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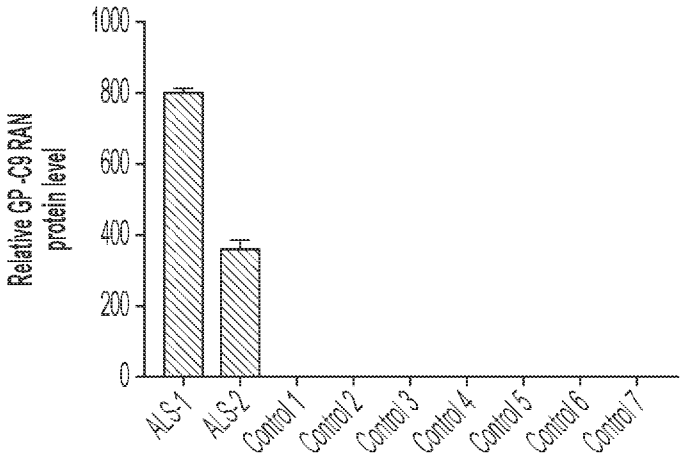

Figure 5

(57) Abstract: Aspects of the disclosure relate to methods and compositions (e.g., kits) for detecting repeat-associated non-ATG (RAN) proteins in a subject (e.g., a subject having or suspected of having a disease associated with RAN protein translation, for example amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia (FTD), or spinocerebellar ataxia type 36 (SCA36), or other diseases that produce poly(PR), poly(GR) or poly(GP) RAN proteins. In some embodiments, methods described by the disclosure comprise detecting one or more RAN proteins in a biological sample obtained from a subject by an electrochemiluminescence-based immunoassay using one or more anti-RAN protein antibodies. In some embodiments, the disclosure relates to kits comprising one or more anti-RAN antibodies and an electrochemiluminescence-based immunoassay plate and/or reagents.

WO 2019/060918 A1

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IMMUNOASSAYS FOR DETECTION OF RAN PROTEINS

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Application Serial No. 62/563,009, filed September 25, 2018, entitled "IMMUNOASSAYS FOR DETECTION OF RAN PROTEINS", the entire contents of which are incorporated herein by reference.

BACKGROUND

Expansion of a GGGGCC hexanucleotide sequence within the intron of the human *C9ORF72* gene is associated with both amyotrophic lateral sclerosis and frontotemporal dementia in humans. Amyotrophic lateral sclerosis (ALS) is a debilitating disease with varied etiology characterized by rapidly progressing weakness, muscle atrophy, muscle spasticity, difficulty speaking (dysarthria), difficulty swallowing (dysphagia), and difficulty breathing (dyspnea). Although the order and rate of symptoms varies from person to person, eventually most subjects are not able to walk, get out of bed on their own, or use their hands and arms. Most subjects with ALS will eventually die from respiratory failure, usually within three to five years from the onset of symptoms. Currently available treatments for ALS are limited. Frontotemporal dementia (FTD) is also a devastating group of disorders resulting from atrophy or shrinkage of the frontal and temporal lobes of the brain. This shrinkage or atrophy results in severe behavioral changes. There is currently no cure for FTD and limited medications for managing the symptoms of FTD. New methods for diagnosing and treating ALS and/or FTD would greatly benefit ALS and FTD subjects.

SUMMARY

Aspects of the disclosure relate to methods and kits for detecting certain biomarkers associated with genomic nucleotide expansions (*e.g.*, associated with one or more disease symptoms, for example associated with a neurological disease or condition). In some embodiments, an immunoassay (*e.g.*, an electrochemiluminescence-based immunoassay) is used to detect or measure levels of one or more repeat associated non-ATG translation proteins in a blood sample obtained from a subject. In some embodiments, methods and kits are provided for detecting certain biomarkers (*e.g.*, pharmacodynamic biomarkers, pharmacokinetic biomarkers, *etc.*) associated with amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia (FTD),

or spinocerebellar ataxia type 36 (SCA36) and methods of treating such diseases based on the same. The disclosure is based, in part, on immunoassays that are capable of sensitively measuring levels of one or more repeat associated non-ATG translation proteins (also referred to as RAN proteins) from cells or tissues of subjects, *e.g.*, subjects having or suspected of having ALS/FTD, or subjects having or suspected of having SCA36. In some aspects, methods described by the disclosure are useful for monitoring (*e.g.*, longitudinally measuring) levels of one or more RAN protein in a subject who has been or is being administered one or more therapeutic agents for treatment of a disease or disorder associated with RAN protein expression, such as ALS, FTD, or SCA36.

In some aspects, the disclosure provides a method comprising detecting in a biological sample (*e.g.*, a blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay. In some embodiments, an electrochemiluminescence-based immunoassay is a Meso Scale Detection (MSD) assay.

In some embodiments, a biological sample is a blood sample or a tissue sample. In some embodiments, a tissue sample is a CNS tissue sample. In some embodiments, a subject is a mammalian subject. In some embodiments, a subject is a human or a mouse. In some embodiments, a subject is a C9-BAC mouse.

In some embodiments, a subject is characterized by: a GGGGCC (G_4C_2) hexanucleotide sequence repeat expansion in the C9ORF72 gene; or a TGGGCC hexanucleotide sequence repeat expansion in the *SCA36* gene; or other neurologic diseases that express poly(GP), poly(GR), or poly(PR) proteins. In some embodiments, a hexanucleotide sequence repeat expansion comprises at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 200, at least 500, at least 1000, or at least 5000 G_4C_2 repeat expansions, or at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 200, at least 500, at least 1000, or at least 5000 TGGGCC repeat expansions. In some embodiments, a nucleic acid sequence comprising in a repeat expansion that encodes a RAN protein (*e.g.*, a homopolymeric RAN protein, a di-amino acid repeat-containing RAN protein, *etc.*) of at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 200, at least 500, at least 1000, or at least 5000 polymer units.

In some embodiments, one or more RAN proteins detected in a sample (*e.g.*, a biological sample) is selected from poly(GP), poly(GR), poly(PR), and poly(PA). In some embodiments, two, three or four RAN proteins are detected in a sample.

In some embodiments, an electrochemiluminescence-based immunoassay comprises a step of contacting the sample with one or more anti-RAN protein antibodies. In some embodiments, one or more anti-RAN antibodies is selected from an anti-poly(GP) antibody, anti-poly(GR) antibody, anti-poly(PR) antibody, and anti-poly(PA) antibody.

In some embodiments, an anti-RAN antibody binds to the di-amino acid repeat region of a RAN protein, for example a is poly(GP), poly(GR), poly(PR), or poly(PA) di-amino acid repeat region.

In some embodiments, an anti-RAN antibody binds to a C-terminal portion of a RAN protein, for example the C-terminal region of a RAN protein comprising a poly(GP), poly(GR), poly(PR), or poly(PA) di-amino acid repeat region.

In some embodiments, one or more anti-RAN protein antibodies is a polyclonal antibody. In some embodiments, one or more anti-RAN protein antibodies is a monoclonal antibody.

In some embodiments, methods of the disclosure further comprise administering a therapeutic agent to the subject if the level of RAN proteins detected in the biological sample is elevated compared to a level of RAN proteins detected in a control sample (*e.g.*, a biological sample obtained from a subject that does not have a repeat expansion in their C9ORF72 gene).

In some embodiments, methods of the disclosure further comprise obtaining a second biological sample from the subject after administration of a therapeutic agent and detecting one or more RAN proteins in the sample using an electrochemiluminescence-based immunoassay.

In some aspects, the disclosure provides a method for measuring pharmacokinetic changes in RAN protein expression, the method comprising: detecting in a first biological sample (*e.g.*, blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay; detecting in a second biological sample (*e.g.*, blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay, wherein the second biological sample is obtained after administration of a therapeutic agent to the subject; and determining that administration of the therapeutic agent to the subject results in a change (*e.g.*, a reduction or inhibition) in one or more RAN protein levels in the subject if the amount of RAN proteins

detected in the second biological sample is less than the amount of RAN proteins detected in the first biological sample.

In some aspects, the disclosure provides a kit comprising: one or more anti-RAN protein antibodies; and an electrochemiluminescence-based immunoassay plate and/or reagents.

