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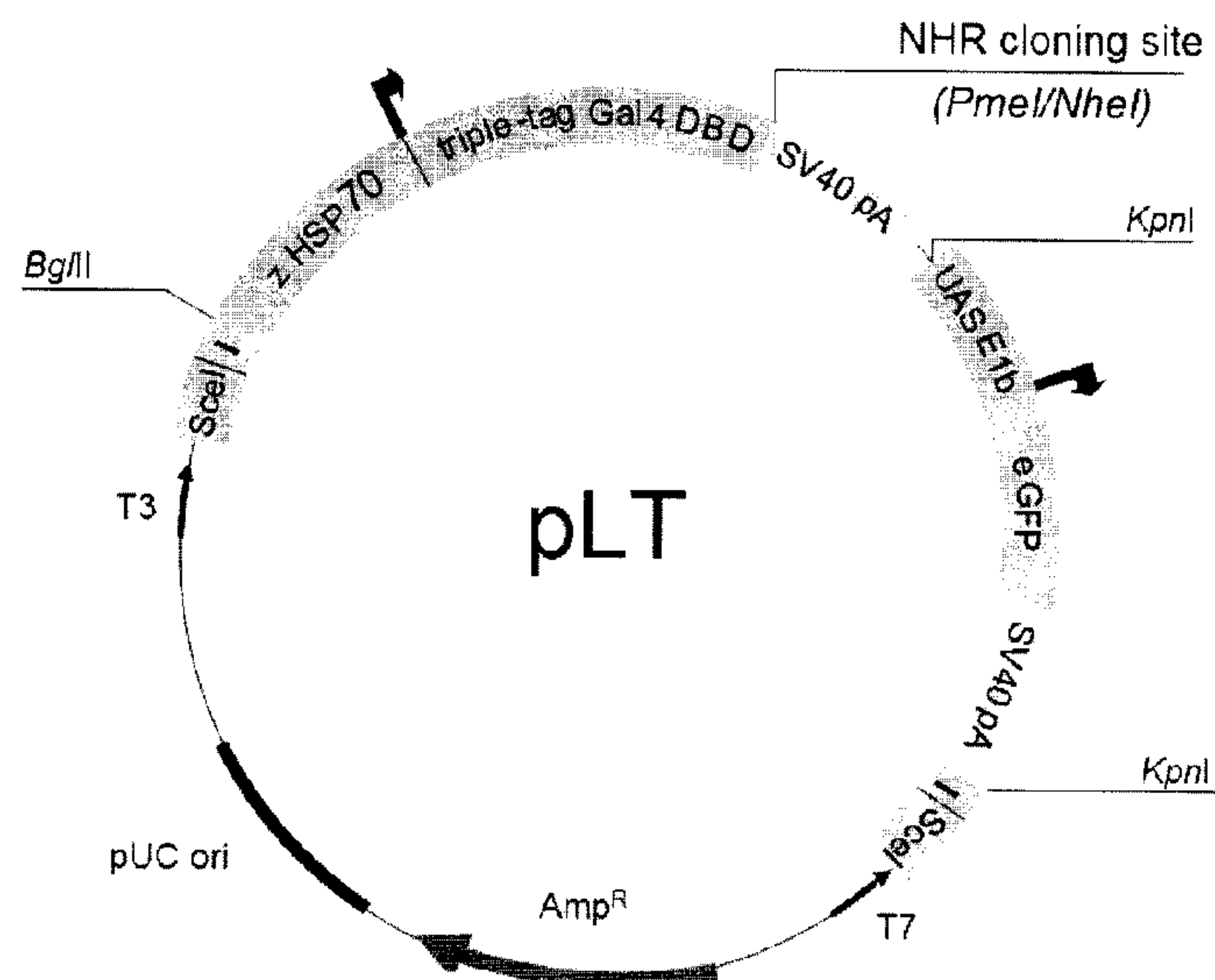
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(54) **Titre : PROCÉDES ET COMPOSITIONS POUR LA DETECTION ET L'ISOLATION DE LIGANDS**
(54) **Title: METHODS AND COMPOSITIONS FOR THE DETECTION AND ISOLATION OF LIGANDS**



(57) **Abrégé/Abstract:**

A method for the detection and isolation of ligands, preferably nuclear receptor ligands, bound to their cognate receptors in live animals, is described. A novel composition comprising 1) a chimeric transcription factor containing a DNA-binding domain, preferably from a non-vertebrate transcription factor, fused to the ligand-binding domain (LBD) of a nuclear receptor, 2) a reporter system, driven by a promoter that contains binding sites for the chosen DNA-binding domain, 3) multiple affinity tags fused to the LBD fusion proteins to facilitate efficient purification, along with specifically associated molecules and 4) sequences required for simultaneous genomic integration of all three components above are described. To make use of the system, expression of the chimeric LBD protein is broadly induced.



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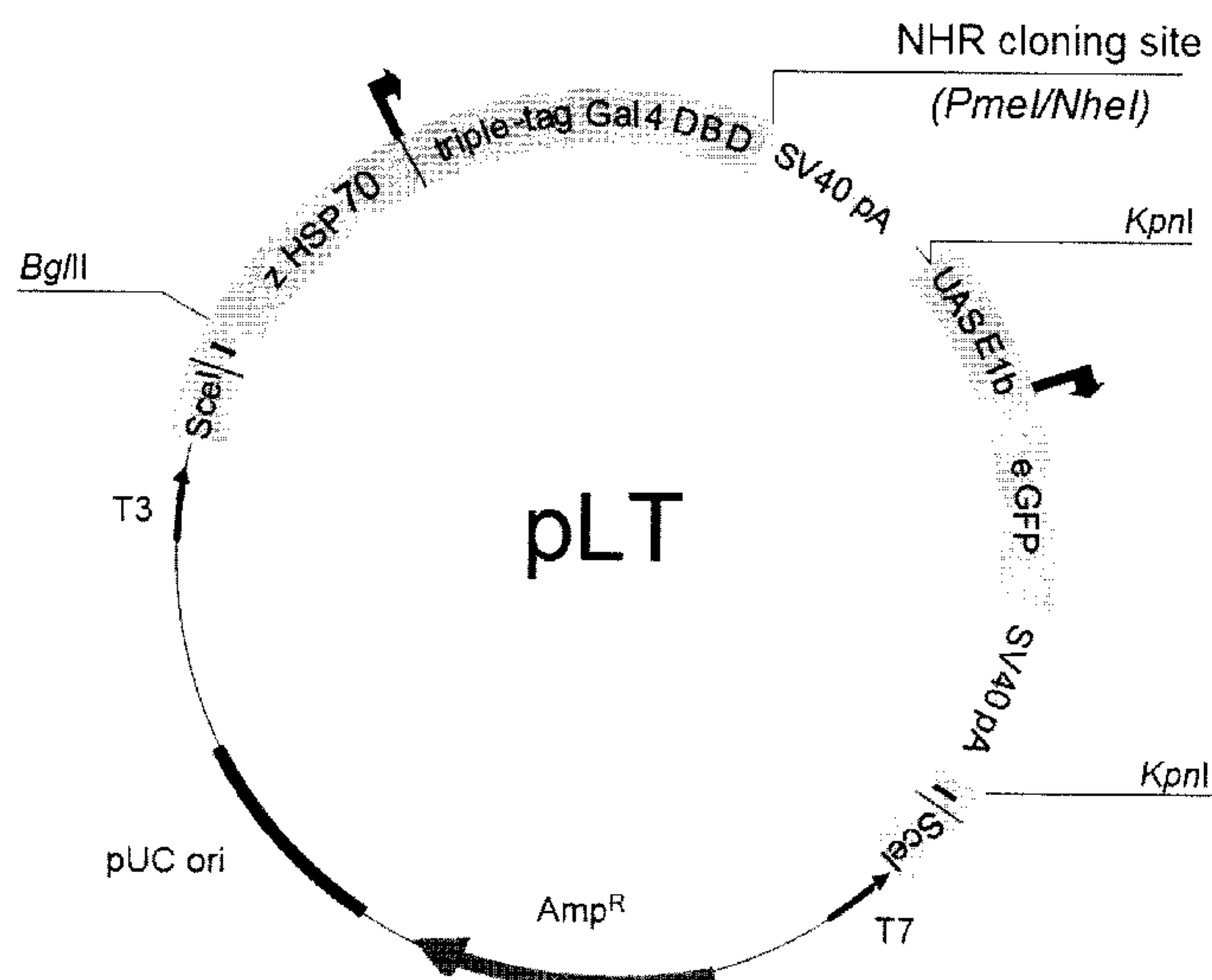
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TITLE: Methods and Compositions for the Detection and Isolation of Ligands

FIELD OF THE INVENTION

The invention relates to methods and compositions for the detection and isolation of ligands. In particular, the invention relates to detection and
5 isolation of ligands in live animals.

BACKGROUND OF THE INVENTION

The activities of many regulatory proteins and RNAs can be modulated by small molecule ligands that move freely within and between living cells. The mobility of these small molecules, and their effects on important cellular
10 targets, make them excellent candidates for pharmaceutical development. Indeed, the National Institute of Health has declared a need to find small molecule ligands for every protein encoded in the genome. The discovery of therapeutic small molecules and their targets is also the primary focus of most pharmaceutical companies. The Nuclear Receptor ("NR") superfamily of
15 transcription factors comprises a particularly attractive set of small molecule targets. Unlike most other transcription factors, NRs are normally switched on and off by small lipids or lipophilic molecules. Furthermore, NRs feature in practically every fundamental biological process, functioning as key control points in key signaling and metabolic pathways {Mangelsdorf, 1995; Chawla,
20 2001}. The pivotal roles played by these proteins, and their potential for functional manipulation by natural and synthetic ligands, make them ideal targets for medical study and drug intervention.

The human genome contains 48 members of the NR superfamily. These proteins share a common structural organization, including a central,
25 zinc finger DNA-binding domain ("DBD"), and C-terminal to this, a structurally conserved ligand-binding domain ("LBD") {Kumar, 1999}. In addition to forming the ligand-binding pocket, the LBD is also involved in homo- and/or hetero-dimerization and possesses ligand-regulated binding sites for transcriptional co-activators and co-repressors {Egea, 2000; Wagner, 1995;
30 Renaud, 1995; Uppenberg, 1998; Bledsoe, 2002; Dhe-Paganon, 2002; Gampe, 2000}. Most LBDs characterized to date are composed of approximately 12 α -helices arranged in three layers to form a hydrophobic

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ligand-binding pocket in the centre. Examples of NR ligands include steroid hormones, thyroid hormones, bile acids, fatty acids, certain vitamins and prostaglandins {Francis, 2003; Bogan, 1998}. Ligand binding induces structural changes in the LBD, such that, in the case of activators, the position
5 of helix 12 is altered resulting in the displacement of co-repressors, recruitment of coactivators and subsequent target gene transcription {Glass, 2000}. Ligands whose binding promotes the transcriptional activation of target genes, termed agonists, induce different structural changes in LBDs than antagonists. Antagonists tend to possess bulky chemical groups that cannot
10 be properly accommodated in the binding pocket, preventing the proper placement of helix 12 for coactivator binding {Glass, 2000}.

NRs play major roles in most physiological processes. These include sex determination {McElreavey, 1999}, maturation {Beuschlein, 2002}, growth control {Zhao, 2001}, metabolism {Basu-Modak, 1999}, neuronal growth and
15 differentiation {Zhou, 1999; Satoh, 2002}, neuroendocrine function {Auger, 2000; Tetel, 2000} electrolyte homeostasis {Turnamian, 1990}, immune responses, xenobiotic responses {Kliwer, 1999; Kliwer, 2002; Willson, 2002}, circadian rhythm and aging {Pardee, 2004}. When NRs malfunction, major diseases ensue, some of which are listed below. By understanding NR
20 functions and the ligands that control them, there is the potential to control the many diseases associated with inappropriate NR activity. There are already many very successful examples of this. One of the more familiar is the control of Estrogen Receptor (ER) activity in breast tumours by the synthetic antagonists tamoxifen and raloxifene {Tonetti, 1999; Osborne, 2000}. RXR-
25 and RAR-directed retinoid analogs have proven successful in the treatment of acute promyelocytic leukemias {Degos, 1995} and glaucoma {Kim, 1990; Stoilov, 2001}. A great deal of attention has also been focused on the development of ligands for LXRs, PPARs and HNF4, which play major roles in hyperlipidaemia, atherosclerosis, diabetes and obesity {Kersten, 2000; Repa,
30 2002; Willson, 2001; Way, 2001; Wakino, 2002; Bogan, 2000}. Until recently, each of these NRs were orphans whose ligand(s) were unknown. Some of the most recent orphan NRs to gather attention are FXR, PXR and CAR, which all

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play major roles in xenobiotic responses {Stoilov, 2001; Willson, 2002; Xie, 2000; Kliwer, 1999; Kliwer, 2002; Kliwer, 2002}. Modulating the activities of these receptors can decrease occurrences of drug resistance and drug-drug incompatibility, which are major problems in drug treatment plans. Other
5 major diseases known to be caused by inappropriate NR activity include Parkinson's disease {Sato, 2002; Lee, 2002; Rawal, 2002}, cardiac myopathies {Zhu, 2003; Huss, 2002} and asthma {Bolt, 2001; Serhan, 2001}.

