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Split Luciferase Binding Assay (SLBA) Protocol

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Protocol status: Working
We use this protocol and it's working

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Abstract

HiBit is an 11 amino acid protein tag that binds with high affinity to a larger subunit called LgBit. The bound complex has luciferase activity and will release luminescent signal in the presence of added furimazine substrate. HiBit is highly quantitative, extremely sensitive, and much faster than conventional immunoassays. When added to C-terminus of protein/peptide, the luminescence from tag ensures entire construct has been translated. The tagged protein/peptide is incubated with patient sera and antibody/antigen complexes are captured with protein A/G beads. Luminescence of captured complexes is measured.

Materials

SLBA buffer (1L)

- 30ml of 5M NaCl
- 10ml of 2M Tris-HCl pH7.4
- 1g sodium azide
- 1g BSA
- 1.5ml Tween-20
- Bring the volume up to 1000ml and filtered using 0.2 micron filter. Store the buffer at 4C in a sterile bottle for up to 3 months.

Sepharose Protein A and Protein G beads

- Add equal volume of Protein A (Millipore Sigma GE17-5280-02) and Protein G (Millipore Sigma GE17-0618-05)
- Wash beads 3x with SLBA buffer
- Resuspend in 1 volume packed beads (e.g. packed beads are 10mL after washing, bring total volume up to 20mL with SLBA buffer)
- Keep prepared beads at 4C for up to 1 month

Note

Protein A/G sepharose is provided in 20% ethanol by manufacturer, so washing the beads removes ethanol which may denature antigens and affect immunoprecipitation

Mouse anti-Hibit Antibody Promega Clone 30E5 (positive control)
Nano-Glo HiBit Lytic Detection System (Promega Cat No. N3040)
96-well Filter Plates 0.2um PVDF Hydrophillic Membrane White Polystyrene (Corning Cat No. 3504)

Note

We have observed that in recent years (2021-2023) these plates have been on a 6 month backorder

White seals for 96-well plates (Perkin Elmer Cat No 6005199)
TnT Quick Coupled Transcription/Translation System (Promega Cat No. L1170)
96-well flat bottom white plates (Corning Cat No 3570)
Wide bore p200 tips (Thermo Scientific Cat No 2069G)
Vacuum manifold (Millipore Sigma Cat No. 575650-U)

Troubleshooting

Before start

Make SLBA buffer

Prepare sepharose beads

Protocol overview

1 **Step 1:** Design insert within Hibit construct, DNA synthesis
Step 2: PCR amplify constructs
Step 3*: In vitro* transcription-translation
Step 4: Quantify protein RLU, normalize protein
Step 5: First run an experiment for the positive control titrations and some other negative controls to determine if the protein of interest have any issues of sticking to the beads or the PVDF membrane. Run a titration for the serum.
·Relevant controls are:
oProtein only
oProtein + beads
oProtein + negative serum or isotype control
Step 6: Run SLBA: experimental samples + positive controls (commercial antibody or known positive serum) + negative controls (healthy control sera) + blank wells.

I. Design insert with Hibit Construct

2 ·Design insert in frame with the construct below

```
aagcagagctcgtttagtgaaccgtcagaattttgtaatacgactcactatagggcggccgggaat
tcgtcgactggatccggtaccgaggagatctgccgccgcgatcgcc[ATG][insert]GGCTCAGGC
TCAGGCTCAGTGAGCGGCTGGAGACTGTTCAAGAAGATCAGCgtttaaacggccggcc
```

This construct contains 5′ CMV promoter, T7 promoter, Kozak. In 3′ contains GS linkers, Hibit (underlined), stop.

