GeneTel Laboratories LLC ***Leader in Proteomics Abs and Abs Arrays***
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A2 ELISA Kit Protocol

Received day: _____
Assay Date:_____
Scientist:_____

Description: To measure A2 beta casein level in milk samples using a direct ELISA kit. A2 beta casein standards (2.5ng/ml-160ng/ml) and diluted milk samples will be coated on the high binding plate and the A2 specific chicken pAbs will be used to detect the A2 beta casein. Rabbit anti-chicken IgY HRP conjugate will be used for the signal amplification. After color development, OD450 will be measured and A2 standard curve will be established. Concentration of A2 beta casein in tested sample will be estimated based on the standard curve. In general, milk sample need to be diluted to 1:200,000 to 1:500,000. For each plate, two columns will be used for standard curve establishment with the detecting range of 2.5ng/ml to 160ng/ml A2.

Materials provided in the kit:

A2 beta casein standard, 40ug/ml (40ng/ul), -20^{o}C
Chicken Anti-A2 beta casein specific pAbs, 0.3mg/ml, -20^{o}C
Rabbit Anti-Chicken IgY HRP conjugate, use at 1:1000 dilution, -20^{o}C
Binding Buffer for standard and milk samples: 100ml
10X TBS 100ml, add 900ml D.I. water to make 1000ml TBS
Blocking Buffer: 1.1 gram BSA, add 20.5ml TBS
Ab dilution buffer: 1% BSA in TBST: 1g BSA, add 100ml TBST
Tween-20: 1ml 50%, add into 900ml TBS to make TBST
Substrate TMB, 6ml
ELISA Plate, 96-well
ELISA Record Sheet

Materials customer needs and prepare:

ELISA Plate Washer and Plate Shaker
ELISA plate Reader
Pipettors and tips
HCl, 1M for stopping reaction in final step
NaOH, 0.5M for milk sample preparation

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ELISA plate Reader
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HCl, 1M for stopping reaction in final step
NaOH, 0.1M for milk sample preparation

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Buffer Preparation:
TBS: add the 100ml 10X TBS into 900ml D.I. water and mix well
Blocking buffer: Tube contains 1.1 gram BSA in TBS, add 20.5ml TBS
TBST: add one tube of provided 50% Tween-20 into the 900ml TBS
Ab dilution buffer: 1% BSA in TBST: 1g BSA in 100ml TBST.

Procedures:

1. Prepare A2 standard in seven 1.5ml tubes. In the first tube (tube#1), add 4ul of 40ug/ml A2 into 996ul Binding buffer to make 160ng/ml A2 standard. Add 500ul coating buffer into each of the rest 6 tubes and do a serial 2X dilution by taking out 500ul from the first tube (with 160ng/ml A2), so that tube#1 has 160ng/ml, tube#2: 80ng/ml, #3: 40ng/ml; #4: 20ng/ml; #5: 10ng/ml; #6: 5ng/ml; #7: 2.5ng/ml. Immediately transfer 100ul/well into ELISA plate after dilution.

2. Prepare the tested milk samples by diluting milk in Binding Buffer accordingly. In general, caseins are not soluble in neutral pH and thus milk sample needs to be diluted at 1:200 to 1:500 in 0.5M NaOH first, then, further dilute in Binding Buffer to obtain 1:200,000-1:500,000. Immediately apply the diluted samples in Binding Buffer to ELISA plate at 100ul/well. Keep the plate at room temperature for one hour and store the plate at 4 ^{o}C overnight to 3 days.

3. Empty coating solution from the wells and block with 200ul/well Blocking Buffer for one hour at room temperature. All the following steps will be performed at room temperature.

4. Empty blocking solution from the wells and wash 3X with TBST.

5. While washing the plate, prepare (10ml per plate) A2 specific detecting pAb (OA0102) in Ab dilution buffer at 1:500 dilution, ie, add 20ul A2 Detecting Abs into 10ml Ab dilution buffer.

6. Add the above prepared A2 detecting pAb at 100ul/well to the plate and shake for 2 hour at RT.

7. Wash 3X with TBST.

8. While washing the plate, prepare Rabbit Anti-Chicken IgY HRP (RCYHRP) conjugate (10ml per plate) solution: dilute the HRP conjugate to 1:1000 in Ab dilution buffer (10ul HRP conjugate into 10ml Ab dilution buffer, mix well). Add 100ul/well to the plate.

9. Mix the plate on the shaker for another 1 hour at RT.

10. Wash 5X with TBST.

11. Add 50ul/well HRP substrate (TMBS). Let the color to develop 3-5 minutes.

12. Stop the reaction with 50ul 1M HCl. and read OD450 immediately.

13. Record the development time on the ELISA recording sheet along with OD450 readout.

14. Make a standard curve using the average OD450 from the two reference standards for each point. Determine the A2 concentration for the tested samples based on their OD450 and standard curve. make sure that the final concentration is obtained by multiplying with dilution factor. Following are A2 standard curve example: color development time 4 minutes.