The potential of future NR-directed pharmaceuticals to control normal and abnormal biological processes is reflected by the percentage of top-
10 selling drugs present on the market (> 10%). This large presence is despite the fact that relatively few NRs have been successfully targeted. The large subfamily of orphan NRs yet to be targeted has the potential to define critical new biological processes and physiological pathways. Hence, they also represent an untapped resource for drug discovery and disease treatment.
15 Even with the NRs that have known ligands, more potent, selective, stage- and tissue-specific agonists/antagonists need to be identified and developed. Further fine-tuning of drug specificities is also required to alleviate the cross-reactivity, cross talk and unwanted side effects of existing ligands. For example, tamoxifen, which is used to inactivate the Estrogen receptor (ER) in
20 breast tumours, also blocks normal and necessary functions of the receptor in other tissues. Tamoxifen also cross-reacts with other NRs. It is likely that new ER agonists and antagonists can be developed that act stage- and tissue-specifically and far more selectively.

The first NR ligands identified were hormones such as the insect
25 metamorphosis-inducing steroid ecdysone and the female-specific steroid estrogen. The powerful effects of these hormones allowed for the large-scale and complex purification schemes necessary for their ultimate identification. However, the complexity of these purification procedures, the lack of suitable assays and the unsuitable biochemical nature of most ligands make
30 conventional purification methods tedious, unsuitable or impossible for the remaining NR ligands.

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Methods currently used to identify NR ligands involve *in vitro* based or cultured cell based screens. Most *in vitro* screening methods depend on ligand-mediated enhancement of coactivator peptide binding. One example of this is the ALPHAScreen, which makes use of time-resolved fluorescence resonance energy transfer (FRET) {Glickman, 2002}. The major drawback of all *in vitro* approaches, however, is that conditions and cofactors required for LBD stability and ligand and/or coactivator binding are likely to be missing. This means that many compounds with potential activity may be passed over. Also, compounds that do prove to be active in the test tube may prove unsuitable *in vivo*. For example, they may be unable to penetrate cells or tissues, they may be rapidly modified or degraded, or they prove to be toxic due to numerous off-target effects.

In the case of cultured cells, the most widely used screening methods involve ligand-stimulated reporter gene activation {Dias, 1998; Grover, 2003}. FRET has also been used to detect ligand-mediated LBD-cofactor interactions {Llopis, 2000; Day, 1998; Weatherman, 2002}. A yeast-based approach, that detects ligand mediated refolding of LBDs that are fused to yellow fluorescent protein (YFP), has also recently been developed {Muddana, 2003}. As with *in vitro* approaches, however, each of these cell-based assays is limited by the presence or absence of appropriate and inappropriate cofactors and conditions restricted to the particular cell type chosen for the assay. For example, alternative cofactors bound by a given NR in one cell type are often absent in others. In addition, the binding, delivery and function of some ligands only works in certain cell types. Also, as with the *in vitro* based assays, these compounds may prove to be unstable or toxic in a whole-animal system. Indeed, the majority of compound hits fail when subsequently tested in live model organisms. Thus *in vitro* and cultured cell based results, while high in throughput, tend not to be predictive of *in vivo* utility.

Further evidence that ligands with tissue-specific efficacy exist and are important is the observation that different ligands for tissue-specific receptors promote distinct changes in LBD conformations. These alternative conformations are likely to have different outcomes in different cell types due

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to the diversity of cofactors and cofactor modifications capable of interacting with these alternative conformations. Thus, a full understanding of *in vivo* NR function requires elucidation of the ligands that are active in each tissue of the living animal. Ultimately, this requires the testing of compounds in the entire
5 organism during all stages of development, as well as adulthood and senescence.

SUMMARY OF THE INVENTION

The present inventors provide a "ligand-trap" assay that uses a novel composition to create transgenic animals that allow for *in vivo* detection of
10 ligand binding and subsequent purification and identification of the bound ligand(s).

Accordingly, the present invention provides a composition comprising

- (a) a first nucleic acid encoding a fusion protein of a DNA-binding domain, a ligand-binding domain and multiple different affinity tags; and
- 15 (b) a second nucleic acid comprising a promoter having binding sites for the DNA-binding domain and encoding a reporter protein.

In another embodiment, the first and second nucleic acids are on a single vector.

The invention further provides an *in vivo* method for detecting an
20 endogenous ligand comprising

- (a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated into its genome;
- (b) inducing expression of the first nucleic acid; and
- 25 (c) detecting a change, if any, in expression of the reporter protein, wherein the change indicates that at least one endogenous ligand is present.

In another embodiment, the invention provides an *in vivo* method for screening a test compound for its ability to interact with a ligand-binding domain comprising

- 30 (a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated into its genome;

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(b) contacting the transgenic animal with the test compound or condition;

(b) inducing expression of the first nucleic acid; and

(c) detecting a change, if any, in expression of the reporter protein,
5 wherein the change indicates that the test compound or condition affects the activity of the ligand-binding domain (directly or indirectly).

The invention further provides a method for purifying the fusion protein, along with bound ligand(s) or test compound(s) that bind to the protein encoded by the first nucleic acid. Accordingly, the invention provides a
10 method for purifying modulators of the reporter system that act via direct molecular interactions, the method comprising

(a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated into its genome;

15 (b) inducing expression of the first nucleic acid;

(c) detecting a signal;

(d) generating a cellular extract from the responding animal or tissues;

and

(e) subjecting the cellular extract generated in step (d) to multiple
20 affinity purification steps, each step comprising binding one affinity tag to an affinity resin capable of selectively binding the one affinity tag and eluting the affinity tag from the affinity resin after substances not bound to the fusion protein have been removed.

In another embodiment, the invention provides a method for purifying a
25 modulator of a reporter system, the method comprising

(a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated into its genome;

(b) contacting the transgenic animal with a test compound;

30 (c) inducing expression of the first nucleic acid;

(d) detecting a signal;

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(e) generating a cellular extract from the responding animal or tissues;
and

(f) subjecting the cellular extract generated in step (e) to multiple affinity purification steps, each step comprising binding one affinity tag to an
5 affinity resin capable of selectively binding the one affinity tag and eluting the affinity tag from the affinity resin after substances not bound to the fusion protein have been removed.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood,
10 however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

15 **BRIEF DESCRIPTION OF THE DRAWINGS**

The invention will now be described in relation to the drawings in which:

Figure 1 shows the bifunctional ligand trap vector (pLT) used to verify the functionality of the invention, referred to as "ligand trapping". The pLT
20 vector encodes a triple-tagged GAL4 DNA-binding domain, under the control of an inducible heat shock promoter and followed by restriction sites for adding in-frame ligand-binding domains. The resulting fusion proteins serve as ligand-regulated transcription factors that can be affinity purified to identify bound ligands. The pLT vector also contains a GFP ligand detection reporter
25 system comprised of multiple GAL4 binding sites upstream of a basal promoter and a GFP reporter gene. Binding of a transcriptionally active GAL4-ligand-binding domain fusion protein to these GAL4 binding sites results in GFP expression. Amp = Ampicillin resistance; T7; T3 = T7 or T3 polymerase promoter; zHSP70 = zebrafish *hsp70* gene promoter; triple tag = 3xFlag tag,
30 Tev cleavage site, Strep II tag, and 6xHis tag. Gal4 DBD = Gal 4 DNA-binding domain, amino acids 1-132. NR = nuclear hormone receptor. SV40 pA = SV

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40 polyadenylation signal. UAS = upstream activating sequence. E1b = adenoviral basal promoter. eGFP = enhanced green fluorescent protein.

Figure 2 describes the function of the ligand detection portion of the ligand trap system. (a) Composition of the ligand-detecting fusion protein. The DNA-binding domain (DBD) of GAL4 is fused N-terminal to a triple-tag and C-terminal to a NR LBD under the control of a heat shock promoter (hsp). (b) Fusion protein transcriptional activity is monitored with a GAL4_{UAS}-regulated GFP reporter. When the GAL4 DBD fusion protein binds to the reporter, and ligands and coactivators are present, GFP is expressed. (c) Example of a ligand sensor system response in a *Danio rerio* (zebrafish) embryo. Sites of GFP fluorescence (white nuclear dots) correspond to cells that contain endogenous activating ligand(s).

Figure 3 shows that different NR ligand trap constructs exhibit unique patterns of activity in developing embryos: GFP expression was monitored after 24 hpf, 48 hpf or 72 hpf. No GFP signal is detected when the GAL4 DNA-binding domain is not fused to a ligand-binding domain (LBD). A, B) F0 fish injected with different ligand trap NRs showing transient GFP expression. C – H) Transgenic F1 ligand-trap embryos. Receptor activity is observed in the yolk, midbrain and forehead of Rev-ErbA α fish (A), the yolk, heart, central nervous system (CNS), eyes and adipose tissue of TR α fish (B, C), the spine of PPAR γ fish (D), the brain of RAR β fish (E) and the brain and CNS of LXR α fish (F).

Figure 4 shows a schematic illustration of the triple-tag (3xFLAG-Strep II-6xHis) affinity purification procedure: The ligand trap bait construct (triple tag-Gal4 DBD-NR LBD) is expressed in cells or animals and is purified using triple affinity purification. The purification can either be performed under gentle (ie: low salt for protein cofactors) or harsh (ie: high salt for lipophilic ligands) conditions. Eluted ligands can be identified by MALD-TOV or ESI mass spectrometry (proteins, peptides), or by non-denaturing, ESI or gas-coupled mass spectrometry (small molecules).

Figure 5 depicts the screening method for exogenous ligands: (a) Transgenic ligand trap fish are mated, producing 200-300 eggs per female.

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Eggs/embryos/fish are distributed to multi-well assay plates after heat shock, with 3-6 specimens per well. (b) A small-molecule library or an extract that contains potential ligands is added to each well. (c) After a period of incubation, reporter (GFP) expression is monitored and compared to controls.

- 5 Agonists induce novel GFP expression, or broaden and strengthen endogenous responses. Antagonists suppress GFP expression induced by endogenous agonists. Referral to the library database reveals the identities of new nuclear receptor agonists or antagonists.

Figure 6 shows the ability of the triple-tag system to copurify and
10 identify specifically associated ligands. A silver stained SDS PAGE gel was loaded with extract containing the ligand trap fusion protein GAL4-TR β expressed, in this case, in cultured ZF4 cells. The GAL4-TR β -containing extract, or mock extract (no TR β induced), was affinity purified in successive steps using the following resins: FLAG M2 monoclonal antibody matrix
15 (Sigma), Strep-Tactin Superflow (IBA) and finally Talon Superflow metal (Clontech). Beads were extensively washed after each interaction and then bound proteins specifically eluted as specified (see Methods). The starting extract and final elutions were loaded on a 10% SDS gel, and after electrophoresis, silver stained for proteins. Each of the bands seen only in the
20 TR β "bait" lane was successfully identified using ESI-MS, including the bait protein itself and two previously known cofactors. Lane 1: Marker; Lane 2: mock extract; Lane 3: Gal4-TR β extract; Lane 4: triple-purified mock eluate; Lane 5: Triple-purified TR β eluate.

Figure 7 shows that Ligand Trap lines can be used to screen for
25 activating substances. Panel A shows transgenic *Drosophila* embryos that express a GAL4-DHR96 (*Drosophila* hormone receptor 96) fusion protein and a UAS-GFP reporter. The embryo on the left was incubated in control media, and the embryo on the right in 10^{-5} molar CITCO. CITCO is a component of a small molecule library used to screen for novel DHR96 ligands. This is the first
30 example of a DHR96-specific ligand (methods described in {Palanker, 2006}). Panel B shows seven day old Thyroid hormone receptor β (TR β) fish embryos grown in the absence (left) or presence (right) of a known TR β ligand (10^{-5}

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molar tri-iodothyroacetic acid). Induced GFP expression is seen in the liver and gut of the ligand-treated embryos (arrow). This shows that fish ligand trap lines can also be used for exogenous ligand screening.

DETAILED DESCRIPTION OF THE INVENTION

5 The present inventors have developed a high throughput approach for the identification and functional testing of ligands, preferably nuclear receptor (NR) protein ligands *in vivo*. The technology makes use of a multifunctional reporter system to signal the presence of ligands in live animals, and to
10 isolate and identify those ligands and cofactors that are directly bound to activated receptors.

 In particular, the inventors have tested and validated a novel "ligand trap" system. The primary components of this system are an NR ligand-binding domain (LBD) fused to the DNA-binding domain of GAL4, a GAL4 promoter-regulated GFP reporter gene and a StrepII/His/FLAG triple-affinity
15 tag fused to the GAL4-LBD fusion protein (Figures 1, 2). In the animal, the localized binding (or disassociation) of ligands converts the GAL4-LBD fusion protein into a transcriptional activator that binds and activates the reporter gene promoter, leading to local GFP expression (Figure 2).

Compositions of the Invention

20 The present invention provides a composition having a first nucleic acid that encodes a chimeric transcription factor that has DNA-binding, ligand-binding and affinity tag portions and a second nucleic acid that encodes a reporter component that is capable of detecting the consequences of ligand association with the chimeric transcription factor.

25 Accordingly, in one embodiment, the present invention provides a composition comprising

- (a) a first nucleic acid encoding a fusion protein of a DNA-binding domain, a ligand-binding domain and multiple different affinity tags; and
- 30 (b) a second nucleic acid comprising a promoter having binding sites for the DNA-binding domain and encoding a reporter protein.