In some embodiments, one or more anti-RAN protein antibodies are selected from anti-poly(GP) antibody, anti-poly(GR) antibody, anti-poly(PR) antibody, and anti-poly(PA) antibody. In some embodiments, the anti-RAN protein antibodies are monoclonal antibodies or polyclonal antibodies.

In some embodiments, an electrochemiluminescence-based immunoassay plate and/or reagents is a MSD assay plate and/or MSD assay reagents.

BRIEF DESCRIPTION OF DRAWINGS

FIGs. 1A-1D show standard curves for detection of RAN proteins using a meso scale detection (MSD) assay. FIG. 1A shows a standard curve for detection of poly(GP) RAN protein. FIG. 1B shows a standard curve for detection of poly(GR) RAN protein. FIG. 1C shows a standard curve for detection of poly(PR) RAN protein. FIG. 1D shows a standard curve for detection of poly(PA) RAN protein.

FIGs. 2A-2B show poly(GP) quantitative analysis using MSD. FIG. 2A shows data from an MSD assay measuring poly(GP) RAN protein level from a brain tissue soluble fraction obtained from a C9Orf72 mouse model (*e.g.*, C9-BAC mouse model) of ALS/FTD. FCX = frontal cortex; CE = cerebellum; error bars represent standard deviation (SD); n=7 for C9(+), 3 for WT. FIG. 2B shows poly(GP) RAN protein level in soluble fractions obtained from transfected and control HEK293T cells. Error bars represent SD; n=3 for each group.

FIGs. 3A-3B show MSD assay detects pharmacokinetic decrease in poly(GP) RAN proteins in C9-BAC mice. FIG. 3A shows a schematic depiction of AAV-EGFP and AAV-PKR(K296R) expression constructs. FIG. 3B shows data indicating a pharmacokinetic decrease in poly(GP) RAN protein in mice treated with a drug that targets the protein kinase R (PKR) pathway and blocks RAN translation.

FIG. 4 shows MSD assay detection of C9 RAN protein levels in soluble fractions obtained from transfected or control cells. GR = poly(GR) RAN protein, PR = poly(PR) RAN protein; PA = poly(PA) RAN protein. Error bars represent SD from three technical replicates.

FIG. 5 shows a Meso Scale Detection (MSD) assay of poly(GP) RAN proteins in blood samples from C9ORF72 ALS patients. Relative levels of poly(GP) RAN protein were quantified by MSD assay using a polyclonal anti-poly(GP) antibody. Significantly higher levels of poly(GP) protein were observed in two study subjects that are positive for the C9ORF72 repeat expansion compared to seven independent control subjects.

FIGs. 6A-6C show representative data for Meso Scale Detection (MSD) assays performed on blood samples at different processing times and temperatures. FIG. 6A shows data indicating an increase in background signal detected by MSD over incubation time in blood held at room temperature (RT) or 4 °C for 1, 2, or 3 days relative to blood processed within 24 hours of collection. FIG. 6B shows data indicating an increase in background signal detected by MSD over incubation time (*e.g.*, 1, 2, or 3 days) at room temperature (RT) relative to blood processed within 24 hours of collection. Higher background was observed in both C9(+) and C9(-) control samples; C9(+) and C9(-) samples were unable to be differentiated in samples held at RT. FIG. 6C shows data indicating an increase in background signal detected by MSD over incubation time (*e.g.*, 1, 2, or 3 days) at 4 °C relative to blood processed within 24 hours of collection. Higher background was observed in both C9(+) and C9(-) control samples; C9(+) samples were able to be differentiated from C9(-) control samples for up to two days incubation at 4 °C. In all MSD assays described in this figure, anti-GP antibody was used for detection of RAN protein.

DETAILED DESCRIPTION

Aspects of the disclosure relate to methods and compositions useful for detecting repeat-associated non-ATG proteins (*e.g.*, RAN proteins) in a biological sample obtained from a subject. The disclosure is based, in part, on longitudinal detection (*e.g.*, detection over a defined time course) of one or more RAN protein levels in a biological sample using electrochemiluminescence-based immunoassays. In some embodiments, methods described by the disclosure are useful for indicating the effectiveness of therapeutic agents which inhibit RAN protein expression and/or RAN protein translation, for example by indicating a reduction in RAN protein levels after administration of one or more therapeutic agents to a subject.

Biological Samples

In some aspects, the disclosure relates to methods of detecting one or more RAN proteins (*e.g.*, detecting the level of one or more RAN proteins) in a biological sample obtained from a subject.

A "subject having or suspected of having ALS and/or FTD" can be a subject that is known or determined to have more than 30 GGGGCC repeats in the *C9ORF72* gene, or a subject exhibiting signs and symptoms of ALS/FTD, including but not limited to motor dysfunction (*e.g.*, spasticity), muscle atrophy, and/or neuropsychiatric manifestations (*e.g.*, compulsive behavior, apathy, anxiety).

A "subject having or suspected of having SCA36" can be a subject that is known or determined to have more than 30 TGGGCC repeats in the *SCA36* gene, or a subject exhibiting signs and symptoms of SCA36, including but not limited to ataxia, muscle atrophy, hyperreflexia, dysarthria, fasciculations, and/or eye abnormalities (*e.g.*, nystagmus, saccades, oculomotor apraxia, ptosis, *etc.*).

A "subject having or suspected of having Huntington's disease" can be a subject that is known or determined to have more than 35 CAG repeats in the HTT gene, or a subject exhibiting signs and symptoms of HD, including but not limited to motor dysfunction (*e.g.*, chorea), diminished executive functions (*e.g.*, cognitive flexibility and abstract thinking), and/or neuropsychiatric manifestations (*e.g.*, compulsive behavior, apathy, anxiety). Generally, the disease status of a subject having or suspected of having HD is classified by the number of CAG repeats present (*e.g.*, detected) in a HTT gene of the subject. Typically, a HTT gene having less than 36 trinucleotide (CAG) repeats produces non-pathogenic cytoplasmic Huntingtin protein. A subject having between 36 and 39 trinucleotide repeats produces mutant Huntingtin protein that is shorter than fully pathogenic forms, and may or may not develop disease. A subject having more than 40 trinucleotide repeats is classified as having fully penetrant HD and will eventually develop HD, also referred to as adult-onset HD. In certain cases of fully penetrant HD characterized by large (>100) repeats, subject can develop juvenile-onset HD, also referred to as akinetic-rigid, or Westphal variant HD. In some embodiments, a subject has or is suspected of having adult-onset HD. In some embodiments, a subject has or is suspected of having juvenile-onset HD.

A "RAN protein (repeat-associated non-ATG translated protein)" is a polypeptide translated from mRNA sequence carrying a nucleotidic expansion in the absence of an AUG

initiation codon. Generally, RAN proteins comprise expansion repeats of one or amino acid, termed poly amino acid repeats (*e.g.*, di-amino acid repeats). For example, in the context of ALS/FTD, which results from a repeat expansion of the hexanucleotide sequence GGGGCC in the *C9ORF72* gene, the following di-amino acid repeat-containing RAN proteins have been identified: poly-(Gly-Ala), poly-(Gly-Pro), poly-(Gly-Arg), poly-(Pro-Ala), or poly-(Pro-Arg), also referred to as poly(GA), poly(GP), poly(GR), poly(PA), and poly(PR), respectively. ALS/RAN proteins are generally described, for example in International PCT Application PCT/US2014/022670, filed on March 10, 2014, published as WO2014/159247, and U.S. Application 14/775,278, filed on September 11, 2015, published as US2016/0025747, the entire contents of each application which are incorporated by reference herein. In the context of SCA36, which results from a repeat expansion of the hexanucleotide sequence TGGGCC in the *SCA36* gene, the following di-amino acid repeat-containing RAN proteins have been identified: poly(GP) and poly(PR). In the context of Huntington's disease (HD), RAN protein translation is caused by a CAG·CTG expansion in the *Htt* gene, which results in translation of RAN proteins polyAlanine, polySerine, polyLeucine, and polyCysteine (polyAla, polySer, polyLeu and polyCys), in addition to poly-Glutamine (polyGln or polyQ).

