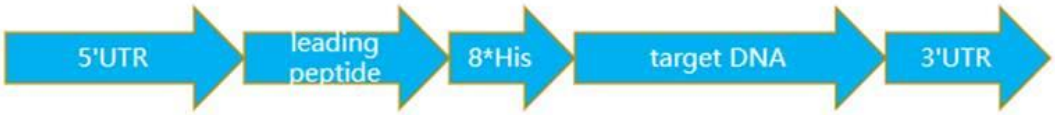
protein expression in vivo



1.Amplify segment.1 (5'UTR+leadingpeptidide+8*His), 346bp.
2.Amplify segment.3 (3'UTR), 126 bp.
3.Amplify segment.2 (from monoclonal colonies,500-550 bp). Introduce the 3'end adaptor of fragment 1to 5'end of segment.2. Introduce the 5'end adaptor of fragment 3to the 3'end of segment.2.
4. Mix and slice fragment 1, 2, and 3 inthe same quantity.
5.Add the purified fragments to a 5mlcell-free expression system at a final concentration of 5ng/ul, and expressovernight at 26°C and 150rpm.
Divide the expressed samples with 2 mlcentrifuge tubes, centrifuge at low temperature, and collect the supernatant.

plasmid vector replacement

1. Plasmid amplification with target gene fragment

Transformation:
Defrost the DH5 cells with the ice bath
Fusion plasmid 4 L was placed into the competent cells, gently mixed and then ice bath for 30 minutes
Heat shock at 42℃ for 45s, then quickly placed on the ice, and stood for 2min.
After the addition of 400 L antibiotic-free ordinary LB medium, the shaking table was oscillated at 37℃ for 30 minutes with a rotating speed of 220rpm
The bacterial solution was coated on solid plate medium with antibiotics, and then inverted for 12h after being put forward for 2h at 37℃
The suspected positive clones were selected and inoculated into a new ANTIBIOtic-containing LB medium for 12h for culture. PCR, sequencing or plasmid extraction were performed to determine whether the transformation was successful
Expanded culture of successfully transformed strains:
The strain was inoculated into 50mL EP tubes, and 15mL LB liquid medium was used for constant temperature culture at 37℃.
The incubation time was about 12h.

2. Plasmid extraction with target gene fragmentwith E.Z.N.A.® Plasmid Mini Kit I

1. Centrifuge at 10,000×g for 1 minute at room temperature. Decant or aspirateand discard the culture media.

2. Resuspend the bacterial pellet by adding 250 µl of Solution MRNase A and vortexing (or pipetting up and down).Complete resuspension (no visible cellclumps) of cell pellet is vital for obtaining good yields.

3. Add 250 µl of Solution ll and gently mix by inverting and rotating the tube several times to obtain a clear lysate. A 2 minutes incubation may benecessary.Avoid vigorous mixing as this will shear chromosomal DNA and lowerplasmid purity. Do not allow the lysis reaction to proceed more than 5 min.(StoreSolution ll tightly capped when not in use to avoid acidification from carbon dioxide in the air.)

4. Add 350 µl of Solution lll and mix immediately by inverting the tube several times until a flocculent white precipitate forms. It is vital that the solution ismixed thoroughly, and immediately after the addition of Solution ll to avoid localizedprecipitation.

5. Centrifuge at 2 10,000×g for 10 minutes at room temperature. A compact white pellet will form. Promptly proceed to the next step.

6. Add the cleared supernatant by CAREFULLY aspirating it into a clean HiBindRMiniprep Column (l) assembled in a provided 2 ml collection tube.Ensure thatthe pellet is not disturbed and that no cellular debris has been carried over into thecolumn.Centrifuge for 1 min at 10,000 × g at room temperature to completelypass lysate through the HiBind" Miniprep Column (l).