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The term "DNA-binding domain" as used herein means a protein domain that is capable of binding a promoter sequence. DNA-binding domains not likely to recognize heterologous host DNA sequences are known in the art, such as those of GAL4, LexA, and Lambda repressor. In a preferred
5 embodiment, the DNA-binding domain is from a non-vertebrate transcription factor that has limited capacity to bind host DNA sequences (to prevent interference with host gene genome function). In a particular embodiment, the DNA-binding domain is from the yeast transcription factor Gal4.

The term "ligand-binding domain", as used herein, means a sequence
10 that is able to interact with another molecule, including proteins, nucleic acids, small molecules or post-translational modifications. A person skilled in the art could use any ligand-binding domain of interest. The composition of the invention is particularly useful for co-purifying (trapping) ligands that are normally difficult to isolate. For example, the ligand-binding domain of a
15 nuclear receptor may be used. Table 1 provides a list of human NR receptors tested thus far. Accordingly, in a particular embodiment, the ligand-binding domain is derived from a nuclear receptor.

Although focused on NRs, the methods and compositions described here can be adapted to many other types of proteins or RNAs. For example,
20 kinases, dehydrogenases, acyl transferases, and PAS domain proteins are small molecule binding proteins that could work equally well. As a more complex example of the invention, one could co-express a Gal4 DNA-binding domain fusion protein containing the MS2 coat protein, along with an RNA sequence comprising an MS2 coat protein binding motif fused to an RNA bait
25 sequence, to discover VP16-coupled RNA binding proteins that bind the bait RNA sequence.

The term "ligand" as used herein can be any molecule that is capable of binding to the ligand-binding domain of the fusion protein. Examples include natural products such as peptides, proteins, nucleic acids, lipids,
30 sugars, metals, as well as assorted synthetic compounds.

The choice of a highly efficient set of affinity tags, such as those used herein, provides the levels of fusion protein purification (up to 10⁹-fold with

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70% yield) required to copurify enough ligand from complex cells or tissues to permit identification by mass spectrometry (procedure diagramed in Figure 4). Besides identifying novel ligands, this approach provides confirmation that agonists or antagonists exert their effects via direct interactions with the ligand-binding domain. For this purpose, the tags must be motifs, fused to the bait fusion protein (DNA-binding domain fused to ligand-binding domain), that bind strongly and specifically to a substrate that can be covalently coupled to a resin. Furthermore, the tag-substrate interaction must be readily dissociated by conditions that do not disrupt interactions between the bait and specifically-interacting molecules.

In one embodiment, the first nucleic acid encodes at least two different affinity tags. In another embodiment, the first nucleic acid encodes at least three different affinity tags, each with an ability to provide approximately 1000-fold purification or higher with yields of 50% or higher. This would yield sufficient levels of bait and ligands/cofactors from animal tissues for identification by mass spectrometry.

In a preferred embodiment, the multiple different affinity tags are the triple-tag comprising StrepII/His/FLAG. The 3X FLAG used binds to an anti-FLAG monoclonal antibody and is released by adding excess FLAG peptide, or optionally, in the case of the triple-tag used here, by TEV protease cleavage of the tag from the bait protein. The StrepII tag binds to streptavidin and is efficiently dissociated from streptavidin by desthiobiotin or biotin. The 6XHis tag binds to metals such as Cu^{++} and can be released using imidazole. Each of these tags fulfills the requirements described above. Furthermore, they work in low salt, high salt or denaturing conditions, allowing the selective purification of ionically associated, hydrophobically associated or covalently bound molecules, respectively.

In another embodiment, the first nucleic acid further comprises an inducible promoter to avoid any deleterious effects caused by expression of the encoded fusion protein. The term "inducible promoter" as used herein means a promoter sequence that allows transcription only in the presence of the inducible element. A person skilled in the art could readily choose an

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appropriate inducible promoter that would not interfere with development or function *in vivo*. For example, a heat- or cold-inducible promoter may be used wherein transcription only occurs upon a shift in temperature. Accordingly, in one embodiment the inducible promoter is heat, transcription factor, or hormone activated. In the preferred embodiment, GAL4 and hormones are already used/involved, making them inappropriate for use. Hence, a heat-inducible *hsp70* promoter is used to control transcription of the chimeric transcription factor.

The term "reporter protein" as used herein means any protein that when expressed is detectable in live cells or animals. Such currently available reporter proteins include green fluorescent proteins, red fluorescent proteins, DS-red and luciferase. In one embodiment, the reporter protein is enhanced green fluorescent protein (eGFP).

In one embodiment, the first and second nucleic acids are on different vectors. In another embodiment, the first and second nucleic acids are on a single vector. In a particular embodiment, the single vector comprises the nucleotide sequence as shown in SEQ ID NO:1 (Table 2).

Methods and Uses of the Present Invention

One use of the compositions of the invention is for *in vivo* ligand detection. In one embodiment, the first and second nucleic acids are used to create two transgenic animals, each expressing one of the nucleic acids. The transgenic animals are then genetically crossed in order to signal the presence of activated LBD fusion proteins. In another embodiment, the first and second nucleic acids are on a single vector used to create a transgenic animal that is capable of expressing both the fusion protein and reporter protein. The compositions of the invention can also be used transiently within cultured cells or non-transgenic animals.

Although it has previously been shown that GAL4 LBD fusions could be used to detect the presence of ligands in cultured cells or fixed tissues these fusions and detection systems were unsuitable for the screening of candidate ligand compounds. Activities could not be monitored unless the animals were sacrificed and the appropriate tissues dissected, sectioned and fixed. These

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methods only provide restricted snapshots of the tissues and stages selected. They are also extremely labor-intensive, making them unsuitable for screening. Furthermore, compounds could not be applied to these previously used transgenic animals unless fed or injected. By combining the use of
5 fluorescent protein reporters (i.e. GFP, RFP, DS-red etc.), and co-expressing LBDs fused to an appropriate DNA binding motif (i.e. GAL4, LexA Nuclear receptor etc), the present inventors have been able to detect the presence of endogenous or exogenously added ligands in live animals, in most tissue types and at all stages of development (eg Figure 5, 7, Table 1 and {Palanker,
10 2006}).

Accordingly, the present invention provides a method of detecting an endogenous ligand *in vivo* comprising

- (a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated
15 into its genome;
- (b) inducing expression of the first nucleic acid; and
- (c) detecting a change, if any, in expression of the reporter protein, wherein the change indicates that at least one endogenous ligand is present.

The term "endogenous ligand" as used herein means any molecule that
20 exists *in vivo* that is capable of interacting with the ligand-binding domain. Such ligands potentially include polypeptides, nucleic acids, lipids, sugars, metal ions, other naturally occurring molecules, post-translational modifications or ingested xenobiotics and pollutants.

The present invention also allows screening of a test compound for its
25 ability to interact with the ligand-binding domain. This provides the ability to screen for new modulators of the target protein. This system also determines whether the added modulators act tissue or stage-specifically, whether they act positively or negatively and what concentration levels are effective. Importantly, it also selects for modulators that are stable, non-toxic and
30 specific. The latter are problems that eliminate the use of 9,999 of every 10,000 modulators identified via *in vitro* or tissue-culture based assays when followed up with *in vivo* testing and clinical trials. Accordingly, in another

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embodiment, the present invention provides an *in vivo* method for screening exogenously provided test compounds for their ability to interact with or otherwise regulate a ligand-binding domain, said method comprising

- (a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated into its genome;
- (b) contacting the animal with the test compound;
- (c) inducing expression of the first nucleic acid; and
- (d) detecting a change, if any, in expression of the reporter protein, wherein the change indicates that the test compound interacts, directly or indirectly, with the ligand-binding domain.

The term "test compound" as used herein means any exogenous compound that is added, applied to, introduced or induced in the animal. Examples include, but are not limited to, any of the ligand types listed above.

The term "transgenic animal" as used herein means an animal that has been made by the addition of exogenous nucleic acid sequences incorporated into its genome. In the present invention, the first and second nucleic acids of the composition of the invention are used to create a transgenic animal. In one embodiment, a first transgenic animal is created using the first nucleic acid of the composition and a second transgenic animal is created using the second nucleic acid of the composition and then the first and second transgenic animals are crossed to produce a transgenic animal that has the first and second nucleic acid incorporated into its genome. Transgenic animals can be made using random integration, homologous recombination, transposable element integration or recombinase assisted integration {Kawakami, 2000; Davidson, 2003}. In one embodiment, the enzyme I-SceI is used to introduce the first and second nucleic acids of the composition into the host genome {Thermes, 2002}. I-SceI provides the advantages of efficient integration, low copy number integration, and large insert size.

Finally, transgenic animals can be generated that carry all components in one vector making genetic crosses unnecessary, and ligand detection and biochemical characterization performed using a single transgenic line.

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Accordingly, in another embodiment, a single transgenic animal is created using a single vector that contains the first and second nucleic acid of the composition.

The methods of the invention can be used in any transgenic animal. In one embodiment, the transgenic animal is selected from the group consisting of *C. elegans*, *Drosophila*, *Xenopus*, mouse or zebrafish.

The term "transiently expressed" as used herein means that the first and/or second nucleic acid(s) have not been incorporated into the host genome or germline.

10 The zebrafish has many key advantages that make it the model organism of choice for the compositions and methods described. Zebrafish develop rapidly, are inexpensive to grow, and thousands of eggs/embryos are easily collected and small enough to be aliquoted into multi-well plates where minute amounts of test compounds can be used (i.e. 10 μ M in 100 μ l per
15 well). Importantly, the eggs, embryos, and adults absorb chemicals directly from water and are DMSO tolerant. Moreover, the embryos are transparent, which makes them ideal for GFP analysis in live animals. Zebrafish also share very high sequence conservation with human genes. In the case of NRs, all human NRs have orthologues in zebrafish, and comparison of their LBDs
20 reveals average identities of about 75% {Maglich, 2003}. Cofactor recruitment and ligand recognition between fish and human NR LBDs is highly conserved. The fish glucocorticoid receptor (GR), for example, is able to induce transcriptional activity in the presence of cortisol and dexamethasone, and shows inhibition of transactivation with the human GR antagonist RU486
25 {Bury, 2003}. Fish estrogen receptors (ER α and β) have also been shown to have high binding affinities for diverse natural and synthetic human ER ligands {Kloas, 2000; Hawkins, 2000}.

Accordingly, in a particular embodiment, the transgenic animal is zebrafish.

30 The multi-tag system fused to the chimeric transcription factor provides a powerful and efficient purification system capable of yielding sufficient levels of purified LBD protein for the identification of specifically associated

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molecules (Figure 5). This provides a new and powerful means to identify endogenous ligands, and to determine whether exogenously provided modulators work directly or indirectly. The identification of endogenous and exogenous nuclear receptor ligands in/from live animals, via purification of the cognate receptor, has never before been suggested or attempted. This is due primarily to the enormous purification factors required to isolate sufficient levels of pure protein from complex tissues. In addition, techniques available for the isolation and identification of small quantities of extracted ligand were unavailable. The present tagging system, coupled with optimized methods of ligand extraction and identification via new and improved mass spectrometry techniques, now makes this approach feasible. Accordingly, in another embodiment, the invention provides a method for purifying a ligand of a reporter system, the method comprising:

- (a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated into its genome;
- (b) inducing expression of the first nucleic acid;
- (c) detecting a signal;
- (d) generating a cellular extract from the animal; and
- (e) subjecting the cellular extract generated in step (d) to multiple affinity purification steps, each step comprising binding one affinity tag to an affinity resin capable of selectively binding one affinity tag and eluting the affinity tag from the affinity resin after substances not interacting with the fusion protein have been removed.

In another embodiment, the method further comprises subjecting the purified material of step (e) to appropriate, high-sensitivity analytical techniques such as MALDI-TOV, ESI, GC or semi non-denaturing mass spectrometry.

Accordingly, in another embodiment, the invention provides a method for purifying a ligand of a reporter system, the method comprising:

- (a) providing an animal having the first and second nucleic acids of the composition of the invention either transiently expressed or incorporated

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into its genome;;

- (b) contacting the transgenic animal with a test compound;
- (c) inducing expression of the first nucleic acid;
- (d) detecting a signal;
- 5 (e) generating a cellular extract from the transgenic animal; and
- (f) subjecting the cellular extract generated in step (e) to multiple affinity purification steps, each step comprising binding one affinity tag to an affinity resin capable of selectively binding one affinity tag and eluting the affinity tag from the affinity resin after substances not bound to the fusion
- 10 protein have been removed.

In one embodiment, the method further comprises subjecting the purified material of step (f) to mass spectrometry to identify the bound ligand.

This system can also be used to identify conditions or genetic mutations that affect LBD activity. For example, stress, heat, diet, aging etc

15 could all be tested. Similarly, mutations could be crossed in genetically or induced with mutagens or specific RNAi/morpholino constructs to understand genetic pathways that control or respond to ligand binding.

