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(54) **SIMULTANEOUOS QUANTIFICATION OF PIG-A ASSOCIATED PROTEINS IN RED CELLS, PLATELETS AND LEUKOCYTE SUBSETS USING A SINGLE MEASUREMENT**

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(57) **ABSTRACT**

A single measurement can quantify phosphatidyl-inositol glycan complementation class A (PIG-A) associated proteins in red cells, platelets and the major leukocyte subsets present in blood (lymphocytes, monocytes, neutrophils and eosinophils). The single measurement relies on flow cytometry to simultaneously evaluate intensity of fluorochrome emissions and light scattering properties.

# SIMULTANEOUOS QUANTIFICATION OF PIG-A ASSOCIATED PROTEINS IN RED CELLS, PLATELETS AND LEUKOCYTE SUBSETS USING A SINGLE MEASUREMENT

**[0001]** The present invention relates generally to a procedure for the simultaneous quantification of at least two different phosphatidyl-inositol glycan complementation class A (PIG-A) associated proteins in red cells, platelets and major leukocyte subsets (including lymphocytes, monocytes, neutrophils and eosinphils) present in blood, using a single measurement.

**[0002]** The present invention may be understood more readily by reference to the following detailed description of particular embodiments of the invention and specific examples. The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

**[0003]** The present invention allows for testing of both normal and pathological samples obtained ex vivo or in vivo, including stored or treated samples. Monoclonal antibodies specific to particular proteins associated with PIG-A are used. For example, the following monoclonal antibodies known in the art can be used to practice the present invention: Anti-CD14, Anti-CD16, Anti-CD24, Anti-CD41, Anti-CD42, Anti-CD45, Anti-CD48, Anti-CD52, Anti-CD55, CD87, Anti-CD90, Anti-CD108, Anti-CD109, and Anti-CD157. A common characteristic of these monoclonal antibodies is their ability to identify peripheral blood subtypes of cells, such as CD41, CD42, CD45, CD61, and CD64, or that they are PIG-A anchored surface proteins. One source for further information in this regard is *Immunobiology: The immune system in health and disease*, Appendix I, Janeway C. A., Travers P., Walport M., and Capra J. D. (Eds.), Elserior Science Ltd./Garland Publishing, 1999.

**[0004]** The monoclonal antibodies are chemically coupled to fluorochromes to permit visualization and quantification of different cell types. This coupling does not alter the binding capacity of the antibodies. When activated by illumination with light of a particular wavelength, the fluorochrome-labeled antibody adsorbs the light energy, inducing a state of excitability followed by emission of light at a characteristic color.

**[0005]** In order to specifically identify antibody binding to cells, any compatible combination of fluorochromes can be used as well as any monoclonal antibody within each cluster of differentiation. For example, FL1 fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (PerCP) and allophycocyanin (APC). Another example of suitable combinations of fluorochromes or fluorochrome tandems is the combination of FITC, PE, PerCP and APC-Cyanin7 (Cy7). Further examples include: FITC, PE, PerCP-Cy5 and APC; or ITC, PE, PE-Cy5 and APC, or FITC, PE, PE-Cy, PE-texas red; or FITC, PE, PE-Cy5 and APC-Cy7. The fluorochromes employed in the practice of the present invention are commercially available and understood by those of skill in the art.

**[0006]** In a first embodiment of the present invention, a blood sample is incubated with a mixture of monoclonal antibodies and fluorochromes as follows:

| Monoclonal Antibody Number | Antibody | Fluorochrome |
|---|---|---|
| 1 | Anti-CD45 | 1 |
| 2 | Anti-CD61 or Anti-CD41 or Anti-CD42 | 1 |
| 3 | Anti-CD64 | 2 |
| 4 | Anti-CD55 | 3 |
| 5 | Anti-CD59 | 4 |

**[0007]** The full complement of monoclonal antibodies allows for a complete analysis of the blood sample. Monoclonal antibodies 1-3 allow for identification of red cells, platelets, and the major leukocyte subsets in the blood sample. Monoclonal antibodies 4 and 5 allow for quantification of each cell subset for the two different PIG-A associated proteins.

**[0008]** A patient's blood sample may be collected in a glass or plastic tube containing an anticoagulant such as EDTA, heparin or ACD. Any reasonable volume may be used, such as from 1-200 □l, the concentration of other reagents to be determined based on the volume of the sample and the saturation conditions of the reagents. The sample should be used as soon as possible, if stored it may be placed at room temperature (approximately 22° C.) or refrigerated (approximately 4° C.).