7.Discard flow-through liquid and re-use the 2 ml collection tube.Add 500 µl of Buffer HB to wash the HiBind° Miniprep Column (l). Centrifuge for 1 min at10,000 x g at room temperature to completely pass solution through theHiBind*Miniprep Column(1).This step ensures that residual protein contaminationsare removed, thus ensuring high quality DNA that will be suitable for downstream applications.

8. Discard flow-through liquid and re-use the 2 ml collection tube.Add 700 ul of DNA Wash Buffer diluted with absolute ethanol to wash the HiBind* MiniprepColumn (1). Centrifuge for 1 min at 10,000 x g at room temperature tocompletely pass solution through the HiBind Miniprep Column(l)and discardflow-through liquid.

Note: DNA Wash Buffer Concentrate must be diluted with absolute ethanol beforeuse.See label for directions. lf refrigerated, DNA Wash Buffer must be brought toroom temperature before use.

9. Optional Step: Repeat wash step with another 700 ul of DNA Wash Buffer diluted with absolute ethanol.

10. Centrifuge the empty column for 2 min at :13,000 xg to dry the column matrix. Do not skip this step, it is critical for good yields.

11. Place the column into a clean 1.5 ml microcentrifuge tube.Add 30 µl to 50 µl(Depending on desired concentration of final product) of Elution Buffer (10mMTris-HCl, pH 8.5) or sterile deionized water directly onto the column matrix andlet it sit at room temperature for 1-2 minutes.Centrifuge for 1 min at : 13,000x g to elute DNA. An optional

second elution will yield any residual DNA, though ata lower concentration.
12. Yield and quality of DNA: Determine the absorbance of an appropriate dilution of the sample at 260 nm and then at 280 nm.
1. Target fragment PCR amplification
Reaction conditions
Predenaturation 94℃ for 2min
Modified 94 ℃ for 30 s
Annealing 55 ℃ for 30 s
The extended 72 ℃ for 30 s
30 cycle
2. Plasmid double enzyme digestion construction
1. Restriction endonuclease A and B were used to conduct enzyme digestion of empty plasmid/target gene sequence intended to be inserted respectively
Enzyme digestion temperature
37 ℃ double enzyme digestion for 1 h
Enzyme inactivation reaction for 30min
The type of restriction endonuclease was determined by plasmid and the type of endonuclease recognition site of the target fragment
2. The enzyme-cleaved plasmid was mixed with the target gene in a ratio of 1:3-1:5 for connection
T4 ligase was used and the reaction time was overnight

protein expression in BL21

1.BL21 cell transformation

2uL plasmid solution was added to the competent cells
Put the competent cells in the refrigerator (4℃), put them in the water bath (42C) and heat 90s immediately after taking them out for 30min. Put them in the refrigerator (4℃) again for 3min
After removal, 200uL LB liquid medium was added to the receptive cells of the transferred plasmid and placed in a shaker (37°C;
In 200rpm), 30nim to 60min
After the corresponding resistance plate was preheated in an incubator (37C) for 20min, the bacterial solution was coated on solid plate medium with antibiotics, and then inverted for 12h after being put forward at 37℃ for 2h
The suspected positive clones were selected and inoculated into a new ANTIBIOtic-containing LB medium for 12h for culture. PCR, sequencing or plasmid extraction were performed to determine whether the transformation was successful

2. Small amount of detection

The 220rpm culture time was 12h

IPTG induced its final concentration to 1mM/mL, and cultured it at XX temperature for XXh after induction

3. Collect the bacterial liquid by centrifugation

HFL-1 cell line culture

PREPARINGCELLCULTURE FLASKS FOR CULTURING HLF

1.Take the Fibroblast Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcoho1in a sterile hood.
2.Pipette 15 ml of Fibroblast Growth Medium* to a T-75 flask.
* Use Corning and Grenier flasks for best results.
* Keep the medium to surface area ratio at 1ml per 5 cm2.For example, 5 ml for a T-25 flask or a 60 mm tissue culture dish.15 ml for a T-75 flask or a 100 mm tissue culture dish.