The following non-limiting examples are illustrative of the present invention:

20 **EXAMPLES**

Example 1 - Zebrafish

METHODOLOGY AND RESULTS

Construction of a bifunctional vector (pLT)

The pLT (Ligand Trap) vector (Figure 1) has two main components, a

25 fusion protein expression system and a fusion protein-dependent reporter system. The vector backbone is derived from pBluescript II (Stratagene), which contains an Ampicillin resistance gene and T7 and T3 promoters. In the example shown, the fusion protein expression system is comprised of an inducible promoter, followed by multiple (consecutive) affinity tags fused to a

30 minimal Gal4 DNA-binding domain (DBD; amino acids 1-132). Following the DBD are a set of restriction sites (MSC; Pme/Nhe) positioned such that the ligand-binding domain of a NR, or any other potential transcriptional activation

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motif, can be cloned in frame C-terminal to the Gal4 DBD to complete the transcription factor fusion gene. A Kozak consensus sequence and a start codon were engineered upstream of the tags for efficient translation. An SV40 polyadenylation signal located after the MCS ensures proper transcript
5 termination, processing and translation. The inducible promoter shown is a minimal zebrafish heat shock promoter (min zHSP70), which can be used to induce expression of the tagged-Gal4 DBD-bait fusion in any tissue or stage in the animal's life cycle. This promoter is flanked by unique restriction sites so that it can be exchanged for any other promoter of choice (e.g. tissue-specific promoters). The "triple tag" shown consists of 3X FLAG, Strep II, and
10 6X His tags. Between the 3X FLAG and the Strep II tag a TEV (tobacco etch virus) cleavage site is engineered.

The reporter part of the ligand trap construct is separated from the fusion protein component by pBluescript II sequence. Unique restrictions sites
15 present in this sequence were used for insertion of the live-animal reporter system. In this case, the reporter is an enhanced green fluorescent protein (eGFP) gene, containing a C-terminal nuclear localization signal (NLS). Multiple DNA binding sites for the Gal4 DBD protein (UAS_{GAL4}) are located upstream of the eGFP sequence, followed by a basal adenoviral E1b
20 promoter. The UAS sites are separated from the eGFP coding sequence by a Kozak sequence followed by an ATG start codon. An SV40 polyadenylation signal was placed after the coding sequence of eGFP.

Expression of the GAL4-LBD fusion proteins at inappropriate times or in inappropriate tissues often causes dominant negative phenotypes. This
25 means that fusion protein expression must be kept silent until the transgenic animals are ready for testing. To accomplish this, a minimal heat-inducible promoter was generated that is not leaky, and that provides high levels of GAL4-LBD fusion protein expression upon short elevations of temperature (Figure 1). Also, to prevent the influence of flanking enhancers and silencers,
30 *Drosophila*-derived gypsy insulator elements were added to each end of the insertable DNA (Figure 1).

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Flanking the protein fusion and reporter system components are a set of I-SceI meganuclease sites, used for optimal genomic integration of the two-component system, and two insulator elements (*gypsy*) to prevent the influences of nearby regulatory elements at the sites of genomic insertion.

5 All recombinant DNA manipulations followed standard procedures (Sambrook et. al., 1989)

Steps of pLT construction:

- 1 Cloning of the Gal DNA-binding domain (DBD) fused to the SV40 polyadenylation signal (SV40 pA); plasmid 1
- 10 2 Creation of a triple-tag; plasmid 2
- 3 Introduction of the triple-tag into plasmid 1 resulting in plasmid 3
- 4 Introduction of two I-SceI meganuclease restriction sites into plasmid 3; resulting in plasmid 4
- 5 Insertion of eGFP fused to the SV40 pA; plasmid 5
- 15 6 Creation of the pLT reporter by cloning Gal4 DBD binding sites (UAS) and a basal promoter (E1b) into plasmid 5; resulting in plasmid 6
- 7 Integrating the reporter into plasmid 4; resulting in plasmid 7
- 8 Cloning of a heat shock inducible promoter (zebrafish Hsp70) into plasmid 7; resulting in plasmid 8
- 20 9 Integration of two *gypsy* insulator elements between the I-SceI sites and the ligand trap components, plasmid 9

Plasmid 1 (pB Gal1-132-pA)

To generate pLT we cloned the Gal4 DBD amino acid 1-132 into the pBluescript II (Stratagene) vector (template pBS SK Gal4VP16 SV40pA
25 {Koster, 2001}) containing a SV40 pA tail. PmeI and NheI restriction sites were introduced into the construct as cloning sites for nuclear receptor ligand-binding domains (NR LBDs). An EcoRV restriction site was designed N-terminal of the Gal4 DBD to introduce the triple tag. The resulting vector was named pB Gal1-132-pA.

30 Oligos used:

5'-ATTCATCTAGAGATATCAAGCTACTGTCTTCTATCGAACAAGC (SEQ ID NO:2)

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3'-ATTATCTAGAGTTTAAACAGCTAGCTGATGATGTCGCACTTATTCTAT
GC (SEQ ID NO:3)

Plasmid 2 (pB II triple tag):

Triple tag oligos were designed that encode the 3xFlag-Tev-Strep-
5 TagII-6xHis tag, and flanked on either site with EcoRI restriction sites. Tev
indicates the consensus cleavage site for the tobacco etch virus (TEV) NIa
proteinase. Oligos were dissolved in STE Buffer (10 mM Tris pH 8.0, 50 mM
NaCl, 1 mM EDTA) and were mixed together in equal molar amounts. The
triple-tag oligonucleotides were cloned into EcoRI pBSII KS (Stratagene)
10 producing the plasmid pB II triple-tag.

Oligos used:

sense

AATTCGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGAC
TACAAGGATGACGATGACAAGGAGAACCTGTACTTCCAGTCCAACCTGGA
15 GCCACCCGCGAGTTCGAAAAGCATCACCATCACCATCACG (SEQ ID NO:4)

antisense

ATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCGAGTTGGACTGGAA
GTACAGGTTCTCCTTGTCATCGTCATCCTTGTCGATGTCAATTCGTG
ATGGTGATGGTGATGCTTTTCGAACTGCGGGTGGCTCC (SEQ ID NO:5).

20 Plasmid 3 (pB triple tag-Gal1-132-pA)

The tag was PCR amplified with primers containing EcoRV restriction
sites and a Kozak consensus sequence followed by an ATG start codon. This
fragment was then introduced into the EcoRV restriction site of pB Gal1-
132-pA resulting in pB triple tag-Gal1-132-pA.

25 Oligos used:

5'-ATTATGATATCgccaccatgGACTACAAAGACCATGACGG (SEQ ID NO:6)
3'-ATTATGATATCGTGATGGTGATGGTGATGC (SEQ ID NO:7)

Plasmid 4 (SceI pB triple tag-Gal1-132-pA)

Next, two I-SceI mega nuclease recognition sites were introduced by
30 site-directed mutagenesis into pB triple tag-Gal1-132-pA. One site was
introduced 3' to the T7 promoter and the other site 5' of the T3 promoter on the

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sense strand. The resulting vector was named Scel pB triple tag-Gal1-132-pA. Correct insertion was verified by digestion.

Oligos used:

T7

5 5'-GACTCACTATAGGGCTAGGGATAACAGGGTAATGAATTGGGTACCG
GG (SEQ ID NO:8)

T7

3'-CCCGGTACCCAATTCATTACCCTGTTATCCCTAGCCCTATAGTGAGTC
(SEQ ID NO:9)

10 T3

5'-CGGTGGAGCTCCAGTAGGGATAACAGGGTAATCTTTTGTTCCTTTA
GTG (SEQ ID NO:10)

T3

3'-CACTAAAGGGAACAAAAGATTACCCTGTTATCCCTACTGGAGCTCCA
15 CCG (SEQ ID NO:11)

Plasmid 5 (pB eGFP-pA)

The eGFP Nls fragment was PCR amplified from the pUAS Stinger vector {Barolo, 2000} and cloned into the pBluescript II (Stratagene) vector containing a SV40 pA (template pBS SK Gal4VP16 SV40pA {Koster, 2001}).

20 The resulting vector was pB eGFP-pA.

Oligos used:

5'-ATTATCTAGAACCATGGTGAGCAAGGGC (SEQ ID NO:12)

3'-ATTATCTAGATTACTTGTACAAGTAGCG (SEQ ID NO:13)

Plasmid 6 (pB UASE1b-eGFP-pA)

25 A fragment containing multi-UAS Gal DBD binding sites followed by the basal adenovirus promoter E1b was PCR amplified from pBUASEIB {Koster, 2001} and inserted into pB eGFP-pA.

Oligos used:

5'-ATTATCCGCGGGGTACCCTCCAAGGCGGAGTACTGTCC (SEQ ID
30 NO:14)

3'-ATAATCGGCCGGTGTGGAGGAGCTCAAAGTGAGGC (SEQ ID NO:15)

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Plasmid 7 (Scel pB triple tag-Gal1-132-pA-UASE1b-eGFP-pA)

The UASE1b-eGFP-pA fragment cut out from plasmid 6 by KpnI digestion and cloned into KpnI digested pB Scel-Tag-Gal1-132-pA (Plasmid 4).

5 Plasmid 8 (pLT ; Scel pB zHsp70 triple tag Gal-1-132-pA-UASE1b-eGFP-pA)

Plasmid 7 was SacII/NotI cut to insert a SacII/NotI fragment (ca 1500 bp) of the minimal zebrafish Hsp70 promoter amplified from pzhSP70/4prom {Shoji, 1998}.

10 Oligos used:

5'-ATTATCCGCGGTCAGGGGTGTCGCTTGG (SEQ ID NO:16)

3'-ATTATGCGGCCGCGATATCGAATTCCTGCAGG (SEQ ID NO:17)

Plasmid 9 (pLT gypsy; Scel gypsy pB zHsp70 triple tag Gal-1-132-pA-UASE1b-eGFP-pA)

15 The gypsy insulator elements from the *Drosophila* transformation vector UAS-Stinger GFP {Barolo, 2000} were PCR-amplified and inserted upstream of the Hsp70 promoter into SacII restriction sites or downstream of the eGFP reporter into HindIII/ApaI.

Oligos used:

20 5'-HSP-ATAACCGCGGTCACGTAATAAGTGTGCG (SEQ ID NO:18)

3'-HSP-ATAACCGCGGAGATCTATACTAGAAATTGATCGGC (SEQ ID NO:19)

5'-GFP-ATAAAAGCTT TCACGTAATAAGTGTGCG (SEQ ID NO:20)

3'-GFP-ATAAGGGCCCATACTAGAAATTGATCGGC (SEQ ID NO:21)

25 Fish maintenance and microinjection of zebrafish embryos and production of transgenic zebrafish lines

Adult zebrafish were originally obtained from the Zebrafish International Resource Center (University of Oregon) and maintained in our own fish facility with a controlled light cycle of 14 h light/10 h dark at 28 C. The fish were fed a combination of dry flake food (Tetra brand) and live brine shrimp nauplia, *Artemia salina*. Mating tanks (groups of six females and three males) were set up in the evening and spawned soon after the onset of the light period, and fertilized eggs were collected at the one-cell stage.

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To generate permanent transgenic ligand trap lines, 3nl of pLT plasmid and I-SceI enzyme in 5 mM Tris, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 100 mM KCl and 0.1% phenol red was injected into the blastomeres of early one-cell stage embryos with a glass micropipette. Eggs were
5 incubated at 28 °C in 0.5 E2 media (17.5 g NaCl, 0.75 g KCl, 2.4 g MgSO₄, 0.41 g KH₂PO₄, 0.12 g Na₂HPO₄ to 1 litre of water to make a 20 × E2 stock. Add 7.25 g CaCl₂ to 100 ml of water. Add 3 g NaHCO₃ to 100 ml of water. To make 2 litre of 0.5 × E2 combine 50 ml 20 × E2 stock, 2 ml of the CaCl₂ solution, 2 ml of the NaHCO₃ solution) in petri dishes. To ensure that sufficient
10 numbers of transgenic fish were generated, at least 150 fish (F0) were raised to adulthood for each injected construct. F0 fish were then bred with wild type fish, and germline integrated transgenic zebrafish were selected by screening progeny by PCR (at least 100 embryos) using primers against GFP. Reporter positive fish were saved for further analysis and breeding. 100 progeny (F1) of
15 a GFP positive fish were raised to sexual maturation and crossed with other offspring of the GFP positive fish. F2 progeny fish were mated with wild type fish to identify homozygous ligand trap fish (F3). After a founder fish was identified by PCR, other methods, such as Southern blotting and visual examination of reporter activity were performed on the progeny to confirm the
20 identity of the founder fish. Germline transgenic fish obtained from the injected founders have continued to express the ligand trap construct for many generations.

Cell culture and temperature treatments

Cell culture was performed with the zebrafish (*Danio rerio*) embryonic
25 fibroblast cell line, ZF4 (ATCC CRL 2050; {Driever, 1993}), which was originally established from 1-day-old zebrafish embryos. The cells were grown to confluency at 28 °C, 5% CO₂, in Dulbecco's modified Eagle's medium/F12 nutrient mix (DMEM/F12) supplemented with 10% FBS, 1% -glutamine, 100 u/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml Fungizone.