In some embodiments, the RAN protein is encoded by a gene associated with Huntington's disease (HD, HDL2), Fragile X Syndrome (FRAXA), Spinal Bulbar Muscular Atrophy (SBMA), Dentatorubropallidoluysian Atrophy (DRPLA), Spinocerebellar Ataxia 1 (SCA1), Spinocerebellar Ataxia 2 (SCA2), Spinocerebellar Ataxia 3 (SCA3), Spinocerebellar Ataxia 6 (SCA6), Spinocerebellar Ataxia 7 (SCA7), Spinocerebellar Ataxia 8 (SCA8), Spinocerebellar Ataxia 12 (SCA12), or Spinocerebellar Ataxia 17 (SCA17), amyotrophic lateral sclerosis (ALS), Spinocerebellar ataxia type 36 (SCA36), Spinocerebellar ataxia type 29 (SCA29), Spinocerebellar ataxia type 10 (SCA10), myotonic dystrophy type 1 (DM1), myotonic dystrophy type 2 (DM2), or Fuch's Corneal Dystrophy (*e.g.*, CTG181).

A subject can be a mammal (*e.g.*, human, mouse, rat, dog, cat, or pig). In some embodiments, the subject is a human. In some embodiments, a subject is a mammalian subject. In some embodiments, a subject is a human or a mouse. In some embodiments, a subject is characterized by a GGGGCC (*e.g.*, G_4C_2) hexanucleotide sequence repeat expansion in the *C9ORF72* gene (*e.g.*, a human *C9ORF72* gene or a gene, such as a mouse gene, corresponding to human *C9ORF72* gene). In some embodiments, a human *C9ORF72* gene comprises or consists of the sequence set forth in any one of NCBI Reference Sequence Numbers NM_145005.6,

NM_018325.4, and NM_001256054.2. In some embodiments, a human *SCA36* gene comprises or consists of the sequence set forth in any one of NCBI Reference Sequence Numbers NM_006392.3, NR_027700.2, and NR_145428.1. In some embodiments, a subject has been determined to have a hexanucleotide sequence repeat expansion (*e.g.*, a GGGGCC (*e.g.*, G_4C_2) repeat expansion in *C9ORF72* or a TGGGCC repeat expansion in *SCA36*) by a genetic assay (*e.g.*, a DNA-based assay, for example a sequencing assay).

In some embodiments, a subject comprises at least 50, at least 100, at least 200, at least 500, at least 1000, or at least 5000 GGGGCC repeat expansions (*e.g.*, repeat expansions of *C9ORF72*). In some embodiments, a subject is characterized by a TGGGCC hexanucleotide sequence repeat expansion in the *SCA36* gene (*e.g.*, a human *SCA36* gene or a gene, such as a mouse gene, corresponding to human *SCA36* gene). In some embodiments, a subject comprises at least 50, at least 100, at least 200, at least 500, at least 1000, or at least 5000 TGGGCC repeat expansions (*e.g.*, repeat expansions of *SCA36*).

Methods of the disclosure are useful in some embodiments, for investigating the efficacy of a therapeutic agent (*e.g.*, a therapeutic agent candidate) in an animal model of a disease or disorder associated with RAN protein translation. A "therapeutic agent candidate" generally refers to an agent (*e.g.*, small molecule, interfering RNA, protein, peptide, *etc.*) that is being tested for the ability to reduce or inhibit RAN protein translation in a cell or subject. Thus, in some embodiments, a subject is a C9-BAC mouse. The C9-BAC mouse model of ALS is described, for example in International PCT Application PCT/US2014/022670, filed on March 10, 2014, published as WO2014/159247, and Liu et al. (2016) *Neuron* 90(3):521-34, the entire contents of each of which are incorporated herein by reference.

Generally, a biological sample can be blood, serum (*e.g.*, plasma from which the clotting proteins have been removed), or cerebrospinal fluid (CSF). However, the skilled artisan will recognize other suitable biological samples, such as tissue (*e.g.*, brain tissue, spinal tissue, *etc.*) and cells (*e.g.*, brain cells, neuronal cells, skin cells, *etc.*). In some embodiments, a biological sample is a blood sample or a tissue sample. In some embodiments, a blood sample is a sample of whole blood, a plasma sample, or a serum sample. In some embodiments, a tissue sample is a CNS tissue sample. In some embodiments, a blood sample is treated to remove white blood cells (*e.g.*, leukocytes), such as the buffy coat of the sample.

The disclosure is based, in part, on the surprising discovery that certain immunoassays (*e.g.*, electrochemiluminescence-based immunoassays) can be used to detect one or more RAN

proteins in a blood sample obtained from a subject. As described further in the Example, it was observed that blood sample processing time and conditions (*e.g.*, incubation time and incubation temperature) affect the amount of background signal observed in a given blood sample. It was observed that if a blood sample is incubated (*e.g.*, held or stored) at room temperature for more than 24 hours after being obtained from the subject, the levels of RAN proteins in the sample are indistinguishable from control samples due to high background signal. Similarly, it was observed that if a sample is stored at 4 °C for more than two days after being obtained from the subject, the levels of RAN proteins in the sample are indistinguishable from control samples due to high background signal.

In some embodiments, an immunoassay (*e.g.*, an electrochemiluminescence-based immunoassay) is performed on a biological sample (*e.g.*, a blood sample) within two days of being obtained from a subject. In some embodiments, an immunoassay (*e.g.*, an electrochemiluminescence-based immunoassay) is performed on a biological sample (*e.g.*, a blood sample) between about 1 minute and about 48 hours after being obtained from a subject. In some embodiments, an immunoassay (*e.g.*, an electrochemiluminescence-based immunoassay) is performed on a biological sample (*e.g.*, a blood sample) between about 60 minutes and about 24 hours after being obtained from a subject.

In some embodiments, a biological sample obtained from a subject is stored at a temperature between -80 °C and about 23 °C (*e.g.*, room temperature). In some embodiments, a biological sample obtained from a subject is stored at a temperature between 0 °C and about 23 °C (*e.g.*, about 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or 23 °C). In some embodiments, a biological sample obtained from a subject is stored at a temperature between 20 °C and about 25 °C (*e.g.*, about 20, 21, 22, 23, 24, or 25 °C).

A biological sample (*e.g.*, a blood sample) may be manipulated or processed prior to being subjected to an immunoassay (*e.g.*, an electrochemiluminescence-based immunoassay). For example, in some embodiments, a biological sample is subjected to an antigen retrieval process prior to being used in an immunoassay. As used herein, "antigen retrieval" (also referred to as epitope retrieval, or antigen unmasking) refers to a process in which a biological sample (*e.g.*, blood, serum, CSF, *etc.*) are treated under conditions which expose antigens (*e.g.*, epitopes) that were previously inaccessible to detection agents (*e.g.*, antibodies, aptamers, and other binding molecules) prior to the process. Generally, antigen retrieval methods comprise steps including but not limited to heating, pressure treatment, enzymatic digestion, treatment with

reducing agents, treatment with oxidizing agents, treatment with crosslinking agents, treatment with denaturing agents (*e.g.*, detergents, ethanol, acids), or changes in pH, or any combination of the foregoing. Several antigen retrieval methods are known in the art, including but not limited to protease-induced epitope retrieval (PIER) and heat-induced epitope retrieval (HIER). In some embodiments, antigen retrieval procedures reduce the background and increase the sensitivity of detection techniques (*e.g.*, electrochemiluminescence-based immunoassays, immunohistochemistry (IHC), immuno-blot (such as Western Blot), ELISA, *etc.*).

RAN protein detection assays

In some aspects, the disclosure provides a kit comprising one or more anti-RAN protein antibodies, and an electrochemiluminescence-based immunoassay. Generally, an "electrochemiluminescence-based immunoassay" refers to an biological assay in which binding of capture antibodies (*e.g.*, one or more anti-RAN protein antibodies) to analytes (*e.g.*, one or more RAN proteins) in a biological sample are detected using electrochemiluminescent labels (*e.g.*, detectable moieties which emit light when stimulated by electricity in the appropriate chemical environment (*e.g.*, in the presence of tripropylamine, TPrA). Electrochemiluminescent labels are described, for example by Muzyka (2014) *Biosens Bioelectron* 15(54):393-407.