PREPARINGCELLCULTURE FLASKS FOR CULTURING HLF

1.Take the Fibroblast Growth Medium from the refrigerator. Decontaminate the bottle with 70% alcoho1in a sterile hood.
2.Pipette 15 ml of Fibroblast Growth Medium* to a T-75 flask.
* Use Corning and Grenier flasks for best results.
* Keep the medium to surface area ratio at 1ml per 5 cm2.For example, 5 ml for a T-25 flask or a 60 mm tissue culture dish.15 ml for a T-75 flask or a 100 mm tissue culture dish.

THAWING AND PLATING HLF

1. Remove the cryopreserved vial of HLF from the liquid nitrogen storage tank using proper protection for your eyes and hands.
2. Turn the vial cap a quarter turn to release any liquid nitrogen that may be trapped in the threads, then re-tighten the cap.
3. Thaw the cells quickly by placing the lower half of the vial in a 37°C water bath and watch the vial closely during the thawing process.
4. Take the vial out of the water bath when only small amount of ice left in the vial. Do not let cells thaw

completely.
5. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.
6.
Remove the vial cap carefully. Do not touch the rim of the cap or the vial.
7. Resuspend the cells in the vial by gently pipetting the cells 5 times with a 2 ml pipette. Be careful not to pipette too vigorously as to cause foaming-
8. Pipette the cell suspension (1ml) from the vial into the T-75 flask containing 15 ml of Fibroblast Growth Medium
9. Cap the flask and rock gently to evenly distribute the cells.
10. Place the T-75 flask in a 37°C, 5% CO2 humidified incubator. Loosen the cap to allow gas exchange. For best results, do not disturb the culture for 24 hours after inoculation.
11. Change to fresh Fibroblast Growth Medium after 24 hours or overnight to remove all traces of DMSO.
12. Change Fibroblast Growth Medium every other day until the cells reach 60% confluent.
13. Double the Fibroblast Growth Medium volume when the culture is >60% confluent or for weekend feedings.
14. Subculture the cells when the HLF reach 80% confluent.

IV. SUBCULTURING HLF

Trypsinize Cells at Room Temperature. Do Not Warm Any Reagents to 37°C.

1. Remove the medium from culture flasks by aspiration.
2. Wash the monolayer of cells with HBSS and remove the solution by aspiration.
    2. Pipette 6 ml of Trypsin/EDTA Solution into the T-75 flask. Rock the flask gently to ensure the solution covers all the cells.
    3. 4. Remove 5 ml of the solution immediately.
    4. 5. Re-cap the flask tightly and monitor the trypsinization progress at room temperature under an inverted microscope. It usually takes about 2 to 4 minutes for the cells to become rounded.
    5. 6. Release the rounded cells from the culture surface by hitting the side of the flask against your palm until most of the cells are detached.
    6. 7. Pipette 5 ml of Trypsin Neutralizing Solution to the flask to inhibit further tryptic activity.
8. Transfer the cell suspension from the flask to a 50 ml sterile conical tube.
9. Rinse the flask with an additional 5 ml of Trypsin Neutralizing Solution and transfer the solution into the same conical tube. 10. Examine the T-75 flask under a microscope. If there are >20% cells left in the flask, repeat Steps 2-9. 11. Centrifuge the conical tube at 220 x g for 5 minutes to pellet the cells. 12. Aspirate the supernatant from the tube without disturbing

the cell pellet. 13. Flick the tip of the conical tube with your finger to loosen the cell pellet. 14. Resuspend the cells in 5 ml of Fibroblast Growth Medium by gently pipetting the cells to break up the clumps. 15. Count the cells with a hemocytometer or cell counter. Inoculate at 10,000 cells per cm2 for rapid growth, or at 7,500 cells per cm2 for regular subculturing.