30 Elevated temperature induces a rapid heat shock transcription factor (HSFs)-mediated expression of heat shock (*hsp*) genes. This cellular response leads to an expression of the ligand trap construct (triple tag Gal

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DBD NHR LBD), which if active, then leads to activation of the reporter. Heat shock treatment (37 °C) was performed by incubating cells or animals in a water bath for 1 h. Control cells or animals were incubated in a water bath at 28 °C. All cell culture medium components were purchased from Gibco BRL
5 Life Technologies Ltd (USA). Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

Detection of fusion proteins by Western blotting

Expression of ligand trap constructs using the pLT system was verified
10 by Western blot. Whole cell extracts were prepared from transfected (cells) or transgenic (animal tissue) cells, and 20 µg protein separated on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) using a wet transfer apparatus (Bio-Rad, CA, USA). Membranes were blocked for 1 h in 5% non-fat dry milk
15 in PBS with 0.5% Tween 20. The tagged fusion proteins were detected using a monoclonal mouse anti-M2 antibody (dilution 1:10 000; Sigma). Horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulin (Amersham Life Sciences, Buckinghamshire, UK) was used as a secondary antibody, and the signals were visualized using enhanced chemiluminescence
20 according to the manufacturer's instructions (Amersham).

Compound screening

Zebrafish were collected from wild type or transgenic ligand trap fish. Embryos were raised in a 28 °C incubator in 0.5 × E2 media in petri-dishes. For ligand screening eggs, embryos, juvenile or adult fish were heat induced
25 (28→37 °C) for 30 min in a water bath. Any developmentally delayed (dead or unfertilized) embryos were removed. Specimens were then combined into clutches of 3-10 and dispensed into multi-well plates in 0.5X E2 media supplemented with 1% DMSO, .05 U/ml penicillin, 50 ng/ml streptomycin. For embryos and juvenile fish 1% DMSO is used as a vehicle for small molecules
30 to facilitate solubility and permeability. Chemicals stored in DMSO or ethanol were diluted appropriately and added individually to different wells. The

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screening concentrations of library compounds ranged from 0.1-20 μ M. Compounds were introduced into adult fish via intraperitoneal injection.

Ligand trap detection

Ligand trap GFP reporter signal can be monitored in live animals 4-6 h
5 after heat induction. For analyzing GFP fluorescent pattern, embryos, larvae and adult fish were anesthetized with 0.05% 2-phenoxyethanol (Sigma) and GFP expression was examined under a fluorescein isothiocyanate (FITC) filter on a Leica CTR MIC.

Affinity purification of proteins and bound molecules

10 Cells or animals were heat induced (37 °C) for 1 h and collected after 12 to 24 h incubation at 28 °C. Fish tissue was minced with scissors and all following procedures were performed at 0°- 4°C. Lysis of cells or animal tissue was performed for non-denaturing purifications in Flag-Strep buffer (100 mM Tris-Cl, 150 mM NaCl, 10 mM of 2-mercaptoethanol, 0.1 % Triton X-100 and
15 1 Roche complete Mine protease inhibitor cocktail tablet). Extracts were sonicated and centrifuged for 10 min at 9000 rpm in a Beckman centrifuge. *Strep II tag* and *Flag tag* incubation and washing was performed in Flag-Strep buffer. *FLAG tag* elution was done in the same buffer with 100 mg/ml 3x FLAG peptide (Sigma) or in Tev-cleavage buffer (100 mM NaCl, 50 mM Tris-
20 HCl pH 8.0, 0.5 mM EDTA, 1 mM DTT and 1 Roche complete Mine protease inhibitor cocktail tablet) with 0.5 mg TEV for 1 h at 4°C. *Strep II tag* elution was done in Flag-Strep buffer with 2.5 mM desthiobiotin. For *HIS tag* incubation and washing His buffer was used (50 mM sodium phosphate, pH 7, 100 mM NaCl, 5 mM of 2-mercaptoethanol , 0.1 % Triton X-100 and Roche
25 complete Mine protease inhibitor cocktail tablet) elution was done in the same buffer with 100 mM imidazole. Elutions were performed in gravity feed disposable columns (Bio-Spin columns, Bio-Rad). Resins used were Talon Superflow metal affinity (Clontech), Strep-Tactin Superflow (IBA), FLAG M2 monoclonal antibody matrix (Sigma, catalog). The extracts were incubated
30 with the resin for 30 min (Talon, Strep-Tactin) or 120 min (anti-FLAG). Figure 6 shows an example of an SDS PAGE gel affinity purification.

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MS and HPLC/ESI-MS

To purify ligand trap bait containing complexes for analysis by mass spectrometry, 5×10^8 pLT-transfected cells (100x15cm confluent culture dishes) or 10g of zebrafish tissue or embryo were lysed and purified as described. Purified samples were precipitated with TCA, separated by SDS/PAGE, and stained with colloidal coomassie blue stain or silver stain (Invitrogen). Individual bands were excised, digested with Trypsin, and analyzed using a quadrupole time-of-flight hybrid tandem mass spectrometer (Q-TOF; Waters-Micromass, Milford, MA) equipped with a Micromass Z-type electrospray ionization source.

The scope of the claims should not be limited by the preferred embodiments and examples, but should be given the broadest interpretation consistent with the description as a whole.

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Table 1

NRNC Symbol	Name	Full Name	GFP signal	F1/F2 fish
NR1A1	TR α	Thyroid hormone receptor-alpha	B, HS, CNSH, Y	Yes
NR1A2	TR β	Thyroid hormone receptor-beta	B, CNS, H AT, Y	Yes
NR 1B1	RAR α	all-trans Retinoic acid receptor-alpha	B, HS, M, S, ;Y	
NR 1B2	RAR β	all-trans Retinoic acid receptor-beta	B, M, Y	Yes
NR 1B3	RAR γ	all-trans Retinoic acid receptor-gamma	B, Y	
NR1C3	PPAR γ	Peroxisome proliferator-activated receptor-gamma	B, AT, Y, CNS	Yes
NR1D1	Rev-erbA α	Rev-erb alpha	M, Y, B	Yes
NR1D2	Rev-erbA β	Rev-erb beta	B, S, H, CNS, HS, E, Y	Yes
NR 1F1	ROR α	RAR-related orphan receptor-alpha	H, S, Y	Yes
NR 1F2	ROR β	RAR-related orphan receptor-beta	H, HS, CNS, S, Y	Yes
NR1F3	ROR γ	RAR-related orphan receptor-gamma	H, M, S, Y	Yes
NR1H3	LXR α	Liver X receptor alpha	Y, M, B	Yes
NR1I1	VDR	Vitamin D receptor	Y	
NR1I3	CAR	Constitutive androstane receptor	S, M, B, Y	
NR 2B1	RXR α	9-cis retinoic acid receptor-alpha	B, HS, M, S, ;Y	
NR2E1	TLX		-	
NR2E3	PNR	Photoreceptor cell-specific nuclear receptor	E, Y	
		Chicken ovalbumin upstream promoter-transcription	-	
NR2F1	Coup-TFI	factor I		
NR2F6	EAR2	erbA-related receptor 2	Y	
NR 3A1	ER α	Estrogen receptor-alpha	B, M, H, Y	
NR3B3	ERR γ	Estrogen-related receptor gamma	B, M, H, Y	
NR3C1	GR	Glucocorticoid receptor	B, H, HS, H, M,	Yes
NR4A1	NGFI		S, Y	Yes
NR4A2	Nurr1		B, CNS, Y	
NR4A3	NOR1	Neruon-derived orphan receptor 1	H, Y	
NR5A1	SF1	Steroidogenic factor 1	Y	
NR6A1	GCNF	Germ cell nuclear factor	-	
		Dosage-sensitive sex reversal-adrenal hypoplasia	Y	
		congenita critical region on the X chromosome, gene		
NR0B1	DAX1	1		
NR0B2	SHP	Short heterodimeric partner	-	

NRNC = Nuclear Receptor Nomenclature; pLT = plasmid Ligand Trap vector; germl. Fish = ligand trap vector stably integrated in the germline; B = brain; M = muscle; HS = hematopoietic system, blood; CNS = central nervous system; S = skin; H = heart; AT = white adipose tissue; Y = Yolk; E = Eye

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Table 2: *SceI* Gypsy(Insulator)zHSP70 Fest-Gall-132-pA-UASE1B-eGFP-pA

5	1	<u>ATTACCCTGT TATCCCTACT GGAGCTCCAC CGCGGTCACG TAATAAGTGT</u>	<i>SceI</i>
	51	<u>GCGTTGAATT TATTCGCAAA AACATTGCAT ATTTTCGGCA AAGTAAAATT</u>	<i>Insulator</i>
10	101	<u>TTGTTGCATA CCTTATCAAA AAATAAGTGC TGCATACTTT TTAGAGAAAC</u>	
	151	<u>CAAATAATTT TTTATTGCAT ACCCGTTTTT AATAAAATAC ATTGCATACC</u>	<i>Insulator</i>
15	201	<u>CTCTTTTAAT AAAAAATATT GCATACTTTG ACGAAACAAA TTTTCGTTGC</u>	
	251	<u>ATACCCAATA AAAGATTATT ATATTGCATA CCCGTTTTTA ATAAAATACA</u>	<i>Insulator</i>
20	301	<u>TTGCATACCC TCTTTTAATA AAAAATATTG CATACGTTGA CGAAACAAAT</u>	
	351	<u>TTTCGTTGCA TACCCAATAA AAGATTATTA TATTGCATAC CTTTCTTGC</u>	<i>Insulator</i>
25	401	<u>CATACCATTT AGCCGATCAA TTAGATCTCC</u>	<i>BglII</i>
	451	GTTATTTCCA AAAATCAAAT TAATTTTATT AAACATTAG AACGAGCATG	<i>HSP</i>
30	501	TTTGTCTAT ATGCTACAGA AGATAAAAAA TAATAGGAGT TAACAGTTAT	
	551	AAAACAACAC ATTTGTTTCT ATTGATTGTT GACCACACTG GGGTCTCATT	
	601	AAGTTAGATT AAAGACACAC TAACTGGGTC AAAAGCAGCA GATTGATTTT	
35	651	ATAGCACCAG GGTAACCTTT CTAACACTTT TACGGCAATC ATATACATTA	<i>HSP</i>
	701	AAATTAAATA CAGACCACGA CTGAACAAGG AGGATGATCT CCAATATTAA	
40	751	ACAAAGAGAC TTGTGCCTAT TTCTCTGAGG GTAAACATGA CCTCTCAAGT	
	801	TAGCAAGTTG TTTTAAACAC TACAAAATA GTTAAGACCT GCAATCCCAG	
	851	AATAAAGTAT TGGTTTTAAC CAATCAATAT AGTACAGTAA ACATCCATTT	<i>HSP</i>
45	901	GTTTTGTTGA AACGTTAAAC AAATCTGACC AAAGCTATTA GCTTATATAA	
	951	AACAGGTTTG CCTTCTATGT AGCTGAAAAC ACCACAGGCC CGATTTTGCT	
50	1001	ACTGTGTAAA ACATTTTCAGC AAGATTTTTT TATTGCATTT TTTTTTACTG	
	1051	AATCGTTCAA ACATTTTATC ATTTTAGTTT GTTCATTCAT TGCAACTGGA	<i>HSP</i>
	1101	AAAACAACAC ATCACACAAC CGCACATATT TCAGCAATAA GTACAATAAA	
55	1151	AACTCAAAT AAAAAAACA TTTTAAATCT CTTTGTATTT TTGACCGCTG	
	1201	TTTCGCGTAA TTTCACGGTA AACTCTGGA AATCTCCACT ACATTCCTCT	

- 30 -

1251 CAGCGGCTCC TCTCAATGAC AGCTGAAGAA GTGACGGCGG CTGCCTGCTG HSP
 1301 TGTTTTGATT GGTCGAATTC ACTGGAGGCT TCCAGAACAG TGTAAGAGTCT
 5 1351 GAACGGGTGC GCGCTCTGCT GTATTTAAAG GCGGAAAGAG AGACCGCAGA
 1401 GAAACTCAAC CGAAGAGAAG CGACTTGACA AAGAAGAAAA GAGCAGCCTG
 10 1451 ACAGGACTTT TCCCCGACGA GGTGTTTATT CGCTCTATTT AAGAATCTAC HSP
 1501 TGTAAGGTAA GTCTCAATAT ATTGTACTCT ATTGGCTAAT CAGAATTATA
 1551 TAGAGATTAT ATGTACTTAA TGTCAAAAAA TCAACTTTGT ATATGTAATC
 15 1601 TTTTACATG TGGACTGCCT ATGTTTCTCT TATTTTAGGT CTACTAGAAA
 1651 ATTATATTTT CCGTTTTTAC AATAAGGATT TTAAAAAAG CAATGAACAG HSP
 1701 ACGGGCATTT ACTTTATGTT GCTGACATTA TTTTATATGA GCATAATAAC
 20 1751 CATAAATACT AGCAAATGTC CTAAATGAAT TTGTGTTAAT GTTGTCTACA
 1801 AAAGAAAATT AGCGTTTTAC TTGTACAACT AATAATAACT TGGTTATTAA
 25 1851 GAGAATTTCA CTTGTTGACT AGAAAAATCC TTTCATAATG AAACAATTGC HSP
 1901 ACCGATAAAT TGTATAAATA TAAAATTAAT TCTAATTGTT TTTTTTTTTT
 30 1951 CTGCAGGAAT TCGATATCGC GGCCGCTCTA GAGATATCGC CACCATGGAC
 EcoRV NotI EcoRV
 M D 3x
 Flag
 35 2001 TACAAAGACC ATGACGGTGA TTATAAAGAT CATGACATCG ACTACAAGGA
 Y K D H D G D Y K D H D I D Y K D Tev
 2051 TGACGATGAC AAGGAGAACC TGTACTTCCA GTCCAACCTGG AGCCACCCGC Strep
 D D D K E N L Y F Q S N W S H P Q
 40 2101 AGTTCGAAAA GCATCACCAT CACCATCACG ATATCAAGCT ACTGTCTTCT
 F E K H H H H H H D I K L L S S 6xHis
 45 2151 ATCGAACAAG CATGCGATAT TTGCCGACTT AAAAAGCTCA AGTGCTCCAA
 I E Q A C D I C R L K K L K C S K Gal
 DBD
 50 2201 AGAAAAACCG AAGTGCGCCA AGTGTCTGAA GAACAACTGG GAGTGTGCTGCT
 E K P K C A K C L K N N W E C R Y
 2251 ACTCTCCCAA AACCAAAAGG TCTCCGCTGA CTAGGGCACA TCTGACAGAA
 S P K T K R S P L T R A H L T E
 55 2301 GTGGAATCAA GGCTAGAAAG ACTGGAACAG CTATTTCTAC TGATTTTTC
 V E S R L E R L E Q L F L L I F P
 2351 TCGAGAAGAC CTTGACATGA TTTTGAAAAT GGATTCTTTA CAGGATATAA
 R E D L D M I L K M D S L Q D I K