**[0009]** The antibody coupled to fluorochrome reagents can be purchased from a number of commercial sources. Upon addition of this reagent to the blood sample, incubation of the sample follows for approximately 30 minutes at room temperature, or for approximately 30-60 minutes under refrigeration.

**[0010]** Fluorescence is measured in a flow cytometer. The scale used for each measured parameter is typically a 4-logarithmic decades scale. Results are expressed in relative linear units scaled from 0 to $10^4$. The techniques and equipment utilized for fluorescence as well as light scattering measurements are well established in the art. The resultant information can De stored in digital format, making it easy to analyze several parameters for a single cell through the use of specialized software programs.

**[0011]** The results of the data indicate which types of cells are present in a sample based on particular characteristics. For example, red cells are identified as being CD45− and CD61− (or CD41− or CD42−) and displaying a high forward scatter (FSC) and sideward light scatter (SSC). Platelets show low FSC and SSC values and they are CD61+(or CD41+or CD42+). Leukocytes appear as high FSC and CD45+: from them lymphocytes show higher CD45+ values and they are CD64−; monocytes are CD64++ at very high levels, they show intermediate SSC characteristics as compared to neutrophils and eosinophils these latter cells being CD64+ with high SSC and CD64− with very high SSC values, respectively. Once these cell populations are identified, expression of PIG-A associated proteins is evaluated on each subset. The evaluation is based on both the presence or absence and the mean fluorescence intensity of unimodal or multimodal populations.

[0012] In a second embodiment of the present invention, a blood sample is incubated with a mixture of monoclonal antibodies and fluorochromes as follows:

| Monoclonal Antibody Number | Antibody | Fluorochrome |
|---|---|---|
| 1 | Anti-CD45 | 2 |
| 2 | Anti-CD61 or | 1 |
| Anti-CD41or | | |
| Anti-CD42 | | |
| 3 | Anti-CD64 | 1 |
| 4 | Anti-CD55 | 3 |
| 5 | Anti-CD59 | 4 |

[0013] In further embodiments of the present invention, all potential combinations of antibodies may be used. For example, CD55 and/or CD59 may be replaced by other antigens, including, but not limited to, CD14, CD16, CD24, CD48, CD52, CD58, CD66bce, CD73, CD87, CD90, CD108, CD109, and CD157.

[0014] It is also possible to follow the teaching of the present invention but to rely on only two or three different fluorochromes. This procedure may be used for analysis of a single PIG-A protein, or when quantification and analysis of a reduced number of blood cell types within a sample is necessary.

What is claimed is:

**1**. A method for quantifying expression of two or more PIG-A associated protein expression levels in different cell populations present in a blood sample including red cells, platelets, lymphocytes, monocytes, neutrophils and eosinophils, comprising the steps of:

incubating the blood sample with a mixture of fluorochrome labeled monoclonal antibodies;

measuring the intensity of fluorochrome emissions in said incubated blood sample;

measuring the light scattering properties in said incubated blood sample; and

quantifying PIG-A protein expression levels in different cell populations from the blood sample, including red cells, platelets, lymphocytes, monocytes, neutrophils and eosinophils,

wherein said mixture of fluorochrome labeled monoclonal antibodies contains at least two different fluorochromes, and

wherein said mixture of fluorochrome labeled monoclonal antibodies contains at least four different monoclonal antibodies.

**2**. The method of claim 1, wherein said fluorochromes or fluorochrome tandems are selected from the group consisting of FITC, PE, PerCP, PE Cy5, PE Cy7, APC, PE-texas red and APC-Cy7.

**3**. The method of claim 1, wherein said monoclonal antibodies are selected from the group consisting of: Anti-CD14, Anti-CD16, Anti-CD24, Anti-CD41, Anti-CD42, Anti-CD45, Anti-CD48, Anti-CD52, Anti-CD55, Anti-CD58, Anti-CD59, Anti-CD61, Anti-CD64, Anti-CD66bcde, Anti-CD73, Anti-CD87, Anti-CD90, Anti-CD108, Anti-CD109 and Anti-CD157.

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| 专利名称(译) | 使用单次测量同时定量红细胞，血小板和白细胞亚群中的PIG-A相关蛋白 | | |
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| 其他公开文献 | US7358059 | | |
| 外部链接 | Espacenet USPTO | | |

摘要(译)
单次测量可以量化红细胞，血小板和血液中存在的主要白细胞亚群（淋巴细胞，单核细胞，嗜中性粒细胞和嗜酸性粒细胞）中的磷脂酰肌醇聚糖A型（PIG-A）相关蛋白。单次测量依赖于流式细胞术来同时评估荧光染料发射的强度和光散射性质。