various antigens (hAQP2/Trx, hAQP2/Trx/Sf-9, and Trx/His control) by Western blotting in Reference Production Example 3.

[FIG. 8] FIG. 8 shows the results of examination of the reactivity of the obtained polyclonal antibodies (OPM21401, OPM 21402) and the control serum (NMS: normal mouse serum) with various antigens (hAQP2/Trx, hAQP2/Trx/Sf-9, and Trx/His control) by Western blotting in Reference Production Example 4.

[FIG. 9] FIG. 9 shows the results of examination of the conditions that do not affect the assay system and under which AQP2 is solubilized from the membrane by using various solubilizers (Urea, Triton X-100, NP-40, Tween-20, guanidine hydrochloride (GuCl), and SDS) in Experimental Example 1(1) (1-1).

[FIG. 10] FIG. 10 shows the results of examination of stabilizers (Urea, Triton X-100, NP-40, Tween-20, guanidine hydrochloride (GuCl), and SDS) added to the first buffer (0.2% BSA/10 mM EDTA/0.2 M Tris-HCl (pH 8.0)) in Experimental Example 1 (1) (1-2).

[FIG. 11] FIG. 11 shows standard curves obtained by performing ELISA using the monoclonal antibody (OPM21401) and using, as standard reference proteins, "Trx fusion AQP2 protein (AQP2/Trx)" produced by the method described in Reference Production Example 1 and "AQP2/Sf9" produced by the method described in Reference Production Example 2 (Experimental Example 1 (1) (1-4)). The dilution ratio shown on the upper abscissa indicates that a stock solution (AQP2/Sf9 lysate) prepared by suspending AQP2/Sf9 in PBS and solubilizing the suspended AQP2/Sf9 with an equal volume of solubilizer was diluted 2K-fold to 1458K-fold (i.e., 2 to 1458 x 1000 times).

[FIG. 12] FIG. 12 shows the results of ELISA using serially diluted 4 urine samples of healthy human volunteers (Nos. 4, 5, 6, and 9) as test samples, versus a standard curve of the standard protein (Trx fusion AQP2 protein) (Experimental Example 1 (1), (1-5)).

[FIG. 13] FIG. 13 (A) shows the results of measuring the amount of human AQP2 in urine samples of 10 healthy human volunteers using the monoclonal antibody (OPM21401) against human AQP2

[0021]

Such biological samples may be freshly obtained, or may be held for a certain period after collection, i.e., collected and stored for a certain period of time, as long as quality degradation or deterioration, such as inactivation of the protein to be measured, does not occur.

[0022]

The method of collecting a biological sample is not particularly limited, insofar as the method does not affect the target protein, preferably membrane protein, contained in the biological sample.

[0023]

Further, the collected biological sample can be subjected to the method of the present invention as is. Alternatively, a portion containing the target protein may be extracted or fractionated from the collected biological sample, and the extract may be subjected to the method of the present invention.

[0024]

The target protein of the present invention is not particularly limited insofar as it is a protein contained in animals or plants as mentioned above. However, the target protein is preferably a membrane protein. The membrane protein is a protein that exists on a cell membrane or a vesicle membrane. Examples of membrane proteins include, but are not limited to, membrane transport proteins, Na^+/K^+-pumps, and ion channels. For example, the membrane protein is preferably aquaporin (Aquaporin:AQP), particularly preferably AQP2 that is one of the 13 types of human AQPs (see "Cell Membrane Water Channel, Aquaporin" The Medical Association of Nippon Medical School, 2009; 5 (2)).

[0025]

AQP is one of the major membrane proteins that belong to the MIP (major intrinsic proteins) family (Agre P (2006), "The aquaporin water channels", Proc Am Thorac Soc 3 (1): 5-13; Agre P, et al., (1993), "Aquaporin CHIP: the archetypal molecular water channel" Am. J. Physiol. 265: (4 Pt 2) F463-76). Aquaporin exists

a method of allowing a frozen biological sample in a container to stand at room temperature, a method of applying running water, a method of immersion in water, and a method of gradual thawing on ice.

[0033]

The freezing and thawing treatments described above in (1) and (2) may be performed once, or repeated more than once. In view of treatment efficiency, each of the freezing and thawing treatments is usually performed once. However, as described in the Experimental Example described below, repetition of the freezing and thawing treatments does not significantly deteriorate immunoassay results, and appears not to adversely affect the protein to be measured. Therefore, repetitively performing freezing and thawing multiple times is not restricted. Accordingly, in a case where it is unknown whether the freezing treatment has been done, for example, when it cannot be confirmed whether the biological sample to be subjected to immunoassay has been frozen at a temperature higher than -80°C, in particular at -70°C or higher, freezing and thawing may be performed again.

[0034]

Alternatively, when a biological sample previously subjected to step (1) is available (for example, when a sample subjected to step (1) by a third party is obtained), the present invention can be carried out by performing the following step (2') instead of steps (1) and (2):

(2') thawing a biological sample frozen at a temperature higher than -80°C, in particular at -70°C or higher.

[0035]

Thus, in another aspect, the present invention provides a method for pretreatment of a biological sample comprising the above thawing step (2') and solubilization step (3). As the freezing temperature and freezing time in step (2'), the same conditions as mentioned above in paragraphs [0029] and [0030] may be used.