Immunocytochemistry and immunofluorescence

Preparingthe slide

1.Coat coverslips with polyethylineimine or poly-L-lysine for 1 h at roomtemperature.
2.Rinse coverslips well with sterile H2O (three times 1 h each).
3.Allow coverslips to dry completely and sterilize them under UV light for atleast 4 h.
4.Grow cells on glass coverslips
5.Rinse briefly in phosphate-buffered saline (PBS).
Forwash buffer we recommend 1x PBS 0.1% Tween 20.

Fixation

Using4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature
Thecells should be washed three times with ice-cold TBS, 5 minutes for each.

Antigenretrieval (optional step)

1.Preheat the antigen retrieval buffer (100 mM Tris, 5% [w/v] urea, pH 9.5) to95°C. This can be done by heating the buffer in a coverglass staining jar whichis placed in a water bath at 95°C.
2.Using a small pair of broad-tipped forceps, place the coverslips carefully inthe antigen retrieval buffer in the cover glass staining jar, making note ofwhich side of the coverslips the cells are on.
3.Heat the coverslips at 95°C for 10 min.
4.Remove the coverslips from the antigen retrieval buffer and immerse them, withthe side containing the cells facing up, in PBS, in the 6-well tissue cultureplates.
5.Wash cells in PBS three times for 5 min.

Permeabilization

1.Incubate the samples for 10 min with PBS containing 0.3% Triton X-100
2.Wash cells in PBS three times for 5 min.

Blockingand immunostaining

1.Incubate cells with 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20)for 30 min to block unspecific binding of the antibodies (alternative blockingsolutions are 1% gelatin or 10% serum).
2.Incubate cells in the diluted antibody in 1% BSA in PBST in a humidifiedchamber for 1 h at room temperature or overnight at 4°C.
3.Decant the solution and wash the cells three times in PBS, 5 min each wash.
4.Incubate cells with the secondary antibody in 1% BSA for 1 h at roomtemperature in the dark.
5.Decant the secondary antibody solution and wash three times with PBS for 5 mineach in the dark.

Counterstaining

1.Incubate cells with 0.1–1 µg/mL Hoechst stain or DAPI (DNA stain) for 1 min.
2.Rinse with PBS.

Mounting

1.Mount coverslip with a drop of mounting medium.

2.Seal coverslip with nail polish to prevent drying and movement undermicroscope.

3.Store in dark at -20°C or +4°C.

8.Streakingand Isolating Bacteria on an LB Agar Plate

Equipment

Steriletoothpicks or wire loop

Bunsenburner (or other small flame source)

Incubator

Marker

Reagents

LBagar plate (with appropriate antibiotic)

Bacterialstab

Procedures:

1. Obtainan LB agar plate with appropriate antibiotic.
2. Labelthe bottom of the plate with the plasmid name and the date. It is also a goodidea to add the antibiotic resistance and your initials. Labeling within alaboratory setting is important for organization, and it is recommended thatyou keep a standard labeling system for all your objects/solutions.
3. Sterilizeyour lab bench by spraying it down with 70% ethanol and wiping it down with apaper towel. Maintain sterility by working near a flame or bunsen burner.
4. Obtainthe appropriate bacterial stab or glycerol stock.
5. Usinga sterile loop, pipette tip or toothpick, touch the bacteria growing within thepunctured area of the stab culture or the top of the glycerol stock.

*Pro-Tip*If you use a wire loop you can sterilize it by passing it through a flame, justbe sure to allow enough time for the loop to cool before touching it to thebacteria.

6. Gently spread the bacteria over a section ofthe plate, as shown in the diagram below, to create streak #1.

*Pro-Tip*Hold your tooth pick at an angle, the way you would hold a pencil, so that youcan make a broad stroke. Only touch the surface of the plate, do NOT dig intothe agar.

*Pro-Tip*Another very popular technique is to draw in discontinuous lines. Start bystreaking a vertical line of bacteria along one edge of the plate. Then streakhorizontal lines in another section of the plate, and then diagonal lines inanother section of the plate. Make sure that

the first line (and only thefirst) in each new section crosses at least one line of the previous section sothat it will contain some bacteria

7. Using a fresh, sterile toothpick, or freshlysterilized loop, drag through streak #1 and spread the bacteria over a secondsection of the plate, to create streak #2.

