Bull 4MID® Kit : Bull proAKAP4

Reference: 4VDX-18K4

1. PRINCIPLE AND GENERAL DESCRIPTION

The Bull 4MID® kit (4VDX-18K4) is a quantitative sandwich ELISA assay to detect and quantify the biomarker proAKAP4 in bull sperm samples. Bull sperm samples that can be used include fresh, frozen or isolated bull spermatozoa. This kit is composed of a 96-well plate coated with a monoclonal antibody specific for proAKAP4. Protocols for sperm sample preparations are provided to be used with the Bull Spermatozoa Lysis Buffer before loading each sample on the 96-well plate of the Bull 4MID® kit. The proAKAP4 protein is recognized by the Capture Antibody coated onto the bottom of the 96-well plate and is then detected using a Detection Antibody covalently coupled to horseradish peroxidase. A Substrate Solution is added to each well and color levels appear proportionally to the concentration of the proAKAP4 present in each sperm sample. The color reaction is stopped by the Stop Solution and the color intensity is measured by spectrophotometry at 450 nm. A positive and a negative control is included in each Bull 4MID® kit. A Lyophilized Standard is provided to perform a reference curve enabling to determine the precise concentration of the proAKAP4 present in the sperm sample.

Please read carefully the user instructions before use

2. MATERIAL REQUIRED - NOT INCLUDED

Multichannel Micropipette of 200 µL.
Micropipette: 20 µL, 200 µL and 1000 µL and pipette tips.
Tubes: Polypropylene tubes for dilution.
Vortex mixer for preparation of samples.
Shaker: Horizontal orbital microplate shaker.
Microplate reader measuring absorbance at 450 nm.
Ultrapure or Double deionized water.

3. REAGENTS AND MATERIALS (INCLUDED)

R1 - Microplate: An ELISA plate of 96-wells (12x8 strips)
R2 - 1 vial of 10x Washing Buffer Solution
R3 - 1 vial of 1x Dilution Buffer
R4 - 1 Tube of Lyophilized Standard
R5 - 1 Vial of 1x Bull Spermatozoa Lysis Buffer
R6 - 1 Tube of Detection Antibody
R7 - 1 Vial of Substrate Solution
R8 - 1 Vial 1x Stop Solution
R9 - 1 Tube of Positive Control
R10 – 2 Plate Sealers

4. STORAGE INFORMATION

The Bull 4MID® kit should be stored at 4°C.

5. GENERAL INSTRUCTIONS OF USE

Before use, bring all reagents except the R6 vial at room temperature (RT). The R6 Detection Antibody vial should be kept at 4°C. Verify the absence of crystals in the R2 and the R5 vials. In the presence of crystals, gently agitate the solution until all crystals are completely dissolved.

A. Buffer and Standard Preparation for Assay

Prepare all solutions in clean recipients and use ultrapure or double deionized water.

1. Prepare the 1x Washing Buffer by a 10-fold dilution of the 10x concentrated R2 Washing Buffer Solution: add 30 mL of the R2 into 270 mL of deionized water. Gently agitate the solution and avoid foaming.

2. Rapidly centrifuge the R4 Lyophilized Standard before opening.

3. Carefully open the vial and reconstitute the R4 vial by adding 1 mL of R3 to obtain a stock solution of Standard.

4. Prepare the highest concentration of the Standard by pipetting 10 µL of the reconstituted Standard Solution into 1990 µL of R3 Dilution Buffer. Change the pipette tip at each following step.