- 31 -

2401 AAGCATTGTT AACAGGATTA TTTGTACAAG ATAATGTGAA TAAAGATGCC
 A L L T G L F V Q D N V N K D A

5 2451 GTCACAGATA GATTGGCTTC AGTGGAGACT GATATGCCTC TAACATTGAG
 V T D R L A S V E T D M P L T L R

 NheI PmeI
 10 2501 ACAGCATAGA ATAAGTGCGA CATCATCAGC TAGCTGTTTA AACTCTAGAA
 Q H R I S A T S S (SEQ ID NO:22)

2551 CTATAGTGAG TCGTATTACG TAGATCCAGA CATGATAAGA TACATTGATG SV40 pA

2601 AGTTTGGACA AACCACAAC T AGAATGCAGT GAAAAAATG CTTTATTTGT

15 2651 GAAATTTGTG ATGCTATTGC TTTATTTGTA ACCATTATAA GCTGCAATAA SV40pA

2701 ACAAGTTAAC AACAACAATT GCATTCATTT TATGTTTCAG GTTCAGGGGG

 ClaI
 20 2751 AGGTGTGGGA GGTTTTTTTAA TTCGCGGCCA TCAAGCTTAT CGATACCGTC

 KpnI
 2801 GACCTCGAGG GGGGGCCCGG TACCCTCCAA GGCGGAGTAC TGTCCCTCCGG

25 2851 GCTGGCGGAG TACTGTCCTC CGGCAAGGTC GGAGTACTGT CCTCCGACAC UAS

2901 TAGAGGTCGG AGTACTGTCC TCCGACGCAA GGCGGAGTAC TGTCCCTCCGG

30 2951 GCTGCGGAGT ACTGTCCTCC GGCAAGGTCG GAGTACTGTC CTCCGACACT UAS

3001 AGAGGTCGGA GTACTGTCCT CCGACGCAAG GTCGGAGTAC TGTCCCTCCGA

3051 CACTAGAGGT CGGAGTACTG TCCTCCGACG CAAGGTCGGA GTACTGTCCT

35 3101 CCGACACTAG AGGTCGGAGT ACTGTCCTCC GACGCAAGGC GGAGTACTGT E1b

3151 CCTCCGGGCT GGCGGAGTAC TGTCCCTCCGG CAAGGGTCGA CTCTAGAGGG

3201 TATATAATGG ATCCCATCGC GTCTCAGCCT CACTTTGAGC TCCTCCACAC

40 3251 CGGCCGCTCT AGAATGGTGA GCAAGGGCGA GGAGCTGTTC ACCGGGGTGG
 M V S K G E E L F T G V V

3301 TGCCCATCCT GGTCGAGCTG GACGGCGACG TAAACGGCCA CAAGTTCAGC
 45 P I L V E L D G D V N G H K F S GFP

3351 GTGTCCGGCG AGGGCGAGGG CGATGCCACC TACGGCAAGC TGACCCTGAA
 V S G E G E G D A T Y G K L T L K

50 3401 GTTCATCTGC ACCACCGGCA AGCTGCCCCGT GCCCTGGCCC ACCCTCGTGA
 F I C T T G K L P V P W P T L V T GFP

3451 CCACCCTGAC CTACGGCGTG CAGTGCTTCA GCCGCTACCC CGACCACATG
 T L T Y G V Q C F S R Y P D H M

55 3501 AAGCAGCACG ACTTCTTCAA GTCCGCCATG CCCGAAGGCT ACGTCCAGGA
 K Q H D F F K S A M P E G Y V Q E GFP

3551 GCGCACCATC TTCTTCAAGG ACGACGGCAA CTACAAGACC CGCGCCGAGG

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R T I F F K D D G N Y K T R A E V

5 3601 TGAAGTTCGA GGGCGACACC CTGGTGAACC GCATCGAGCT GAAGGGCATC
K F E G D T L V N R I E L K G I GFP

3651 GACTTCAAGG AGGACGGCAA CATCCTGGGG CACAAGCTGG AGTACAATA
D F K E D G N I L G H K L E Y N Y

10 3701 CAACAGCCAC AACGTCTATA TCATGGCCGA CAAGCAGAAG AACGGCATCA
N S H N V Y I M A D K Q K N G I K GFP

3751 AGGTGAACTT CAAGATCCGC CACAACATCG AGGACGGCAG CGTGCAGCTC
V N F K I R H N I E D G S V Q L

15 3801 GCCGACCACT ACCAGCAGAA CACCCCCATC GCGACGGCC CCGTGCTGCT
A D H Y Q Q N T P I G D G P V L L GFP

20 3851 GCCCGACAAC CACTACCTGA GCACCCAGTC CGCCCTGAGC AAAGACCCCA
P D N H Y L S T Q S A L S K D P N

3901 ACGAGAAGCG CGATCACATG GTCCTGCTGG AGTTCGTGAC CGCCGCCGGG
E K R D H M V L L E F V T A A G GFP

25 3951 ATCACTCTCG GCATGGACGA GCTGTACAAG AGCAGGCACA GAAGGCATCG
I T L G M D E L Y K S R H R R H R

4001 CCAGCGCTCT AGGAGCCGCA ATCGCAGCCG AAGTCGCAGC AGTGAACGAA
Q R S R S R N R S R S R S S E R K GFP

30 NLS
4051 AACGCCGTCA ACGGAGCCGA AGTCGCAGCA GTGAACGAAG ACGTACTTG
R R Q R S R S R S S E R R R Y L

35 4101 TACAAGTAAT CTAGAACTAT AGTGAGTCGT ATTACGTAGA TCCAGACATG
Y K * (SEQ ID NO:23) GFP-
NLS

4151 ATAAGATACA TTGATGAGTT TGGACAAACC ACAACTAGAA TGCAGTGAAA

40 4201 AAAATGCTTT ATTTGTGAAA TTTGTGATGC TATTGCTTTA TTTGTAACCA

4251 TTATAAGCTG CAATAAACAA GTTAACAACA ACAATTGCAT TCATTTTATG

45 4301 TTTCAGGTTC AGGGGGAGGT GTGGGAGGTT TTTTAATTCG CGGCCATCAA

4351 GCTTTCACGT AATAAGTGTG CGTTGAATTT ATTCGCAAAA ACATTGCATA
Insulator

50 4401 TTTTCGGCAA AGTAAAATTT TGTTGCATAC CTTATCAAAA AATAAGTGCT

4451 GCATACTTTT TAGAGAAACC AAATAATTTT TTATTGCATA CCCGTTTTTA
Insulator

55 4501 ATAAAATACA TTGCATACCC TCTTTTAATA AAAAATATTG CATACTTTGA

4551 CGAAACAAAT TTTCGTTGCA TACCCAATAA AAGATTATTA TATTGCATAC
Insulator

4601 CCGTTTTTAA TAAAATACAT TGCATACCCT CTTTTAATAA AAAATATTGC

- 33 -

4651 ATACGTTGAC GAAACAAATT TTCGTTGCAT ACCCAATAAA AGATTATTAT
Insulator

5 4701 ATTGCATACC TTTTCTTGCC ATACCATTTA GCCGATCAAT TCTAGTATGG

KpnI

4751 GCCCGGTACC CAATTCATTA CCCTGTTATC CCTA (SEQ ID NO:1)

SceI

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DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

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WE CLAIM:

1. A composition for live animal ligand detection and purification comprising
 - (a) a first nucleic acid comprising an inducible promoter and encoding a fusion protein of a DNA-binding domain, a ligand-binding domain and at least three different affinity tags that are suitable for use in succession and are sufficient for purification and subsequent identification of bound ligands; and
 - (b) a second nucleic acid encoding a fluorescent reporter system, said second nucleic acid comprising a promoter having binding sites for the DNA-binding domain and encoding a fluorescent reporter protein detectable in live animals.
2. The composition of claim 1, wherein the DNA-binding domain is Gal4.
3. The composition of claim 1 or 2, wherein the ligand-binding domain is derived from a Nuclear Receptor protein.
4. The composition of any one of claims 1-3, wherein the at least three different affinity tags are StrepII, His and FLAG.
5. The composition of any one of claims 1-4, wherein the inducible promoter is heat, transcription factor or hormone activated.
6. The composition of claim 5, wherein the inducible promoter is hsp70.
7. The composition of any one of claims 1-6, wherein the reporter protein is selected from the group consisting of green fluorescent protein, red fluorescent protein, DS-red and luciferase.
8. The composition of claim 7, wherein the reporter protein is enhanced green fluorescent protein.

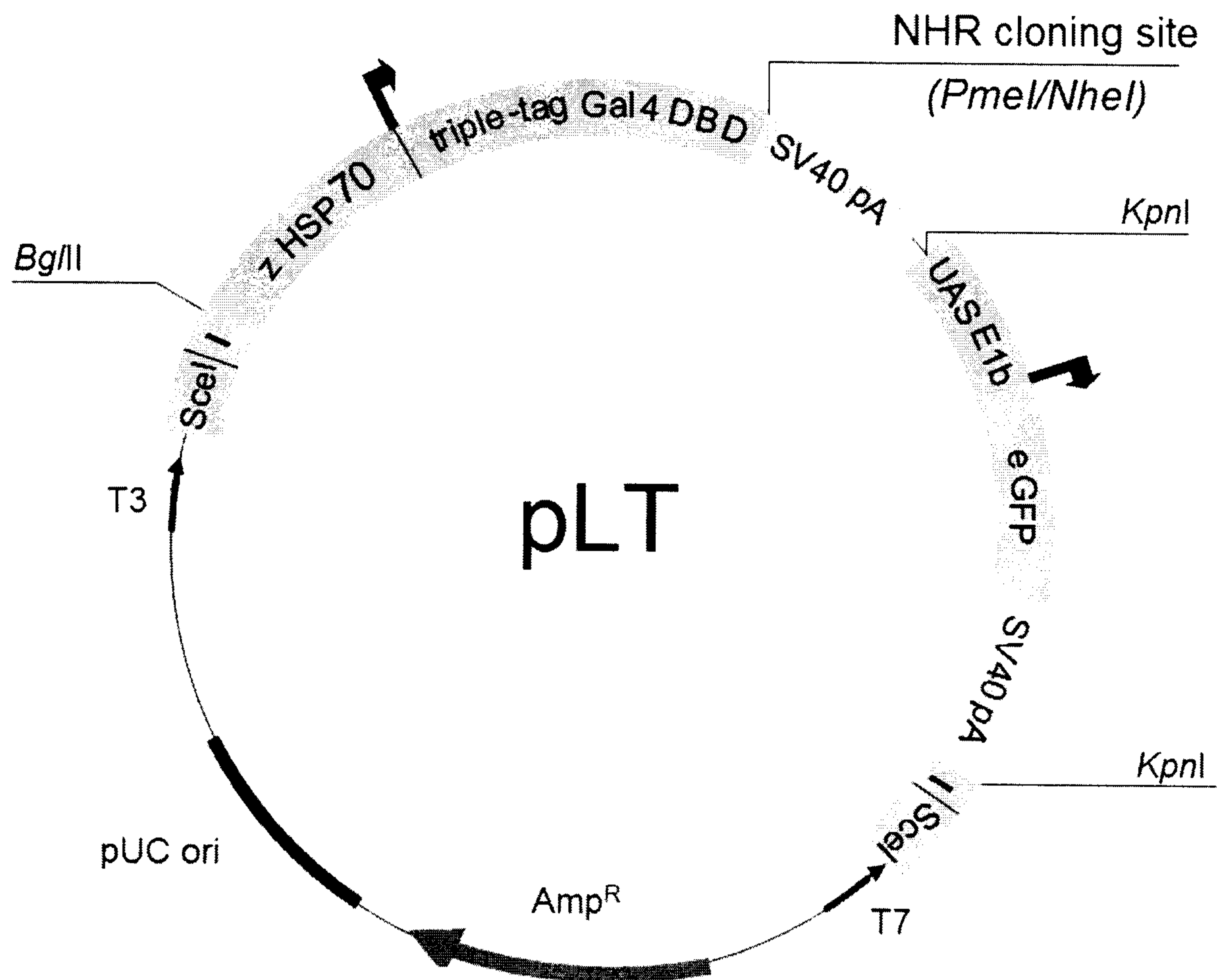
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9. The composition of any one of claims 1-8, wherein the first and second nucleic acids are on a single vector.
- 5 10. The composition of claim 10, wherein the single vector comprises the nucleotide sequence as shown in SEQ ID NO:1.
11. The composition of any one of claims 1-10, wherein each affinity tag provides at least 1000-fold purification with at least 50% yield activity.
- 10 12. The composition of any one of claims 1-11, wherein the DNA-binding domain is unlikely to recognize host protein and DNA sequences.
13. A use of the composition of any one of claims 1-12 for *in vivo* ligand
15 detection and purification.
14. A method for purifying a ligand of a reporter system, the method comprising:
- 20 (a) providing a live animal, said animal having the first and second nucleic acids of the composition of any one of claims 1-12 transiently expressed or incorporated into its genome;
- (b) inducing expression of the first nucleic acid;
- (c) detecting a signal;
- (d) generating a cellular extract from the animal; and
- 25 (e) subjecting the cellular extract generated in step (d) to multiple affinity purification steps, each step comprising binding one affinity tag to an affinity resin capable of selectively binding one affinity tag and eluting the affinity tag from the affinity resin after substances not bound to the fusion protein have been removed;
- 30 wherein the purified substance that is bound to the fusion protein is a ligand of the reporter system.