8. Using a third sterile pipette tip, toothpick,or sterilized loop, drag through streak #2 and spread the bacteria over thelast section of the plate, to create streak #3.

9. Incubate plate with newly plated bacteriaovernight (12-18 hours) at 37 °C.

*Pro-Tip*Some plasmids or bacteria need to be grown at 30 °C instead of 37 °C. This isoften true for large unstable plasmids, which sometimes recombine at 37 °C. Besure to check this before incubating your plate.

10. In the morning, single colonies should bevisible. A single colony should look like a white dot growing on the solidmedium. This dot is composed of millions of genetically identical bacteria thatarose from a single bacterium. If the bacterial growth is too dense and you donot see single colonies, re-streak onto a new agar plate to obtain singlecolonies.

11. Once you have single colonies, you canproceed to Recovering Plasmid DNA or use the individual colonies for otherexperiments.

SDS PAGE-Preparation

SDS PAGE-Preparation:

An intact SDS PAGE electrophoresis system should include: a tank, lid with power cables, electrode assembly, cell buffer dam, casting stands, casting frames, combs(usually 10-well or 15-well), and glass plates (thickness 0.75mm or 1.0mm or 1.5mm). (Bio-rad brand one is recommended)

The SDS PAGE gel in a single electrophoresis run can be divided into stacking gel and separating gel. Stacking gel (acrylamide 5%) is poured on top of the separating gel (after solidification) and a gel comb is inserted in the stacking gel. The acrylamide percentage in SDS PAGE gel depends on the size of the target protein in the sample. (details shown below)

Acrylamide %	M.W. Range
7%	50 kDa - 500 kDa
10%	20 kDa - 300 kDa
12%	10 kDa - 200 kDa
15%	3 kDa - 100 kDa

Volumes of stacking gel and separating gel differ according to the thickness of gel casting:

Thickness of the gel	Vol. of stacking gel	Vol. of separating gel
0.75mm	2ml	4ml
1.0mm	3ml	6ml
1.5mm	4ml	8ml

- **For a 5 ml stacking gel:**

H_2O	2.975 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate (AP)	0.05 ml
TEMED	0.005 ml

For a 10ml separating gel:

Acylamide percentage	6%	8%	10%	12%	15%
H_2O	5.2ml	4.6ml	3.8ml	3.2ml	2.2ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2ml	2.6ml	3.4ml	4ml	5ml
1.5M Tris(pH=8.8)	2.6ml	2.6ml	2.6ml	2.6ml	2.6ml
10% (w/v)SDS	0.1ml	0.1ml	0.1ml	0.1ml	0.1ml
10% (w/v) ammonium persulfate (AP)	100μ l	100μ l	100μ l	100μ l	100μ l
TEMED	10μ l	10μ l	10μ l	10μ l	10μ l

Note: AP and TEMED must be added right before each use.

- **5X Sample buffer (loading buffer):**

10% w/v	SDS
10 mM	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenolblue

Make sure your target protein dissolved in the liquid phase, and no inappropriate ingredients present (e.g. guanidine hydrochloride can interact with SDS and cause precipitation) Generally, to treat your unprepared sample, you can use sonicator, lysis buffer or both to sufficiently make your target protein released, and centrifuge to make supernatant and pellet separated.

- **1x Running Buffer:**

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

(Approximately vol. of less than 1 liter is needed depending on the type of your electrophoresis system.)