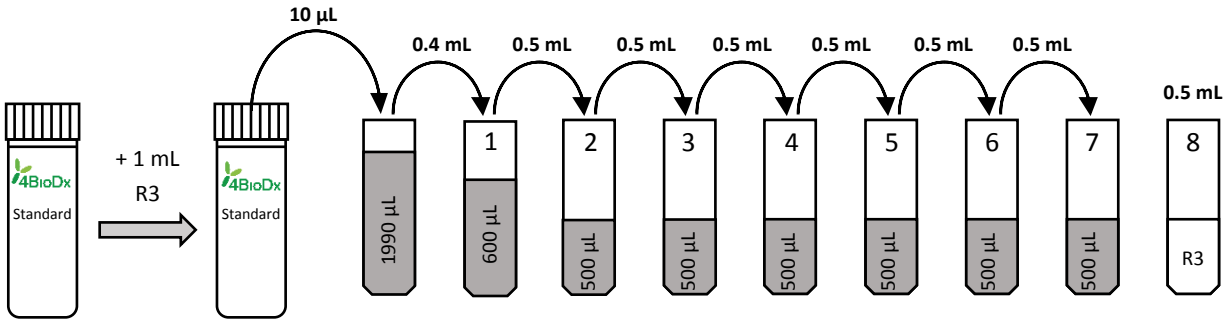
5. Add then 400 µL of the highest concentration of Standard to 600 µL of R3 (tube n°1).

5. You need at that point, to add 0.5 mL of R3 Dilution Buffer into 6 new tubes that we called tube n°2 to tube n°8 (always use new polypropylene tubes).

6. Perform then a serial dilution by pipetting 500 µL of tube n°2 and adding it to the third tube already containing 500 µL of Buffer R3 (2-fold dilution). Mix thoroughly.

7. Then take 500 µL from the tube n°3 and add it to tube n°4. Mix carefully. Reproduce until the tube n°7.

Remark: Change the pipette tip at each step of the process.



Through serial dilution the final proAKAP4 quantity will be of 60, 30, 15, 7.5, 3.25, 1.62, 0.81 and 0 ng of proAKAP4 in the wells of the 96-wells plate used for the standard curve. These values are used to perform the standard curve.

Standard	1	2	3	4	5	6	7	8
ng of proAKAP4 in each well	60	30	15	7.5	3.25	1.62	0.81	0

Please note that tube n°8 is the **negative control** of the assay.

B. Semen Sample Preparation and Dilution

For the assay, proAKAP4 biomarker should be first extract from spermatozoa flagellum by using a specific R5 Bull Spermatozoa Lysis Buffer.
Never put sperm lysed samples on ice.
Do not forget before pipetting any semen sample (fresh ejaculate or diluted semen) to resuspend cells by gentle shaking of the tube containing the semen, as spermatozoa cells will pellet by gravity.

Fresh Ejaculate:

1. In a 0.5 mL conic tube add 100 µL of R5 Bull Spermatozoa Lysis Buffer.
2. Resuspend spermatozoa cells in the semen by gently shaking the semen before pipetting for homogenizing the sample.
3. Add 25 µL of semen to the R5 Bull Spermatozoa Lysis Buffer to reach a volume of 125 µL.
4. Carefully Vortex precisely during 1 min at maximum speed.
5. Add 125 µL of R3 Dilution Buffer.
6. Vortex 2 min at maximum speed.
7. Keep at ambient temperature (17°C – 25°C) until use

Remark: For storage longer than 1 hour, please store at -20°C (for up to one week).

Frozen semen:
Unfroze the semen sample as usual, resuspend spermatozoa by gently mixing the semen and proceed as for fresh ejaculate.

Frozen semen:

1. In a 0.5 mL conic tube add 150 µL of R5 Bull Spermatozoa Lysis Buffer.
2. Resuspend spermatozoa cells in the semen by gently shaking the semen before pipetting for homogenizing the sample.
3. Add 50 µL of Semen to the R5 Bull Spermatozoa Lysis Buffer to reach a volume of 200 µL.
4. Carefully Vortex precisely during 1 min at maximum speed.
5. Add 200 µL of R3 Dilution Buffer.
6. Vortex 2 min at maximum speed.
7. Keep at ambient temperature (17°C – 25°C) until use

Remark: For storage longer than 1 hour, please store at -20°C (for up to one week).

