Protocol
Calretinin – ELISA

Products

30% H2O2 solution: Merck, 8222887.1000
3,3’ ,5,5’-tetramethylbenzidin (TMB): Fluka, 87748
Aceton: Fluka, 00570
Bovine serum albumin: Fluka, 05480
Citric acid monohydrate: Fluka, 27490
Ethanol: Fluka, 02860
Kathon MW/WT: Christ AG, Aesch, Switzerland
NaHCO3: Fluka, 71628
Phenol: Merck, 206
Sulfuric acid 97%: Fluka, 84720
Tris/HCl (Tris-(hydroxymethyl)-aminoethan): Fluka, 93352
Tween-20: Fluka, 93773

Reagents

REAGENT 1. Rabbit CR 7679 antibody solution

- Tris/HCl pH 7.5 20 mmol/L
- NaCl 150 mmol/L
- Kathon MW/WT 0.2 ml/L
- Amaranth 10 mg/L
- **Added only before use:** rabbit CR 7697 antibody (1:5)

This antibody solution is used for sensitization of micro-well plate. Preparation: CR 7697 antibody is added ONLY directly before running the test at a concentration of 1:5! REAGENT 1 will be diluted at 1:1’000 in SOLUTION #3.1. -> end concentration of the antibody 1:5’000.
Stability: Kathon allows this solution to be stored at least 6 month at 2-8°C without significant loss of immunological activity

REAGENT 2. Mouse CR 6B3 antibody solution

- Tris/HCl pH 7.5 0.1 mol/L
- Bovine serum albumin (BSA) 5.0 g/L
- Phenol 1.0 g/L
- Kathon (MW/WT) 0.2 ml/L
- Evans Blue 10 mg/L
- **Added only before use:** mouse CR 6B3 antibody (1:10 – 1:20)

Preparation: monoclonal mouse CR 6B3 *antibody is added ONLY directly before running the test at a concentration of 1:10! REAGENT 2 will be diluted 1:500 in REAGENT 4 -> end concentration of the antibody 1:5’000.*
Stability: at least 6 months at 2-8°C

REAGENT 3. Calretinin standard solution

1000 ng/mL recombinant rat Calretinin in:

- Potassium-phosphate pH 6.5 0.1 mol/L
- Bovine serum albumin (BSA) 5.0 g/L
- Kathon MW/WT 0.2 ml/L

The recombinant Calretinin is ONLY added to the solution directly before use at a concentration of 1ug/ml!

Stability: at least 6 months at 2-8°C

REAGENT 4. Test dilution buffer

- Tris/HCl pH 7.5 0.2 mol/L
- Bovine serum albumin (BSA) 5.0 g/L
- Kathon MW/WT 0.2 ml/L
- Phenol 1.0 g/L

Preparation: add 6.05 g of Tris/HCl, 0.1 ml kathon MW/WT, 0.5 g phenol and 2.5 BSA in 400 ml ddH2O. Adjust pH to 7.5 with 4 N HCl, and complete the volume to 500 ml with ddH2O.

Stability: at least 6 months at 2-8°C

REAGENT 5. Tetramethylbenzidin-H2O2 solution (TMB)

- 3,3' ,5,5'-tetramethylenzidin 20 mmol/L
- Aceton 100 ml/L
- Ethanol 900 ml/L
- H2O2 50 mmol/L

Preparation: dissolve 240 mg TMB in 5 ml aceton. Further add 45 ml ethanol and then 300 µl of 30% H2O2 solution.

Stability: at least 6 month at 15-25°, in a well closed, light-protected glass container

REAGENT 6. Substrate buffer

- Potassium-citrate buffer pH 4.1 30 mmol/L
- Kathon MW/WT 0.2 ml/L

Preparation: dissolve 6.3 g citric acid monohydrate in about 800 ml ddH2O. Adjust pH to 4.1 with 4 N KOH. Complete the volume to 1000 ml with ddH2O and add 200 µl kathon MW/WT

Stability: Few months at 15-25°C

Other solutions

Sensitization buffer (SOLUTION #3.1.)

- NaHCO3 pH 9.5 100 mmol/L

Preparation: dissolve 4.2 g NaHCO3 in 400 ml ddH2O. Adjust pH to 9.5 with 4 N NaOH. Complete the volume to 500 ml with ddH2O

Stability: at least 6 months at 2-8°C

Blocking buffer (SOLUTION #3.2.)