SDS PAGE Protocol:

1. Make the separating gel:
Set the casting frames (clamp two glass plates in the casting frames) on the casting stands.
Prepare the gel solution (as described above) in a separate small beaker.
Swirl the solution gently but thoroughly.
Pipet appropriate amount of separating gel solution (listed above) into the gap between the glass plates.
To make the top of the separating gel be horizontal, fill in water (either isopropanol) into the gap until a overflow.
Wait for 20-30min to let it gelate.
Make the stacking gel:
Discard the water and you can see separating gel left.
Pipet in stacking gel untill a overflow.
Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it gelate.
2. Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow untill the

buffer surface reaches the required level in the outer chamber.
3. Prepare the samples:
Mix your samples with sample buffer (loading buffer).
Heat them in boiling water for 5-10 min.
4. Load prepared samples into wells and make sure not to overflow. Don't forget loading protein marker into the first lane. Then cover the top and connect the anodes.
5. Set an appropriate volt and run the electrophoresis when everything's done.
6. As for the total running time, stop SDS-PAGE running when the downmost sign of the protein marker (if no visible sign, inquire the manufacturer) almost reaches the foot line of the glass plate. Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel posessing higher percentage of acylamide, the time will be longer.
Note: Various factors affect the properties of the resulting gel.
• Higher concentration of ammonium persulfate and TEMED will lead to a faster gelation, on the other hand, a lower stability and elasticity.
• The optical temperature for gel gelation is 23°C-25°C. Low temperature will lead to turbid, porous and inelastic gels.
• The pH is better to be neutral and the gelation time shoud be limited in 20-30 min.

Western Blot Protocol:

1. Run gel as usual. Take gel out of electrophoresis apparatus. Cut into segments as required; Part of gel can be stained directly in Coomassie brilliant blue R-250 (2.5 g Coomassie Brilliant Blue R-250, 450 mls methanol, 100 mls glacial acetic acid, water to 1 liter). Part to be used for electroblotting is put into tap water on shaker, after first having marked it unambiguously to identify top/bottom, left and right etc.
2. Leave in water on shaker for 5 minutes. This step can be substituted by washing the gel in electro-transfer buffer (see below) for 5 minutes.
3. We use a semidry blotter, which we have found to be quicker, more economical and easier than fully submerged blotting methods. We cut Whatman 3M filter papers to the size of our gels, and place three of these onto the semi dry blotter. These are then wet with transfer buffer (we routinely use 3.03 g Tris base, 14.4 g Glycine, 10% Methanol per liter). The gel is put onto the filters and a prewetted nitrocellulose filter is put ontop of the gel. Alternately put a PVDF membrane on top; if you are using PVDF remember it is essential to prewet the PVDF in 100% methanol. Great care should be taken to ensure that no air bubbles are anywhere in this stack of membranes. Then three more wetted Whatman 3M filters should be placed ontop of the pile, again taking great care not to have any bubbles in pile. Put the top onto the apparatus and screw it down. Proteins in transfer buffer are negative in charge mostly due to residual SDS and they therefore move from -ve to +ve pole. So the +ve electrode is above the nitrocellulose and the -ve side is below the gel.
4. Run for 30 minutes to 1 hour at ~100mA. The most reliable way of doing this is to use a powerful power supply 200-500mA and put it on constant voltage, with a setting of 5 to 10 Volts. Low molecular weight proteins (20kDa or less) will transer in 30 minutes at 5 Volts, while higher molecular weight (150kDa or more) transfer in 60 minutes at 10 Volts.
5. After running disassemble the apparatus and remove nitrocellulose filter. Stain this for 5

minutes on shaker in Ponceau reagent (0.25% Ponceau S in 40% methanol and 15% acetic acid). Destain with regular SDS-PAGE gel destain solution (7.5% methanol, 10% acetic acid). If you transferred efficiently, the proteins can be seen as pale pink bands. This tells you whether the transfer was O.K. or not and also exactly where the bands are. You can photograph, photocopy or mark the position of the bands directly with a pencil. If you can't see any bands at this stage, it's probably smart to try to optimize steps 3 and 4. The gel may be discarded or may be stained as usual in coomassie, to see how much protein is left behind.