In some embodiments, an electrochemiluminescence-based immunoassay is a Meso Scale Detection (MSD) assay. As used herein the term "meso scale detection (MSD) assay" refers to an immunoassay used for detection of analytes by electrochemiluminescence (*e.g.*, using one or more detectable reagents, such as SULFO-TAG™ labels (*e.g.*, labels comprising one or more Ruthenium complexes) that emit light upon electrochemical stimulation), for example as described by Moxness et al. (2005) *Clin. Chem.* 51(10):1983-5, and U.S. Patent No. 7,008,796, which is incorporated by reference with respect to description of MSD assay steps.

Generally, a MSD assay comprises contacting a solid substrate, for example a multi-well assay plate comprising one or more capture antibodies (*e.g.*, one or more anti-RAN protein antibodies) attached to the substrate, with a biological sample (*e.g.*, a blood sample obtained from a subject), under conditions under which RAN proteins present in the biological sample bind to the one or more capture antibodies to form a complex, and subsequently contacting the complexes with one or more secondary antibodies (*e.g.*, an antibody that binds to the RAN protein portion of the complex or an antibody that binds to the capture antibody, such as an anti-mouse antibody, anti-rabbit antibody, *etc.*) that are conjugated to a detectable reagent. In some

embodiments, a detectable reagent comprises an electrochemiluminescent moiety, for example as described in U.S. Patent No. 5,310,687, which is incorporated herein by reference with respect to disclosure regarding such electrochemiluminescent moieties. In some embodiments, a detectable reagent comprises a Ruthenium complex, for example Ruthenium (II) tris-bipyridine-(4-methylsulfone), also referred to as $[Ru(Bpy)_3]^{+2}$, or a salt thereof.

A detectable reagent (*e.g.*, a detectable moiety, for example a Ruthenium complex, such as a SULFO-TAG™) may be conjugated to a primary antibody (*e.g.*, a capture antibody, such as an anti-RAN protein antibody) or a secondary antibody (*e.g.*, a detection antibody, such as an antibody that binds to a RAN protein or an antibody that binds to a capture antibody).

In some aspects, the disclosure provides a method for measuring one or more RAN proteins in a sample (*e.g.*, using a kit as described herein), the method comprising: detecting in a biological sample (*e.g.*, a blood sample, CNS tissue sample, *etc.*) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay. In some embodiments a subject is diagnosed as having or being at risk of developing ALS and/or FTD based upon detection of the one or more RAN proteins in the biological sample. In some embodiments a subject is diagnosed as having or being at risk of developing SCA36 based upon detection of the one or more RAN proteins in the biological sample. In some embodiments, a subject has previously been determined (*e.g.*, diagnosed by a medical professional such as a doctor) as having ALS, FTD, or SCA36 based upon a genetic test (*e.g.*, a nucleic acid-based test, such as a PCR-based test, identifying the presence of a repeat expansion of *C9ORF72* or *SCA36* of the subject).

In some embodiments, one or more RAN proteins detected in a sample is selected from poly(GP), poly(GR), poly(PR), and poly(PA). In some embodiments, two, three or four RAN proteins are detected in a sample (*e.g.*, poly(GP, poly(GR), poly(PR), and poly(PA), or any combination thereof).

In some embodiments, an electrochemiluminescence-based immunoassay comprises a step of contacting the sample with one or more anti-RAN protein antibodies. In some embodiments, an anti-RAN antibody is selected from an anti-poly(GP) antibody, anti-poly(GR) antibody, anti-poly(PR) antibody, and anti-poly(PA) antibody. In some embodiments, an anti-RAN antibody targets poly-Alanine, poly-Leucine, poly-Serine, or poly-Cysteine, for example as described in PCT Publication No. WO 2017/176813.

In some embodiments, an anti-RAN antibody binds to a di-amino acid repeat region of a RAN protein or binds to a C-terminal portion of a RAN protein. In some embodiments, an anti-RAN antibody targets a homopolymeric amino acid repeat region or a C-terminus of a RAN protein translated from a *Htt* gene.

In some embodiments, an anti-RAN antibody may target any portion of a RAN protein that does not comprise the poly amino acid repeat. Examples of anti-RAN antibodies targeting the C-terminus of RAN protein are disclosed, for example, in U.S. Publication No. 2013/0115603, the entire content of which is incorporated herein by reference. In some embodiments a set (or combination) of anti-RAN antibodies (*e.g.*, a combination of two or more anti-RAN antibodies selected from anti-poly(GP), anti-poly(PR), anti-poly(PA), and anti-poly(GR)) is used to detect one or more RAN proteins in a biological sample.

An anti-RAN antibody can be a polyclonal antibody or a monoclonal antibody. Typically, polyclonal antibodies are produced by inoculation of a suitable mammal, such as a mouse, rabbit or goat. Larger mammals are often preferred as the amount of serum that can be collected is greater. An antigen is injected into the mammal. This induces the B-lymphocytes to produce IgG immunoglobulins specific for the antigen. This polyclonal IgG is purified from the mammal's serum. Monoclonal antibodies are generally produced by a single cell line (*e.g.*, a hybridoma cell line). In some embodiments, an anti-RAN antibody is purified (*e.g.*, isolated from serum).

Numerous methods may be used for obtaining anti-RAN antibodies. For example, antibodies can be produced using recombinant DNA methods. Monoclonal antibodies may also be produced by generation of hybridomas (see *e.g.*, Kohler and Milstein (1975) Nature, 256: 495-499) in accordance with known methods. Hybridomas formed in this manner are then screened using standard methods, such as enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (*e.g.*, OCTET or BIACORE) analysis, to identify one or more hybridomas that produce an antibody that specifically binds with a specified antigen. Any form of the specified antigen (*e.g.*, a RAN protein) may be used as the immunogen, *e.g.*, recombinant antigen, naturally occurring forms, any variants or fragments thereof. One exemplary method of making antibodies includes screening protein expression libraries that express antibodies or fragments thereof (*e.g.*, scFv), *e.g.*, phage or ribosome display libraries. Phage display is described, for example, in Ladner et al., U.S. Pat. No. 5,223,409; Smith (1985) Science 228:1315-1317; Clackson et al. (1991) Nature, 352: 624-628; Marks et al. (1991) J. Mol.

Biol., 222: 581-597WO92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; and WO 90/02809.

In addition to the use of display libraries, the specified antigen (*e.g.*, one or more RAN proteins) can be used to immunize a non-human animal, *e.g.*, a rodent, *e.g.*, a mouse, hamster, or rat. In one embodiment, the non-human animal is a mouse.

In another embodiment, a monoclonal antibody is obtained from the non-human animal, and then modified, *e.g.*, made chimeric, using recombinant DNA techniques known in the art. A variety of approaches for making chimeric antibodies have been described. See *e.g.*, Morrison *et al.*, Proc. Natl. Acad. Sci. U.S.A. 81:6851, 1985; Takeda *et al.*, Nature 314:452, 1985, Cabilly *et al.*, U.S. Pat. No. 4,816,567; Boss *et al.*, U.S. Pat. No. 4,816,397; Tanaguchi *et al.*, European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B.

Antibodies can also be humanized by methods known in the art. For example, monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; and Oxford Molecular, Palo Alto, Calif.). Fully humanized antibodies, such as those expressed in transgenic animals are within the scope of the invention (see, *e.g.*, Green *et al.* (1994) Nature Genetics 7, 13; and U.S. Patent Nos. 5,545,806 and 5,569,825).

For additional antibody production techniques, see Antibodies: A Laboratory Manual, Second Edition. Edited by Edward A. Greenfield, Dana-Farber Cancer Institute, ©2014. The present disclosure is not necessarily limited to any particular source, method of production, or other special characteristics of an antibody.

In some embodiments, an anti-RAN antibody is purified (*e.g.*, isolated from serum). In some embodiments, an anti-RAN antibody is labeled (*e.g.*, comprises a fluorescent label, luminescent label, radiolabel, enzymatic label, or any other detectable label, *etc.*).

In some embodiments, an anti-RAN antibody has a Kd value (equilibrium dissociation constant between the antibody and the RAN antigen) ranging from the low micromolar (10^{-6}) to nanomolar (10^{-7} to 10^{-9}). In some embodiments, an anti-RAN antibody has a Kd value in the low nanomolar range (10^{-9}). In some embodiments, an anti-RAN antibody has a Kd value in the picomolar range (10^{-12}).