3 ·Order from oligo synthesis company (e.g. IDT, Twist)

II. PCR amplify construct

4 Resuspend lyophilized DNA to 1uM (can do less, but test PCR first)

5 Prepare 100uL PCR reaction per construct as below:

	A	B
		1x
	Water	72
	5x PhusionHF Buffer	20
	10nM dNTP	2
	10uM Ultra Hibit FW	2
	10uM Ultra Hibit REV	2
	Phusion HF	1

Recipe for 1x PCR reaction

PCR primers:

```
FW: aagcagagctcgtttagtgaaccgtcaga
REV:ggccggccgtttaaacGCTGATCTT
```

Add 1uL 1uM lyophilized DNA into 99uL of master mix (can use a lot less, test depending on your construct)

6 Run PCR program in thermocycler:

	A	B	C
	Temp	**Time**	**No. Cycles**

	A	B	C
	98	2 min	1
	98	30s	25
	68	30s	
	72	30s	
	72	5 min	1
	10	inf	1

PCR program

7 Optional: Purify PCR with Ampure XP SPRI beads, using 1x volume beads : PCR ratio following manufacturer's instructions. Elute in 50uL water.
Optional: Using Qubit HS DNA quantification kit, quantify DNA following manufacturer's instructions.
Optional: run gel to confirm product is correct size (recommended if first time)

Note

PCR success can be measured by luciferase activity in the next step

III. In vitro transcription translation (TNT)

8 Optional: Normalize purified amplicon DNA to 0.125ug/uL

Note

We have observed good luciferase yield without PCR purification and quantification

9 Using rabbit reticulocyte lysate (Promega quick couple Kit) prepare master mix. One TNT master mix is good for 4 rxn + 1 neg control (no DNA)

	A	B	C
	TNT rabbit reticulocyte master mix	40	
	PCR enhancer	1	
	Methionine (non-radiolabeled)	1	
		42	ul MM /well
		8	DNA (0.125 ug/uL)

TNT recipe for one reaction. MM is master mix

Note

We include an in-frame stop codon as a negative control to measure background luminescence in the TNT reaction

10 Transfer to the PCR tubes in the thermocycler to proceed with transcription/translation. Incubate for 3h at 30C

Note

We have observed better yield by extending incubation time

IV. Normalize protein RLU

11 Dilute protein 1:100 in SLBA buffer, perform each RLU measurement in triplicate

12 Add 50uL diluted protein/well to 96-well white flat bottom plate

13 Add 50uL luciferase reagent/well. Prepare master mix (shown here for 1 reaction):

	A	B
	Nano Glo Hibit Lytic Reagent	50
	Hibit substrate	1
	Lg Bit protein	0.5

Recipe for 1 reaction of split luciferase reagent.

14 Incubate 30 min RT dark, measure counts using luminometer

15 Dilute protein with SLBA buffer to add 2e6-2e8 RLU per well

Note

Input RLU will need to be optimized with positive and negative controls for each antigen.

V. SLBA Day 1

16 Calculate the amount needed for 2e6-2e8 RLU protein per well and RLBA buffer in total volume of 50uL per well. Dispense into PCR plate.

17 Add 1-5ul of serum to antigen+buffer

Note

Determine optimal amount of serum input experimentally

18 Orbital shake 15 min 500rpm at 4C, incubate overnight 4C.

19 Block the 0.2um PVDFFilter 96-well Plates PVDF plate with 200ul of RLBA buffer overnight at RT

VI. SLBA Day 2

20 Remove wash buffer from blocked PVDF plates by placing on vacuum manifold, seal bottom of plate.

21 Using wide bore P200 tips, add 25ul of beads/well (1:1 protein A:G; SLBA buffer to beads should be 1:1 ratio) to filter plate

22 Add 55ul of serum + Ag mix to each well that contains the beads

23 Seal the plate with foil cover and orbital shake (500rpm) in the cold room for 45mins.

24 Place PVDF plate on vacuum fold with gentle vacuum to remove unbound antigen.

25 Add 200ul of SLBA buffer to each well, repeat for a total of 3 washes

26 Add 150uL SLBA buffer. Seal the bottom plate with foil cover.

27 Orbital shake (500rpm) for 5 mins at 4C.

28 Connect the vacuum seal and wash it with 200ul of SLBA buffer, repeat for a total of 3 washes

29 Release the pressure after wash and put the paper towel on the vacuum seal and applied pressure to remove excess fluid. Do it until almost little buffer are seen on the paper towel from the vacuum pressure.

30 Gently place a white seal on bottom of plate

31 Resuspend beads in 50uL SLBA buffer

32 Add 50uL luciferase reagent (prepared as above) to each well, mix

33 Incubate 1h room temperature in the dark

34 Read counts using luminometer