6. After Ponceau staining put the nitrocellulose filter into blocking solution, such as 1% bovine serum albumin (BSA) or 1% Carnation non fat milk (NFM), for 20 minutes to 1 hr at RT or 37°C. Since the NFM works just as well as BSA but is much cheaper, there is really no good reason to use BSA. Ponceau staining will fade to become completely invisible. Carry on with antibody incubations etc.

Antibody Incubations:

1. Put in antibody solutions. Volume should be enough to cover blot and allow it to float freely when you agitate. In initial experiments, antibody concentration should generally be about 1:100 - 1:1,000 for ascites, CL350 tissue culture supernatant or antiserum, undiluted to 1:10 for monoclonal supernatant, and about 1-10µg/ml for a pure IgG. If dilution brings antibody concentration to less than 50 µgs/ml, add some BSA or NFM to act as carrier protein (e.g. make BSA or NFM concentration 1mg/ml). Incubate for at least 1 hour with shaking (can be room temperature or at 37°C, can also do overnight at 4°C).

2. Wash membranes in TBS (10mM Tris, 154mM NaCl, pH=7.5 plus 0.1% Tween 20) for 3 times at least five minutes each time with extensive agitation.

3. Incubate in second antibody (peroxidase-conjugate, phosphatase conjugate or radioactive). Add BSA or NFM carrier as before if necessary. Incubate for at least one hour at room temperature or 37°C can also do overnight at 4°C with shaking as before.

4. Wash membranes in TBS (10mM Tris, 154mM NaCl, pH=7.5 plus 0.1% Tween 20) for 3 times at least five minutes each time with extensive agitation.

Alkaline Phosphatase Blot System

1. Incubate in alkaline phosphatase conjugated antibody against the primary antibody (e.g. Goat anti-mouse, rabbit or chicken; buy from Sigma or some other trusted source). Typical concentration is 1:1,000 in TBS (10mM Tris/HCl, 154mM NaCl, pH=7.5). Add a small amount of BSA or NFM to act as carrier. Incubate for 1 hour at room temperature (or 37°C) with shaking.

2. Wash in TBS three times 5 minutes each. (N.B. the alkaline phosphatase enzyme is inhibited by EDTA, which chelates zinc and magnesium, and by phosphate, which inhibits forward reaction. Make sure therefore you use TBS which is EDTA and phosphate free- Don't make up developer in PBS!)

3. Put into developer. Buffer is 100mM Tris/HCl, 100mM NaCl, 5mM MgCl2 pH=9.5. To 10ml of this add 33µl BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt, make up 50mg/ml in water or Dimethyl formamide; in water makes a yellow suspension) and 33µl of NBT (Nitro Blue Tetrazolium, also 50mg/ml in water). Can store these solutions at -20°C. Can buy this solution made up already from Sigma. Reaction product is purple, and appears in a few minutes; can incubate for up to an hour if the signal is weak. Watch development of reaction and stop with water. Some of background disappears on drying.

Horse Radish Peroxidase Staining

After washing of blots in TBS or PBS (must not have azide in wash buffer! This inhibits the peroxidase enzyme) add reaction mixture. This is; 20 mls 0.1M Tris/HCl pH=7.2 (Vecta stain buffer). 200 µl NiCl (80 mg/ml), 6 µl 30% hydrogen peroxide, 1ml of 5mgs/ml diaminobenzidine. (Wear gloves, DAB is carcinogenic). Alternate protocol; Make 20 mls ammonium acetate buffer (50mM, pH=5.0). Add 1 ml of 10mg/ml Diaminobenzidine, 40µl 30% hydrogen peroxide. Brown reaction product is seen in 1-10 minutes, not quite so nice as above method.

Chemiluminescence Staining

Chemiluminescence has an advantage of perhaps an order of magnitude greater sensitivity than the dye based methods above. In addition, several films may be exposed from a single blot, giving an advantage in interpretation of weak and strong signals on the same membrane. However it requires a darkroom to perform and is more expensive in reagents. Reagents are generally bought in a kit, and we recommend simply following the kit instructions.