Accordingly, in some embodiments, methods and kits described by the disclosure are capable of measuring levels of RAN proteins in a subject over a specified time period (*e.g.*. longitudinally over a course of treatment), thereby providing an assessment of therapeutic

efficacy of certain ALS/FTD or SCA36 treatments (*e.g.*, therapeutic agents for treating ALS/FTD or SCA36). Without wishing to be bound by any theory, measuring a reduced level of one or more RAN proteins in a subject after administration of a therapeutic agent for treatment of ALS/FTD or SCA36 (*e.g.*, relative to the level of RAN proteins measured in the subject prior to the administration) is indicative of the therapeutic agent effectively treating the subject for ALS/FTD or SCA36.

In some aspects, the disclosure provides a method for measuring pharmacokinetic changes in RAN protein expression, the method comprising detecting in a first biological sample (*e.g.*, blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay; detecting in a second biological sample (*e.g.*, blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay, wherein the second biological sample is obtained after administration of a therapeutic agent to the subject; and determining that administration of the therapeutic agent to the subject results in a change (*e.g.*, an elevation or decrease) in one or more RAN protein levels in the subject if the amount of RAN proteins detected in the second biological sample is less than the amount of RAN proteins detected in the first biological sample.

As used herein, "elevated" means that the level of one or more di-amino acid-repeat-containing proteins or a hexanucleotide repeat-containing RNA is above a control level, such as a pre-determined threshold or a level of one or more di-amino acid-repeat-containing proteins or a hexanucleotide repeat-containing RNA in a control sample. Controls and control levels include RAN protein levels obtained (*e.g.*, detected) from a subject that does not have or is not suspected of having ALS/FTD, or SCA36 (*e.g.*, a subject having 30 or less repeats of a GGGGCC expansion or a TGGGCC expansion). An elevated level includes a level that is, for example, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 400%, 500%, or more above a control level. An elevated level also includes increasing a phenomenon from a zero state (*e.g.*, no or undetectable di-amino acid-repeat-containing protein expression or hexanucleotide repeat-containing RNA expression) to a non-zero state (*e.g.*, some or detectable di-amino acid-repeat-containing protein expression or hexanucleotide repeat-containing RNA).

As used herein, "decreased" means that the level of one or more di-amino acid-repeat-containing proteins or a hexanucleotide repeat-containing RNA is below a control level, such as a pre-determined threshold or a level of one or more di-amino acid-repeat-containing proteins or

a hexanucleotide repeat-containing RNA in a control sample. Controls and control levels include RAN protein levels obtained (*e.g.*, detected) from a subject that does not have or is not suspected of having ALS/FTD, or SCA36 (*e.g.*, a subject having 30 or less repeats of a GGGGCC expansion or a TGGGCC expansion). A decreased level includes a level that is, for example, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 300%, 400%, 500%, or more below a control level. A decreased level also includes decreasing a phenomenon from a non-zero state (*e.g.*, some or detectable di-amino acid-repeat-containing protein expression or hexanucleotide repeat-containing RNA) to a zero state (*e.g.*, no or undetectable di-amino acid-repeat-containing protein expression or hexanucleotide repeat-containing RNA expression). In some embodiments, a decrease (*e.g.*, decrease in the level of one or more RAN protein in the sample relative to a control or a prior sample) can be indicative of therapeutic efficacy of a therapeutic agent (*e.g.*, therapeutic efficacy in the subject from which the sample was obtained).

The time between which a first biological sample and a second biological sample are obtained may vary. In some embodiments, a first biological sample is obtained between 1 week and 1 minute prior to administration of a therapeutic agent (*e.g.*, the first administration of a therapeutic agent). In some embodiments, a first biological sample is obtained between 1 day (*e.g.*, 24 hours) and 1 minute prior to administration of a therapeutic agent (*e.g.*, the first administration of a therapeutic agent). In some embodiments, a second biological sample is obtained from the subject between 1 minute and six months after administration of a therapeutic agent (*e.g.*, the first administration of a therapeutic agent). In some embodiments, a second biological sample is obtained from the subject between 1 day and 1 week after administration of a therapeutic agent (*e.g.*, the first administration of a therapeutic agent). In some embodiments, a second biological sample is obtained from the subject between 1 day and 1 week after administration of a therapeutic agent (*e.g.*, the most recent or last administration of a therapeutic agent).

In some embodiments, a second biological sample may be collected about 1 hour, 5 hours, 10 hours, 24 hours (*e.g.*, 1 day), 48 hours (*e.g.*, 2 days), 120 hours (*e.g.*, 5 days), 30 days, 45 days, or six months after administration of the therapeutic agent. In some embodiments, several biological samples (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more biological samples) are obtained from the subject, for example over a specified timeframe (*e.g.*, during a therapeutic course) and one or more RAN proteins are detected.

Methods of treating diseases and disorders associated with RAN protein translation

The disclosure relates, in some aspects, to methods of monitoring a therapeutic treatment course for a disease associated with RAN protein translation, for example ALS/FTD, SCA36, HD, *etc.* In some aspects, the disclosure provides methods of treating a disease associated with RAN protein translation comprising administering an effective amount of a therapeutic agent to a subject who has been determined to exhibit increased RAN protein translation (*e.g.*, relative to a subject not having a disease or disorder associated with RAN protein translation) as measured by an electrochemiluminescent immunoassay. In some embodiments, the subject has previously been administered a therapeutic agent (*e.g.*, prior to the determining). In some embodiments, the therapeutic agent administered to the subject is different from the previously administered therapeutic agent. In some embodiments, a subject is administered an increased or decreased dose of a therapeutic agent based on detection of an elevated or reduced level of RAN proteins in a biological sample as measured by an electrochemiluminescent immunoassay.

In some embodiments, methods described by the disclosure comprise a step of administering a therapeutic agent (*e.g.*, an agent for treatment of ALS/FTD or an agent for treatment of SCA36) to the subject if the level of RAN proteins detected in the biological sample is elevated compared to a level of RAN proteins detected in a control sample.

Controls and control levels include RAN protein levels obtained (*e.g.*, detected) from a subject that does not have or is not suspected of having a disease or disorder associated with RAN protein translation, for example ALS/FTD, or SCA36 (*e.g.*, a subject having 30 or less repeats of a GGGGCC expansion or a TGGGCC expansion). In some embodiments, a control sample refers to a sample obtained from a subject having or suspected of having a disease associated with RAN protein translation prior to the administration of a therapeutic agent to the subject.

As used herein, "treat" or "treatment" refers to (a) preventing or delaying the onset of a disease or disorder associated with RAN protein translation; (b) reducing the severity of a disease or disorder associated with RAN protein translation; (c) reducing or preventing development of symptoms characteristic of a disease or disorder associated with RAN protein translation; (d) preventing worsening of symptoms characteristic of a disease or disorder associated with RAN protein translation; and/or (e) reducing or preventing recurrence of

symptoms in subjects that were previously symptomatic for a disease or disorder associated with RAN protein translation.

For example, in the context of ALS/FTD, "treat" or "treatment" refers to (a) preventing or delaying the onset of ALS and/or FTD, or SCA36; (b) reducing the severity of ALS and/or FTD, or SCA36; (c) reducing or preventing development of symptoms characteristic of ALS and/or FTD, or SCA36; (d) preventing worsening of symptoms characteristic of ALS and/or FTD, or SCA36; and/or (e) reducing or preventing recurrence of ALS and/or FTD, or SCA36 symptoms in subjects that were previously symptomatic for ALS and/or FTD, or SCA36. Examples of therapeutic agents for the treatment of ALS/FTD include but are not limited to Riluzole (Rilutek, Sanofi-Aventis), trazodone (Desyrel, Oleptro), selective serotonin reuptake inhibitors (SSRIs), baclofen, diazepam, phenytoin, trihexyphenidyl, amitriptyline, anti-RAN antibodies, *etc.*

In another example, "treating HD" refers to (a) preventing or delaying the onset of HD; (b) reducing the severity of HD; (c) reducing or preventing development of symptoms characteristic of HD; (d) preventing worsening of symptoms characteristic of HD; and/or (e) reducing or preventing recurrence of HD symptoms in subjects that were previously symptomatic for HD. Examples of therapeutic agents for the treatment of tetrabenazine, haloperidol, chlorpromazine, risperidone, quetiapine, amantadine, levetiracetam, clonazepam , citalopram, fluoxetine, sertraline, olanzapine, alproate, carbamazepine, lamotrigine, cysteamine, PBT2, PDE10A inhibitor, pridopidine, laquinimod, anti-RAN antibodies, *etc.*

A subject may be administered a therapeutically effective amount of one or more therapeutic agents. As used herein, an "effective amount" is a dosage of a therapeutic agent sufficient to provide a medically desirable result, such as treatment or amelioration of one or more signs or symptoms caused by a disease or disorder associated with RAN protein translation or accumulation (e.g., a neurodegenerative disease). The effective amount will vary with the age and physical condition of the subject being treated, the severity of the disease or disorder (e.g., the amount of RAN protein accumulation, or cellular toxicity caused by such an accumulation) in the subject, the duration of the treatment, the nature of any concurrent therapy, the specific route of administration and the like factors within the knowledge and expertise of the health practitioner.