C. ELISA PROTOCOL

1. Open carefully the reusable aluminum foiled package containing the R1 96-well Plate using scissor.
2. Add 100 µL from the highest to the lowest concentrations of the Standard Dilution (tube 1 to tube 8) to establish the standard curve on the first two columns (from A1, A2 to H1, H2) of the R1 96-well plate, in two wells, for duplicate.
3. Add then 100 µL directly of the R9 positive control in duplicate (A3, A4).
4. Add 100 µL of each semen sample as prepared above, always in duplicate (from B3, B4 to H3, H4 and the following columns until H11, H12).
5. Cover the R1 plate with one of the plate sealers and incubate for 1h30 at room temperature on a shaker with gentle agitation (250 rpm).
6. Eliminate the samples before adding the washing buffer.
7. Wash each well by adding 300 µL of R2 Washing Buffer 1x solution. Then discard the washing solution. Repeat two times more. Please tap down gently the microplate on an absorbent dry paper to remove residual washing buffer droplet between each washing step and before loading the R6 detection antibody.

Remark: The results of the assay are markedly influenced by the proper performance of washing.

8. Dilute the 60 µL of R6 Detection Antibody in the 12 mL of R3 Dilution Buffer Vial.
9. Add 100 µL of Detection Antibody solution prepared in step 7 each well of the 96-well plate.
10. Cover the plate with a new plate sealer and incubate for 30 min at RT with gentle agitation (250 rpm).
11. Eliminate the Antibody solution before adding the washing buffer.
12. Wash each well by adding 300 µL of R2 Washing Buffer 1x solution. Then discard the washing solution. Repeat two times more. Please tap down gently the microplate on an absorbent dry paper to remove residual washing buffer droplet between each washing step and before adding the R7 Substrate.
13. Add 100 µL of R7 Substrate Solution to each well (keep carefully away from light by protecting with an aluminum sheet).
14. Protect from light and incubate under gentle agitation (300 rpm) for 30 minutes at RT.
15. Add 50 µL of R8 Stop Solution to each well and mix 2 min at 200 rpm before reading the plate.
16. Determine the optical density using a microplate reader set to 450 nm. Always perform the measure a maximum 5 min after adding the STOP solution.

D. Calculation of Results – ProAKAP4 Concentration Determination

1. Average the duplicate optical density measures for each Standard. Then subtract optical density of zero point of Standard dilution to the optical density of each optical density of Standard or of Samples.
2. Create a standard curve by reducing the data using a computer software generating a two-degree polynomial regression equation expressing the optical densities in function the proAKAP4 quantities in nanogram. If Semen have been diluted, the concentration calculated from the standard curve must be adjusted by multiplying the values by the dilution factor. **A template can be provided on demand.**
3. To express the proAKAP4 concentration in ng / µL, calculate the concentration using the two-degree polynomial regression equation.
4. If the spermatozoa concentration is provided in million / mL, divide the results of the concentration of proAKAP4 by the number of million and then multiply by one thoursand.

6. PRACTICAL ADVICES AND CAUTIONS

- Wear protection glasses and follow the good laboratory practice.
- Use the whole reagents before the expiration limit.
- The Substrate Solution can be irritating for the skin.
- The Stop Solution can be harmful in case of ingestion and could lead when in contact with the skin to irritation. Please avoid contact with skin.
- Do not expose the substrate solution to light nor to oxidative substances.
- Observe all federal, state, and local regulations for disposal.
- The user should calculate the possible amount of the samples used in the whole test. Please make sure that sufficient samples are available.
- The 4MID® kit cannot assay the samples which contain sodium azide (NaN3), because NaN3 will inhibit the activity of horseradish peroxidase (HRP).
- Please place the unused wells to the foil pouch containing the desiccant pack and reseal with tape. All remaining reagents need to be stored at 2°C - 8°C.
- Protect all reagents from strong light during storage and incubation.
- All bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism.

- Any variation in ambient temperature, pipetting, washing method, incubation time can cause variation in results. Each user should obtain his own standard curve.

REFERENCES

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- **Moss et al. (1999)** Biology of Reproduction. Vol.61: 335-34

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