[0036]

(3) Solubilization step

invention, particularly urine, and that determines the amount of a protein contained in the biological sample, for example, a membrane protein such as aquaporin (AQP), preferably human AQP2, as shown in the Experimental Examples.

[0048]

ELISA is preferably used as the immunoassay. For example, the antibody to AQP used in ELISA can be produced by a standard method, or with reference to Reference Production Examples 2 and 3 described below. The ELISA using urine as a test sample can be performed according to the protocol described in Experimental Example 1 described below. As described in Experimental Example 2, the pretreatment of the present invention enables highly accurate and efficient detection of the amount of a membrane protein, such as AQP, contained in urine.

[Examples]

[0049]

Hereinafter, a configuration of the present invention and effects thereof will be described based on Reference Production Examples and Experimental Examples. However, the present invention is not limited to any degree by such Experimental Examples and the like.

[0050]

Reference Production Example 1 Production of *E. coli* expressed Trx/His fused human AQP2

Human aquaporin 2 (AQP2) gene was cloned and expressed in *E. coli*. Human AQP2 protein is a five-transmembrane protein that is composed of 271 amino acids, and whose protein domain has a molecular weight of about 30 kD (Sasaki S, et al., Cloning, characterization, and chromosomal mapping of human aquaporin of collecting duct. Clin. Invest.. 1994; 93(3): 1250-1256.). Here, human AQP2 protein was expressed in *E. coli* in a state of having its N-terminal tagged with Trx.

[0051]

The method is summarized as follows.

[0052]

[0080]

FIG. 6 shows the results of ELISA assays performed in a similar manner after the whole blood collection performed after the seventh immunization. In FIG. 6, (A) shows antibody titers against the immunogen (hAQP2/Trx), and (B) shows antibody titers against the Sf-9 cell expressed hAQP2 (hAQP2/Trx/Sf-9). The respective polyclonal antibodies obtained at this time were named OPP21401, OPP21402, OPP21403, and OPP21404. The volumes of the antisera were 50 mL, 52 mL, 50 mL, and 68 mL, respectively. Further, final antibody titers were confirmed by ELISA, and it was found that OPP21403 showed the highest antibody titer.

[0081]

Further, the reactivity of each polyclonal antibody against each type of antigen (hAQP2/Trx, hAQP2/Trx/Sf-9, and Trx/His control) was examined by Western blotting. Specifically, each antigen (hAQP2/Trx, hAQP2/Trx/Sf-9, and Trx/His control) transferred to a nitrocellulose membrane was allowed to react with each antiserum diluted 1,000-fold for coloring, using a 4-chloro-1-naphthol method. FIG. 7 shows the results. As shown in FIG. 7, all antibodies (OPP21401, OPP21402, OPP21403, and OPP21404) reacted not only to hAQP2/Trx but also hAQP2/His/Sf-9. OPP21403 had the strongest reaction with hAQP2/His/Sf-9.

[0082]

From this, among the four types of polyclonal antibodies obtained above, it was considered that OPP21403, which had the strongest reaction with hAQP2 ~~been~~ expressed in Sf-9 cells ~~and which had the strongest reaction with hAQP2~~, was appropriate for detection of AQP2 in a living body-derived sample.

[0083]

solution (hybridoma culture solution). About 40 mL of this solution was transferred to a 50 mL centrifugation tube, and centrifuged at room temperature at 1200 rpm for 5 minutes to collect the cells (hybridoma). The cells were suspended in 3 mL of a cell bunker (JUJI FIELD, Cat. No. BLC-1), and the suspension was dispensed into three cryotubes by 1 mL and preserved in liquid nitrogen. A portion of the centrifuged supernatant was preserved at 4°C and subjected to antibody typing and Western blotting.

[0097]

(1-5) Ascitic fluid collection

In order to obtain a purified antibody to be used for establishing an ELISA assay system, the hybridoma obtained above was injected into mice and ascites containing large amount of antibodies was collected.

[0098]

Specifically, about 10 mL of the cell culture solution (hybridoma culture solution) prepared in (3-4) was centrifuged at room temperature at 1200 rpm for 5 minutes, to collect the cells (hybridoma). The cells washed once with PBS(-) were re-suspended in 1.5 mL of PBS(-), and 0.5 mL aliquots of the suspension were injected into the peritoneal cavities of three male mice. Each of the mice had been intraperitoneally injected in advance with ~~pristine~~ pristane (2, 6, 10, 14-tetramethylpentadecan; Sigma, Cat. No. T7640) by 0.5 mL/body at least 3 days before the cell injection.

[0099]

Ascites were collected when the sizes of the abdomens of the mice injected with the cells were at a maximum. Ascites of a single mouse was taken sterilely, and was centrifuged at room temperature at 1200 rpm for 5 minutes to collect a supernatant. Then, red blood cells in the precipitate were hemolyzed using 0.747% NH_4Cl, as in the feeder cell preparation. The cells were washed with RPMI 1640, and then suspended in 3 mL of a cell bunker. The suspension was dispensed into three cryotubes by 1 mL, to be preserved in liquid nitrogen. Ascites of the other two mice