Generally, a therapeutic agent can be a small molecule (*e.g.*, metformin or a metformin derivative), an interfering RNA (*e.g.*, dsRNA, siRNA, miRNA, amiRNA, ASO, aptamer, *etc.*),

protein or fragment thereof, peptide, antibody, *etc.* In some embodiments, a therapeutic agent modulates RAN protein expression, for example by modulating a pathway that controls RAN protein expression, such as protein kinase R (PKR) pathway, EIF2 pathway or EIF3 pathway. In some embodiments, a therapeutic agent is delivered by a viral vector, for example a lentiviral vector, retroviral vector, adenoviral vector, or adeno-associated virus (AAV) vector.

The identification and selection of appropriate additional therapeutic agents is within the capabilities of a person of ordinary skill in the art, and will depend upon the disease from which the subject is suffering. For example, in some embodiments one or more therapeutic agents for Huntington's disease (e.g. tetrabenazine, amantadine, chlorpromazine, *etc.*), Fragile X Syndrome (e.g., selective serotonin reuptake inhibitors, carbamazepine, methylphenidate, Trazodone, *etc.*), Spinocerebellar Ataxia(e.g., baclofen, riluzole, amantadine, varenicline, *etc.*), or amyotrophic lateral sclerosis (ALS) (e.g., riluzole, *etc.*), myotonic dystrophy type 1 (tideglusib, mexiletine, *etc.*) are administered to the subject.

Administration of a treatment may be accomplished by any method known in the art (see, *e.g.*, Harrison's Principle of Internal Medicine, McGraw Hill Inc.). Administration may be local or systemic. Administration may be parenteral (*e.g.*, intravenous, subcutaneous, or intradermal) or oral. Compositions for different routes of administration are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences by E. W. Martin). Dosage will depend on the subject and the route of administration. Dosage can be determined by the skilled artisan.

Without wishing to be bound by any particular theory, detection (*e.g.*, quantification of RAN proteins) in the biological samples can be used to determine the effectiveness of a therapeutic agent or regime in the subject from which the samples are obtained.

Kits

In some aspects, the disclosure provides a kit comprising a first container containing one or more anti-RAN antibodies and a second container containing one or more detectable reagents. In some embodiments, the one or more anti-RAN antibodies bind to one or more RAN proteins selected from poly(GP), poly(GR), poly(PR), and poly(PA). In some embodiments, the one or more anti-RAN antibodies bind to one or more RAN proteins selected from targets poly-Alanine, poly-Leucine, poly-Serine, or poly-Cysteine. In some embodiments, the one or more detectable reagents comprise a Ruthenium complex, for example Ruthenium (II) tris-bipyridine-(4-methylsulfone), also referred to as $[Ru(Bpy)_3]^{+2}$, or a salt thereof. In some embodiments, a

kit comprises a third container containing a control sample. A control sample may be a negative control sample (*e.g.*, a control sample that does not contain or lacks, one or more RAN proteins) or a positive control sample (*e.g.*, a control sample that comprises one or more RAN proteins, optionally wherein the amount of the one or more RAN proteins in the sample is known).

EXAMPLE

Use of a meso scale detection (MSD) assay for detection of RAN protein translation from the C9ORF72 gene was investigated. FIGs. 1A-1D show standard curves for detection of RAN proteins (poly(GP) in FIG. 1A, poly(GR) in FIG. 1B, poly(PR) in FIG. 1C, and poly(PA) in FIG. 1D) using a meso scale detection (MSD) assay. Each of the anti-RAN protein antibodies tested in this example was a polyclonal antibody that binds to the di-amino acid repeat region of the respective RAN protein.

The MSD assay was tested for detection of poly(GP) RAN protein *in vitro* and on biological samples obtained from mammalian subjects. FIG. 2A shows data from an MSD assay measuring poly(GP) RAN protein level from a brain tissue soluble fraction obtained from a C9Orf72 mouse model (*e.g.*, C9-BAC mouse model, "C9(+)") of ALS/FTD. Data indicate detection of increased poly(GP) protein in both frontal cortex (FCX) and cerebellum (CE) of C9(+) relative to wild-type (WT) mice by MSD. Increased expression of poly(GP) protein was also detected by MSD in HEK293 cells that had been transfected with a poly(GP)expression construct relative to untransfected HEK293 cells (FIG. 2B).

RAN translation in a mouse model of ALS/FTD (*e.g.*, C9-BAC mice) after administration of a therapeutic agent was also investigated by MSD assay. Briefly, C9(+) or C9(-) control mice were administered an rAAV configured to express EGFP (control) or therapeutic agent protein kinase R (PKR) variant K296R, which is a dominant negative variant of PKR observed to inhibit RAN translation. FIG. 3A shows a schematic depiction of AAV-EGFP and AAV-PKR(K296R) expression constructs. MSD data indicate a pharmacokinetic decrease in poly(GP) RAN protein in C9(+) mice treated with a PKR K296R compared to control mice administered the EGFP construct.

Antibodies targeting additional RAN proteins were also tested in the MSD assay system. HEK 293 cells were transfected with expression constructs encoding poly(GR), poly(PR), and poly(PA) RAN proteins. MSD assay was performed and RAN protein expression levels of transfected cells were compared to untransfected control cells. FIG. 4 shows MSD assay

detection of C9 RAN protein levels (poly(GR), poly(PR), poly(PA); left to right) in soluble fractions obtained from transfected or control cells. Increased RAN protein level was detected by MSD assay in each transfected cell sample relative to control cells.

The ability of MSD assay to detect levels of RAN proteins in biological samples obtained from human subjects was investigated. FIG. 5 shows a Meso Scale Detection (MSD) assay of poly(GP) RAN proteins in blood samples obtained from C9ORF72 ALS patients. Relative levels of poly(GP) RAN protein were quantified by MSD assay using a polyclonal anti-poly(GP) antibody. Significantly higher levels of poly(GP) protein were observed in two study subjects that are positive for the C9ORF72 repeat expansion compared to seven independent control subjects (*e.g.*, subjects not having the C9ORF72 repeat expansion).

Processing conditions for biological samples obtained from subjects were investigated. FIGs. 6A-6C show representative data for Meso Scale Detection (MSD) assays performed on blood samples obtained from subjects at different processing times and temperatures. Anti-poly(GP) antibody was used in this study. Generally, data indicate an increase in background signal detected by MSD over incubation time in blood held at room temperature (RT) or 4 °C for 1, 2, or 3 days relative to blood processed within 24 hours of collection (FIG. 6A). An increase in background signal detected by MSD over incubation time (*e.g.*, 1, 2, or 3 days) was observed at room temperature (RT) relative to blood processed within 24 hours of collection. Higher background was observed in both C9(+) and C9(-) control samples. Indeed, C9(+) and C9(-) samples were unable to be differentiated in samples held at RT for 1, 2, or 3 days. A similar but less significant trend was observed at 4 °C (FIG. 6C). However, at 4 °C, although higher background was observed in both C9(+) and C9(-) control samples, C9(+) samples were able to be differentiated from C9(-) control samples for up to two days incubation.

CLAIMS

What is claimed is:

1. A method comprising detecting in a biological sample (*e.g.*, a blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay.

2. The method of claim 1, wherein the biological sample is a blood sample or a tissue sample, optionally wherein the tissue sample is a CNS tissue sample.

3. The method of claim 1 or 2, wherein the subject is a mammalian subject, optionally wherein the subject is a human or a mouse.