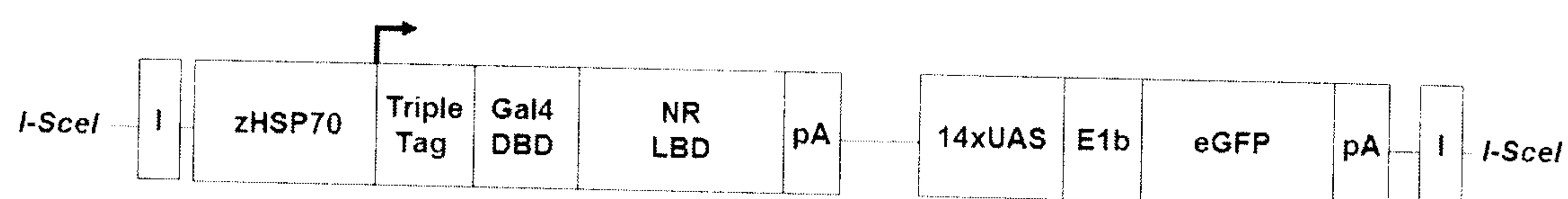
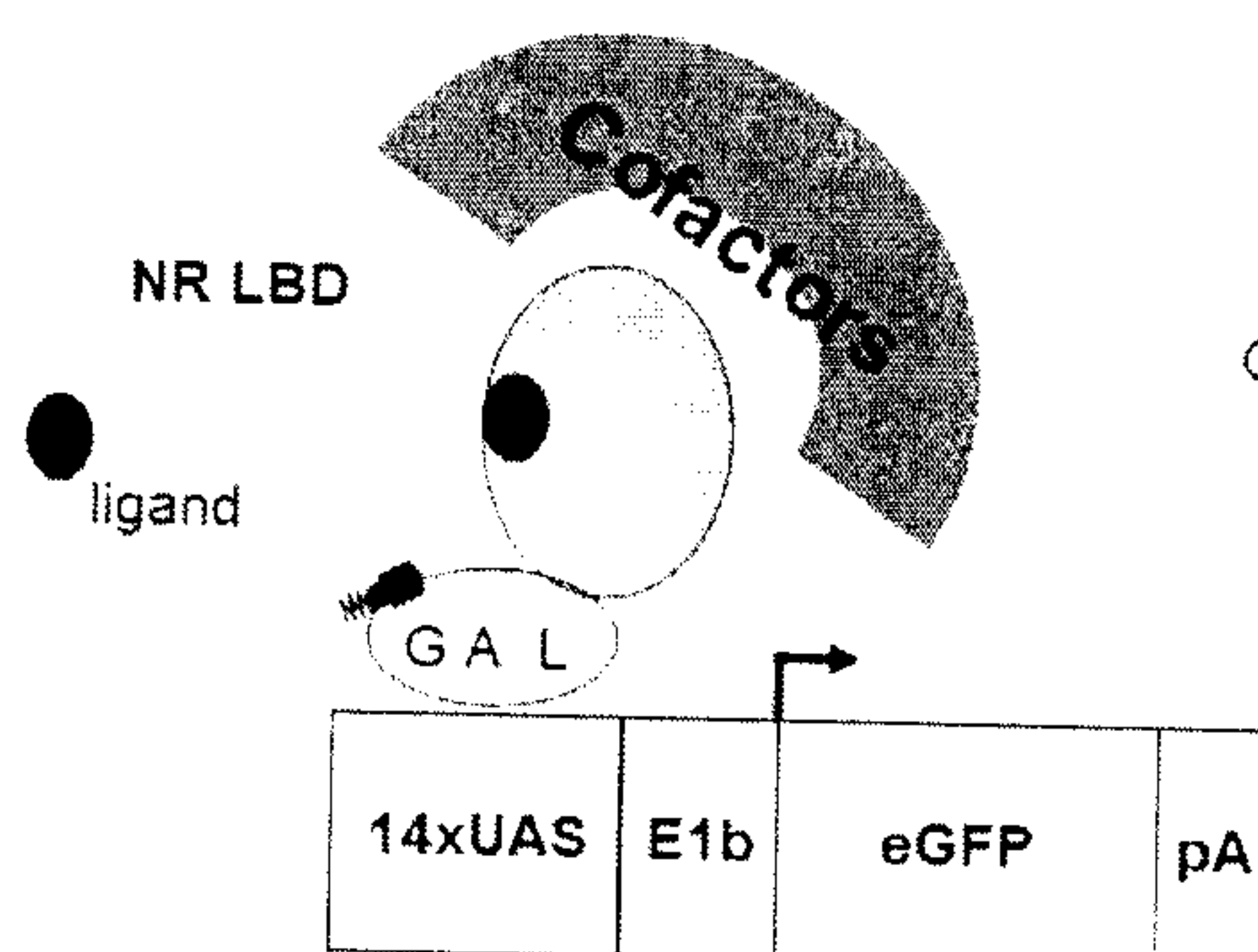
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15. The method of claim 14, further comprising subjecting the purified substance of step (e) to MALDI-TOV, ESI, GC or semi non-denaturing mass spectrometry.
- 5 16. A method for purifying a ligand of a reporter system, the method comprising the steps of:
- (a) providing a live animal, said animal having the first and second nucleic acids of the composition of any one of claims 1-12 transiently expressed or incorporated into its genome;
 - 10 (b) contacting the animal with a test compound;
 - (c) turning on the first promoter;
 - (d) detecting a signal;
 - (e) generating a cellular extract from the animal; and
 - (f) 15 subjecting the cellular extract generated in step (e) to multiple different affinity purification steps, each step comprising binding one affinity tag to an affinity resin capable of selectively binding one affinity tag and eluting the affinity tag from the affinity resin after substances not bound to the fusion protein have been removed;
- 20 wherein the purified substance that is bound to the fusion protein is a ligand of the reporter system.
17. The method of claim 16, further comprising subjecting the purified substance of step (e) to MALDI-TOV, ESI, GC or semi non-denaturing mass spectrometry.
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18. The method of any one of claims 14-17, wherein the live animal is selected from the group consisting of *C. elegans*, *Drosophila*, *Xenopus*, mouse and zebrafish.
- 30 19. The method of claim 18, wherein the live animal is zebrafish.

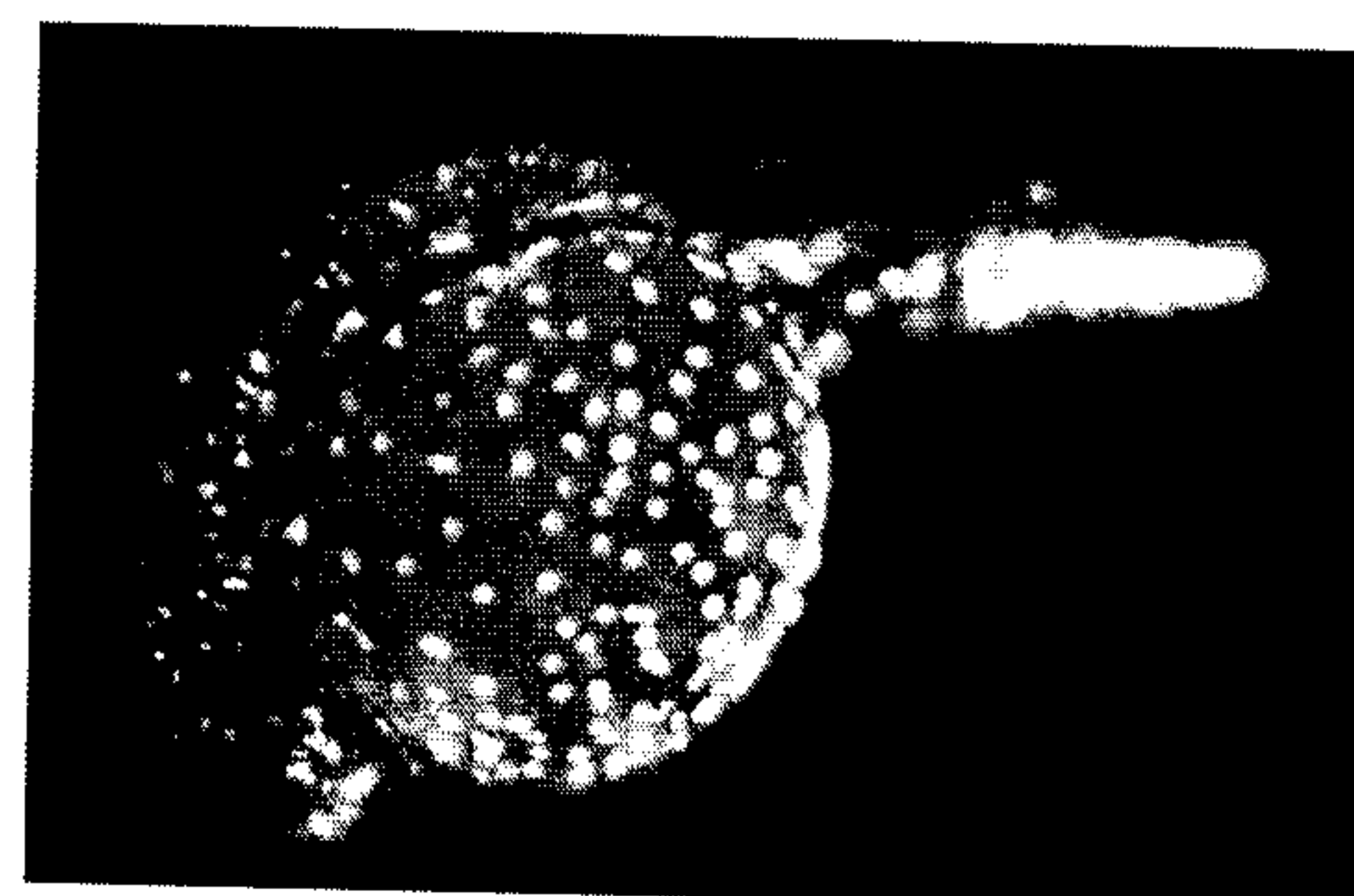
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FIGURE 1