- Tris/HCl pH 7.5 200 mmol/L
- Bovine serum albumin (BSA) 10 g/L
- Kathon MW/WT 0.2 ml/L

Preparation: dissolve 12.1 g of Tris-HCl and 0.1 ml Kathon MW/WT in 400 ml ddH2O. Adjust pH to 7.5 with 4 N HCl. Further add 5 g BSA. Complete the volume to 500 ml with ddH2O
Stability: at least 6 months at 2-8°C

Stop buffer (SOLUTION #3.3.)

- Sulfuric acid 1.0 mol/L

Preparation: add 28 ml sulfuric acid to 500 ml ddH2O

➔ *Warning: do not add water in acid*

Washing buffer (SOLUTION #3.4.)

- Tween-20 0.5 ml/L

Preparation: dilute 500 µl Tween-20 in 1000 ml ddH2O

Other materials

Micro-well plates: polystyrol, flat bottom, 96-wells, NUNC-immunoplate, MAXISORP, 4-42 404
Multichannel photometer: filter 450 nm

ELISA running

Sensitization of micro-well plate with rabbit CR 7679 antibody

20 ml sensitization solution is required per micro-well plate

a) A corresponding amount of antibody solution (REAGENT 1) is diluted 1:1000 in sensitization buffer (SOLUTION #3.1.): for example 20 µl REAGENT 1 in 20 ml SOLUTION #3.1.
   ➔ rabbit CR 7679 antibody (end concentration 1:5'000) in 100 mmol/L $NaHCO_3$ pH 9.5
b) Pipette 200 µl of this solution per well
c) Incubate 16-24 hrs at 15-25°C
d) Remove sensitization solution and wash 3x with ddH2O (300 µl/well)
e) Pipette 200 µl of blocking buffer (SOLUTION #3.2.) per well
f) Close the plate with parafilm and incubate at least one day at 15-25°C

The micro-well plates sensitized with CR 7679 antibody can be stored (under humidified conditions at 2-8°C) in the blocking buffer until 2 months without significant loss of immunological binding capacity

Preparation of solutions for standard curve and loading of samples

g) Dilute Calretinin standard solution 1000 ng/ml (REAGENT 3) in test dilution buffer (REAGENT 4)
   ➔ 1:200 for 5.0 ng/ml
   ➔ 1:400 for 2.5 ng/L

- ➔ 1:800 for 1.25 ng/L
- ➔ The null-value is given by the test dilution buffer alone

h) Prepare mouse CR 6B3 antibody test solution: Dilute mouse CR 6B3 antibody solution (REAGENT 2) 1:500 in test dilution buffer (REAGENT 4) (mouse CR 6B3 antibody end concentration 1:5'000)

5 ml of this preparation is required per micro-well plate (5 ml REAGENT 4 and 10 µl REAGENT 2)

i) Remove blocking buffer from the wells
j) Pipette 200 µl of standard curve solutions and samples (user-defined dilution with REAGENT 4) in duplicates in each well, and add 50 µl of CR 6B3 antibody test solution prepared in step h)
k) Close the plate and incubate it for 16-24 hrs at 2-8°C (possible to do it over the week end)
l) Empty the wells and wash 3x with washing buffer (SOLUTION #3.4.) (300µl/well)
m) Incubation with 200 µl of rabbit anti-mouse-HRP (DAKO P0260, 1:1000 in REAGENT 4) for 4-5 hrs at room temperature.
n) Empty the wells and wash 3x with washing buffer (SOLUTION #3.4.) (300µl/well)
o) Wash 2-3x with dd. H2O (300µl/well).

Enzyme reaction

p) Mix 1 volume of TMB-H2O2 solution (REAGENT 5) with 20 volumes of substrate buffer (REAGENT 6)

21 ml of this solution is required per micro-well plate

This solution should not be vortexed (could cause precipitation) and has to be prepared just before use (stability 1 hr at 15-25°C)

q) Pipette 200 µl of this TMB-H2O2 solution per well
r) Incubate 10 minutes at 15-25°C
s) Stop the reaction by adding 100 µl stop buffer (SOLUTION #3.3.)
t) Within 1 hr, analyze the plate with the photometer at 450 nm