4. The method of claim 3, wherein the subject is characterized by

(i) a GGGGCC hexanucleotide sequence repeat expansion in the C9ORF72 gene, optionally wherein the subject comprises at least 100, at least 200, at least 500, at least 1000, or at least 5000 GGGGCC repeat expansions; or

(ii) a TGGGCC hexanucleotide sequence repeat expansion in the C9ORF72 gene, optionally wherein the subject comprises at least 100, at least 200, at least 500, at least 1000, or at least 5000 TGGGCC repeat expansions.

5. The method of claim 3, wherein the subject is a C9-BAC mouse.

6. The method of any one of claims 1 to 5, wherein the one or more RAN proteins detected in the sample is selected from poly(GP), poly(GR), poly(PR), and poly(PA).

7. The method of any one of claims 1 to 6, wherein two, three or four RAN proteins are detected in the sample.

8. The method of any one of claims 1 to 7, wherein the electrochemiluminescence-based immunoassay comprises a step of contacting the sample with one or more anti-RAN protein antibodies, optionally wherein the anti-RAN antibody is selected from an anti-

poly(GP) antibody, anti-poly(GR) antibody, anti-poly(PR) antibody, and anti-poly(PA) antibody.

9. The method of claim 8, wherein the anti-RAN antibody binds to the di-amino acid repeat region of a RAN protein, optionally wherein the di-amino acid repeat region is poly(GP), poly(GR), poly(PR), or poly(PA).

10. The method of claim 8, wherein the anti-RAN antibody binds to a C-terminal portion of a RAN protein, optionally wherein the RAN protein comprises a poly(GP), poly(GR), poly(PR), or poly(PA) di-amino acid repeat region.

11. The method of any one of claims 8 to 10, wherein the one or more anti-RAN protein antibodies is a polyclonal antibody.

12. The method of any one of claims 8 to 10, wherein the one or more anti-RAN protein antibodies is a monoclonal antibody.

13. The method of any one of claims 1 to 12, wherein the electrochemiluminescence-based immunoassay is a Meso Scale Detection (MSD) assay.

14. The method of any one of claims 1 to 13, further comprising a step of administering a therapeutic agent to the subject if the level of RAN proteins detected in the biological sample is elevated compared to a level of RAN proteins detected in a control sample.

15. The method of claim 14, comprising the step of obtaining a second biological sample from the subject after administration of the therapeutic agent and detecting one or more RAN proteins in the sample using an electrochemiluminescence-based immunoassay.

16. The method of any one of claims 1 to 14, wherein the electrochemiluminescence-based immunoassay is performed within two days of the biological sample being obtained from the subject.

17. The method of claim 16, wherein the electrochemiluminescence-based immunoassay is performed within 24 hours of the biological sample being obtained from the subject.

18. The method of claim 16 or 17, wherein the biological sample is stored at a temperature below 23 °C prior to performing the electrochemiluminescence-based immunoassay, optionally wherein the biological sample is stored at a temperature at or below 4°C.

19. A method for measuring pharmacokinetic changes in RAN protein expression, the method comprising:

(i) detecting in a first biological sample (*e.g.*, blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay;

(ii) detecting in a second biological sample (*e.g.*, blood sample) obtained from a subject one or more RAN proteins using an electrochemiluminescence-based immunoassay, wherein the second biological sample is obtained after administration of a therapeutic agent to the subject; and

(iii) determining that administration of the therapeutic agent to the subject results in a change in one or more RAN protein levels in the subject if the amount of RAN proteins detected in the second biological sample is less than the amount of RAN proteins detected in the first biological sample.

20. The method of claim 19, wherein the first biological sample is obtained between 1 week and 1 minute before the administration of the therapeutic agent.

21. The method of claim 19 or 20, wherein the second biological sample is obtained between 1 hour and 1 week after the administration of the therapeutic agent.

22. A kit comprising:

(i) one or more anti-RAN protein antibodies; and

(ii) an electrochemiluminescence-based immunoassay plate and/or reagents.

23. The kit of claim 22, wherein the one or more anti-RAN protein antibodies are selected from anti-poly(GP) antibody, anti-poly(GR) antibody, anti-poly(PR) antibody, and anti-poly(PA) antibody.

24. The kit of claim 22 or 23, wherein the anti-RAN protein antibodies are monoclonal antibodies or polyclonal antibodies.

25. The kit of any one of claims 22 to 24, wherein the electrochemiluminescence-based immunoassay plate and/or reagents is a MSD assay plate and/or MSD assay reagents.

