-IP and SPR here.

2. The lysates containing FKBP51/52 or real samples without purification should be analyzed for IC50 from the analysis curves

- ✓ Our method can mainly be used for high-throughput screening of molecules *in vitro*. For the real samples, such as cell lysates, co-IP and proximity ligation assay can be applied to detect the Hsp90-FKBP51 or Hsp90-FKBP52 interactions. We have discussed this shortcoming of our method in the discussion section.

Minor Concerns:

1.The analysis curves used for the quantity analysis could be obtained from Fig 3.

- ✓ Yes, the dose-response curves of the inhibitor are generated using nonlinear regression analysis by GraphPad Prism software. The values of IC_{50} were calculated based on the generated dose-response curves. We have added this in the result section.

2.The pixel solution is too low for Fig 1.

- ✓ We have updated the high-resolution Figure 1 in the revised manuscript.

We would like to thank the reviewers and the editorial board for the opportunity to improve our manuscript. We believe that the revised version of this manuscript may be accepted for publication in JoVE.

With best regards,

Pavel Pavlov, PhD