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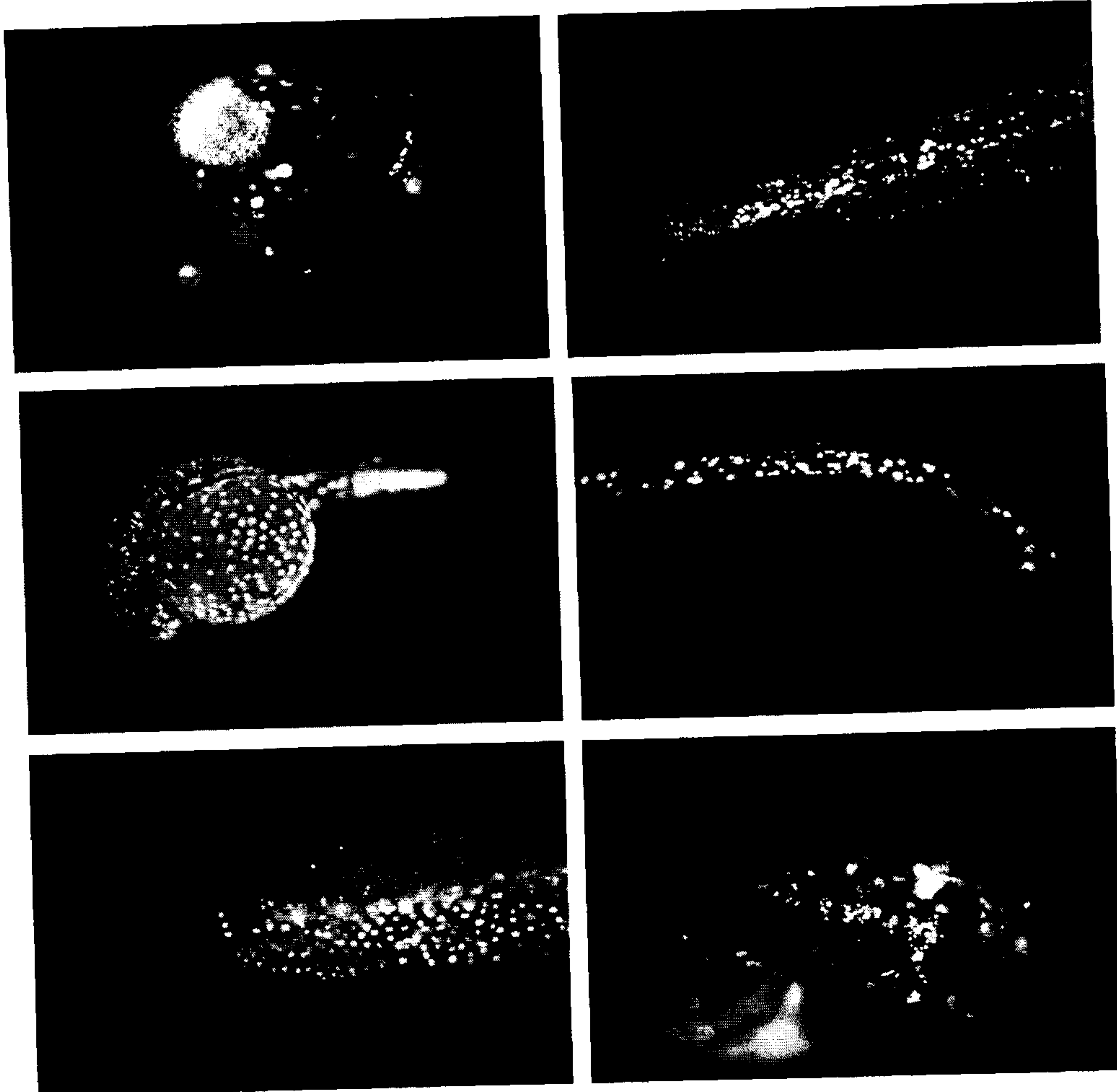
FIGURE 2**B****C**

GFP expression

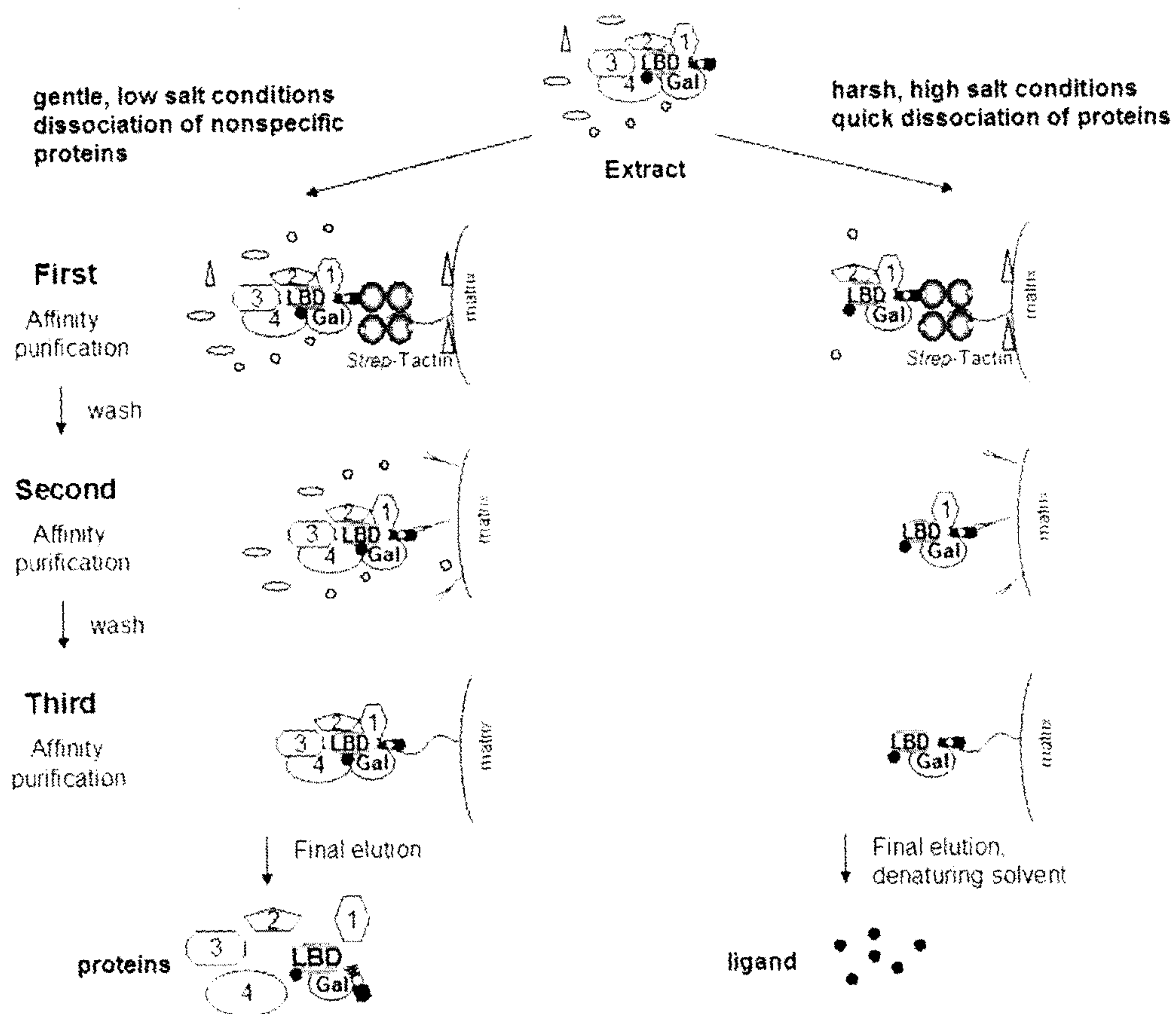


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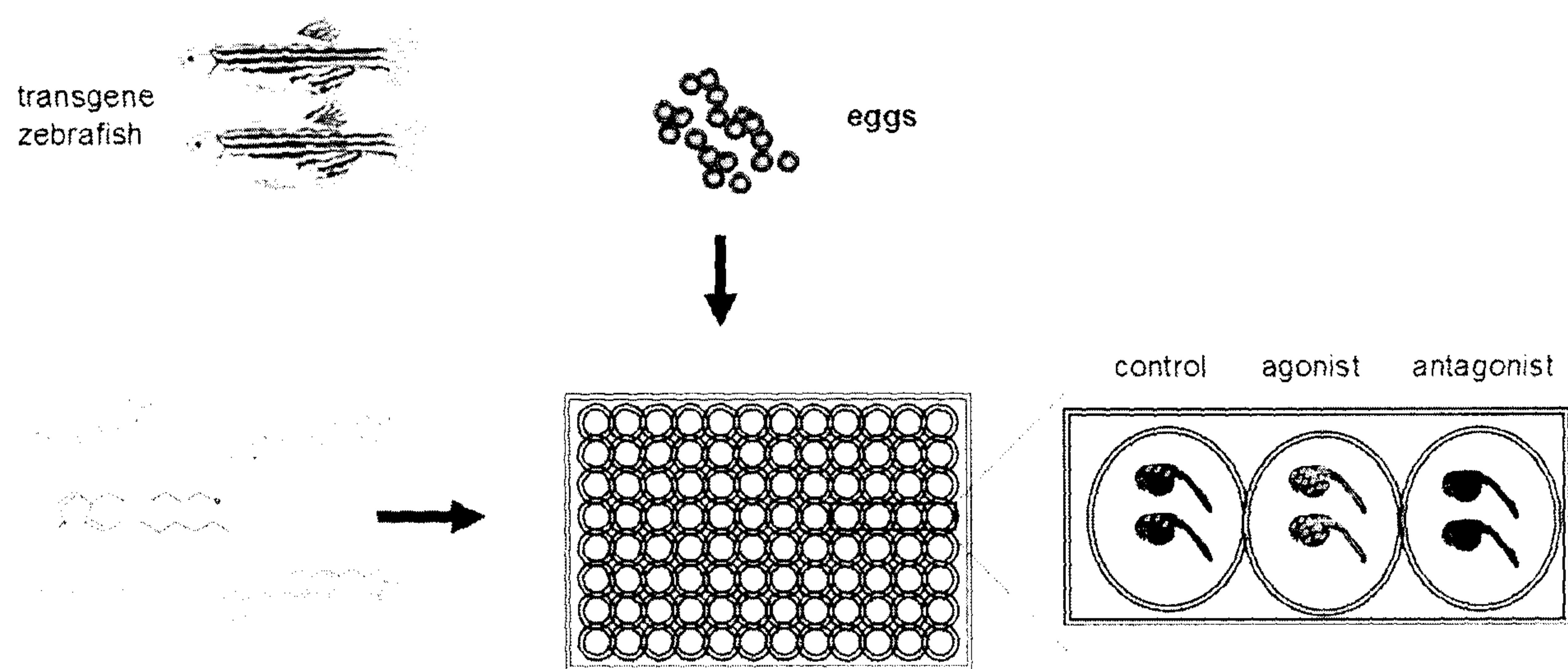
FIGURE 3



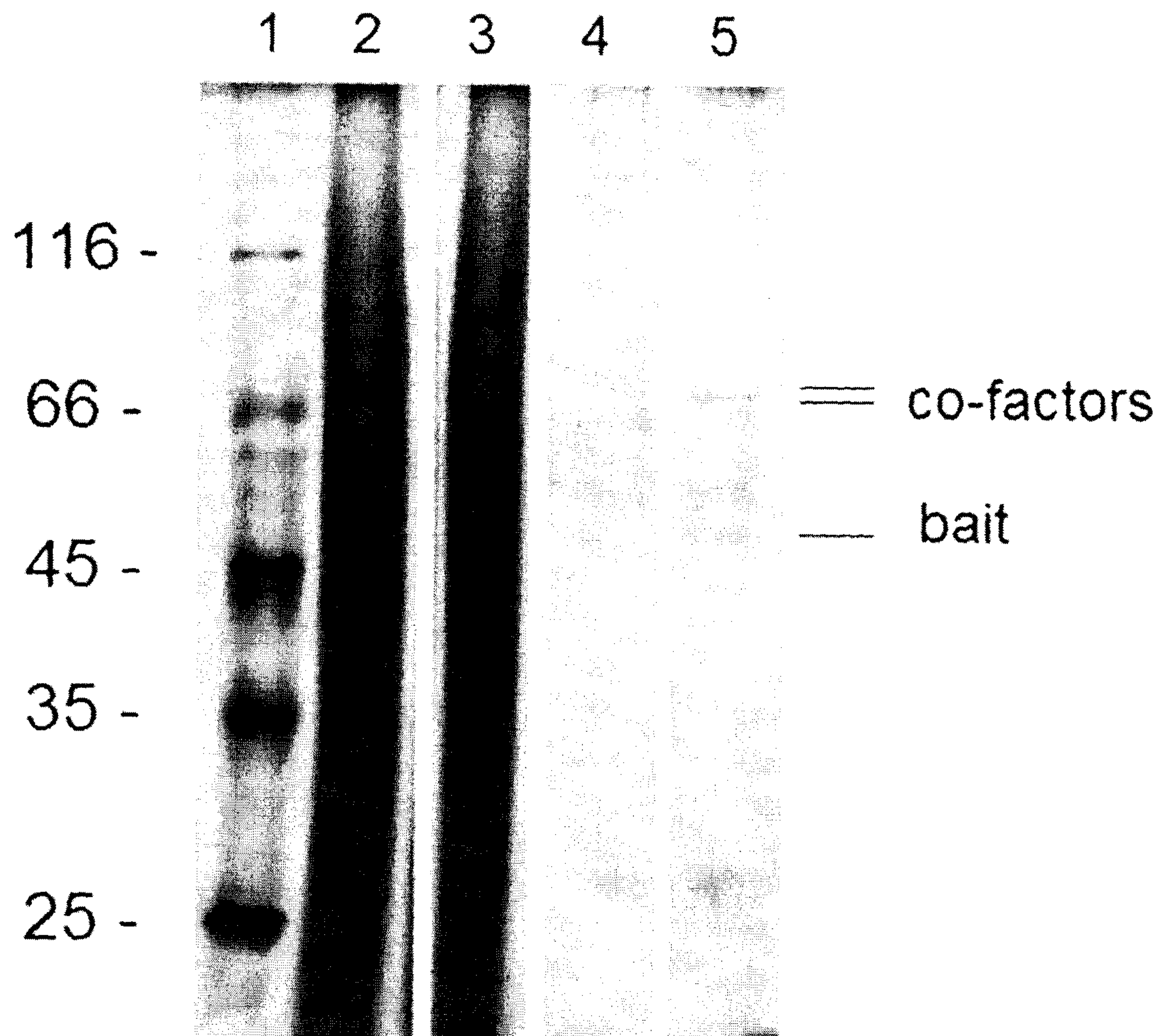
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FIGURE 4

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FIGURE 5

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FIGURE 6

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FIGURE 7