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Direct Competitive ELISA (cytochrome c)

REAGENTS

10X PBS (1 liter)

NaCl	80 g
KCl	2 g
$Na_2HPO_4 \cdot 7H_2O$	11.5 g
KH_2PO_4	2 g

Mix well and store at room temperature.
Add H_2O to 1 liter (Working solution pH ~7.3).

PBSN

1X PBS	100 ml
NaN_3	50 mg

BBS (2 liters)

H_3BO_4	21 g
NaCl	14g

Dissolve in H_2O, pH to 8.5 and volume to 2 liters and store at room temperature.

Blocking Reagent

BBS	500 ml
Tween 20	250 μl
0.5 M EDTA	1 ml
BSA	1.25 g
NaN_3	0.25 g

Store at 4°C.

Homogenization Buffer

1 M KCl	17.5 ml

Ingredients for 100 ml of H.Bx for luciferase and CAT.

1 M H_2SO_4

18 M H_2SO_4	22.22 ml

Add to H_2O to final volume of 400 ml.

PROCEDURE

Criss-Cross Serial Dilution Analysis

1. Prepare antigen (Ag) (rat heart cytochrome c, Sigma C-7892) in PBSN by 1:2 serial dilution. Add 50 μl of Ag to each well of a row of a 96-well EIA microplate (Bio-Rad 224-0096). The highest concentrion (10 μg/ml) to row A, the second highest concentration to row B and the lowest concentration (0 μg/ml) to row H. Incubate at room temperature overnight.
2. Rinse the coated plate with double distilled water three times.
3. Add 150 μl of blocking reagent to each well and incubate at room temperature for 30 min.
4. Rinse the plate with double distilled water three times.
5. Add 50 μl of blocking reagent to each wells except the wells in the first column.
6. Add 100 μl of primary antibody (Ab) (mouse monoclonal Ab against rat cytochrome c, 6H2.B4, IgG 2a, K, 0.7 mg/ml from Dr. R. Jemmerson, 1:100 in blocking reagent) to the wells in the first column and incubate at room temperature for 2 hr.
7. Rinse the plate with double distilled water three times.
8. Add 50 μl of secondary Ab (goat anti-mouse IgG conjugated to horse radish peroxidase in blocking reagent, Axell #JGM035003 diluted in PBS-Tween, 1:5000 in blocking reagent) and incubate at room temperature for 2 hr.
9. Rinse the plate with double distilled water three times.
10. Add 75 μl of blocking reagent and vortex and incubate at room temperature for 10 min.
11. Rinse the plate with double distilled water three times.
12. Add 100 μl of TMB substrate (Bio-Rad, 172-1066, mix A solution with B solution at 9:1 before use) and incubate at room temperature for 10 min.
13. Add 100 μl of 1 M H_2SO_4 and read absorbance at 450 nm in a microplate reader (Bio-Rad) using the mean of the row H values as zero.
14. Plot the data for each Ag dilution (A450 for Y-axis and log1/D for X-axis). The concentrations of Ag that gives a maximal A450 value would be appropriate for coating the plate. The dilution of primary Ab that

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gives the 1/2 of the maximal reading at A450 would be used for sensitive detection of cytochrome c in the direct competitive ELISA.

Microplate coating

1. Add 400 μl of cytochrome c (500 μg/ml in H.Bx. for luciferase and CAT) to 50 ml of PBSN and the final concentration for cytochrome c is 4 μg/ml.
2. Add 50 μl of the Ag in PBSN to each well of a microplate and incubate at room temperature overnight. The coated plate can be stored at 4°C for several months.

Muscle homogenization

1. Weigh out ~100 mg frozen muscle while frozen and put into a 15 ml polyethylene tube (Sarstedt 60.541).
2. Add 10 ml/g 1X *homogenization buffer* (H.Bx for luciferase and CAT with 175 mM KCl).
3. When the muscle is thawed on ice, homogenize the sample at setting 7 with a Polytron homogenizer for 10'' x 3 with 10'' intervals in ice-water bath.
4. Freeze-thaw the samples three times using -80°C freezer or liquid nitrogen and ice.
5. Centrifuge the samples at 11,000 rpm (15,000 x g) for 15' at 4°C.
6. Transfer the supernatant to an eppendorf tube and store at -20°C.

ELISA

1. Rinse the coated plate with double distilled water three times.
2. Add 150 μl of blocking reagent to each well and incubate at room temperature for 30 min.
3. Rinse the plate with double distilled water three times.
4. Prepare cytochrome c standard in homogenization buffer (100 ng/μl is the highest concentration and 2:3 series dilution for the rest 9 tubes and homogenization buffer as the 0 ng/μl control).
5. To wells in a NEW microplate, add 100 μl of blocking reagent containing 1:9000 primary Ab and add 11.11 μl of standard or sample in duplicates to each well. Incubate at room temperature for 30 min.
6. To coated well add 50 μl of the above mixture or 50 μl of blocking reagent (as negative control to zero the reading) and incubate at room temperature for 2 hr.
7. Rinse the plate with double distilled water three times.
8. Add 50 μl of secondary Ab and incubate at room temperature for 2 hr.
9. Rinse the plate with double distilled water three times.
10. Add 75 μl of blocking reagent and vortex and incubate at room temperature for 10 min.
11. Rinse the plate with double distilled water three times.
12. Add 100 μl of TMB substrate and incubate at room temperature for 10 min.
13. Add 100 μl of 1 M H_2SO_4 and read absorbance at 450 nm in a microplate reader.

REFERENCES

1 Frederick M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J. G. Seidman, John A. Smith, and Kevin Struhl. *Current Protocols in Molecular Biology*, Chapter 11: Immunology. John Wiley & Sons, Inc., 1994.

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