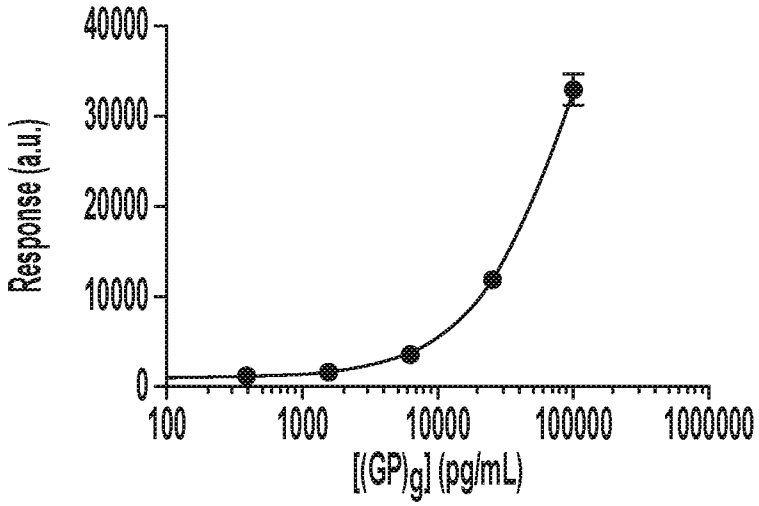


Figure 1A

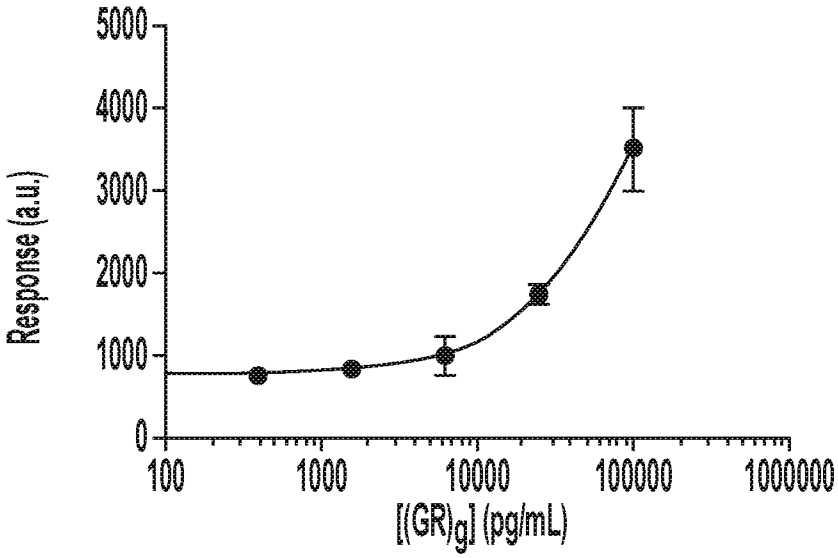


Figure 1B

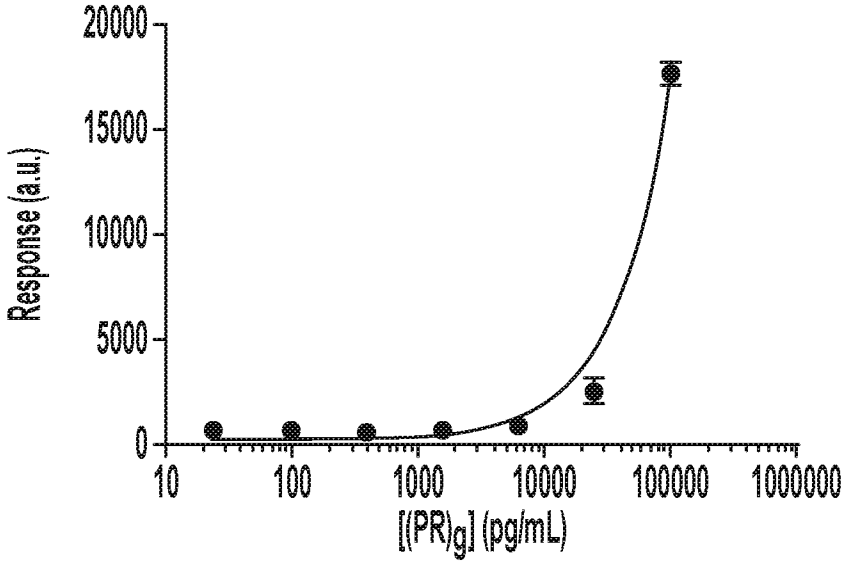


Figure 1C

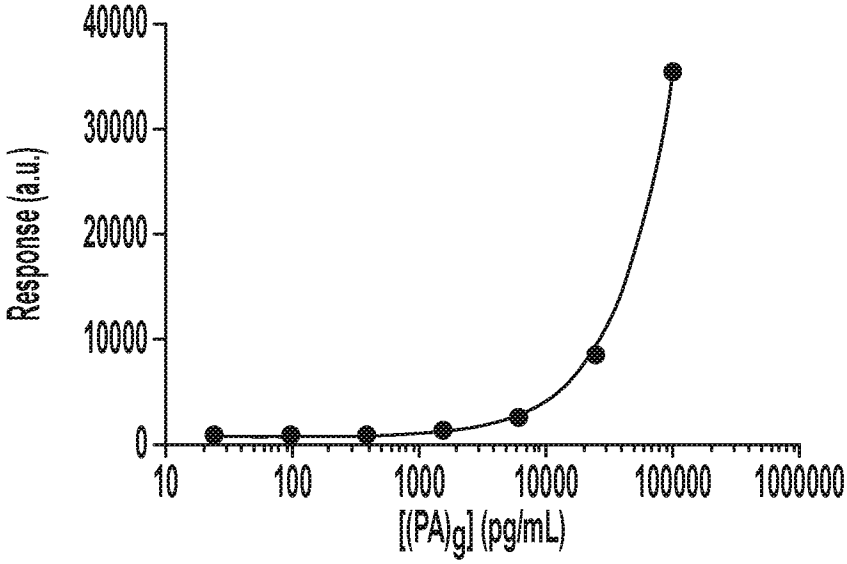


Figure 1D

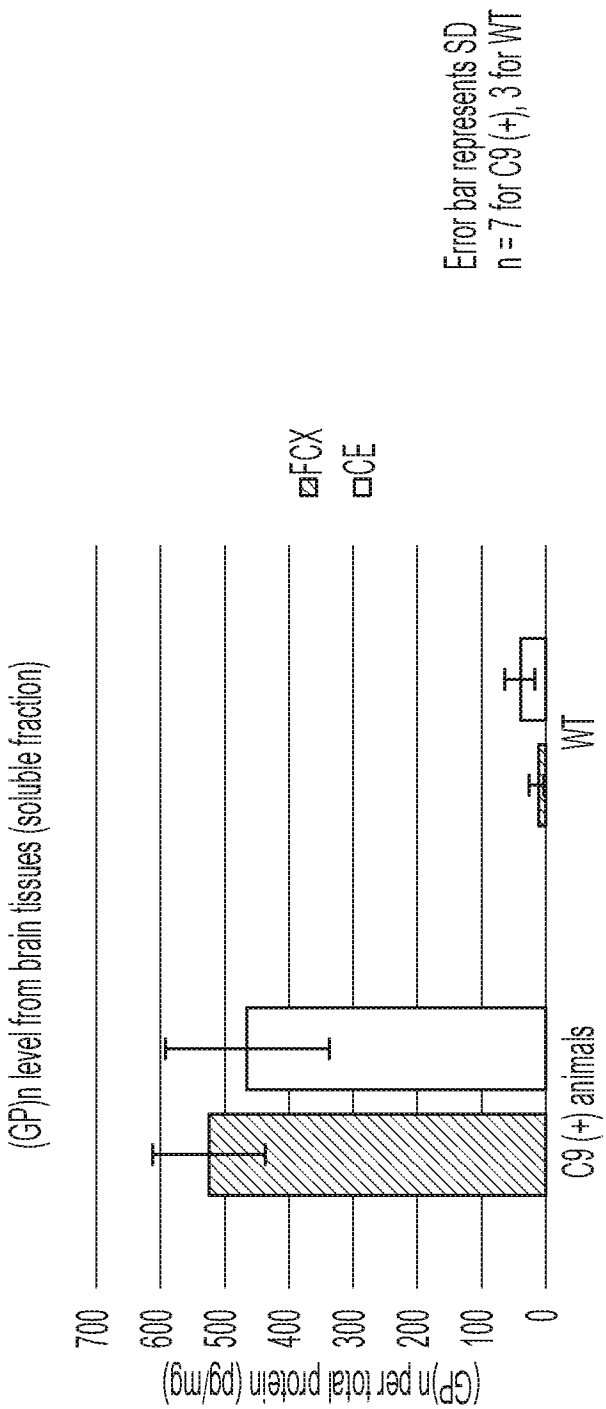

Error bar represents SD
n = 7 for C9 (+), 3 for WT
FCX
CE
(GP)n level from brain tissues (soluble fraction)
(GP)n per total protein (pg/mg)
700
600
500
400
300
200
100
0
C9 (+) animals
WT

Figure 2A

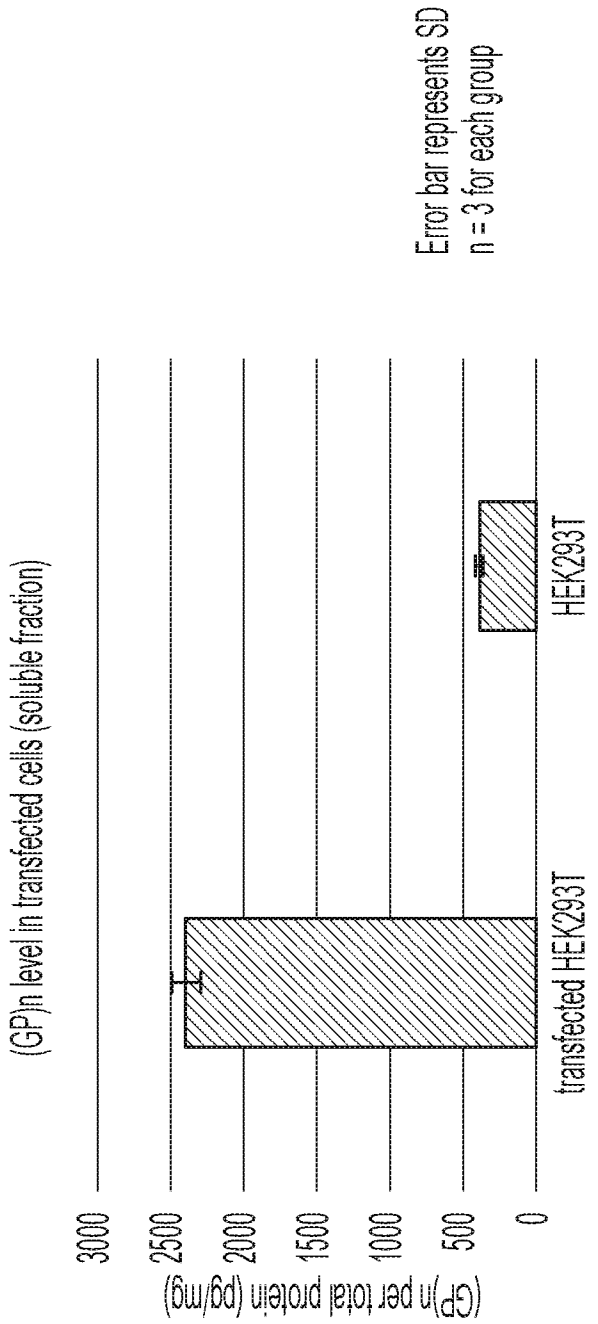

Error bar represents SD
n = 3 for each group
(GP)n level in transfected cells (soluble fraction)
(GP)n per total protein (pg/mg)
3000
2500
2000
1500
1000
500
0
transfected HEK293T
HEK293T

Figure 2B

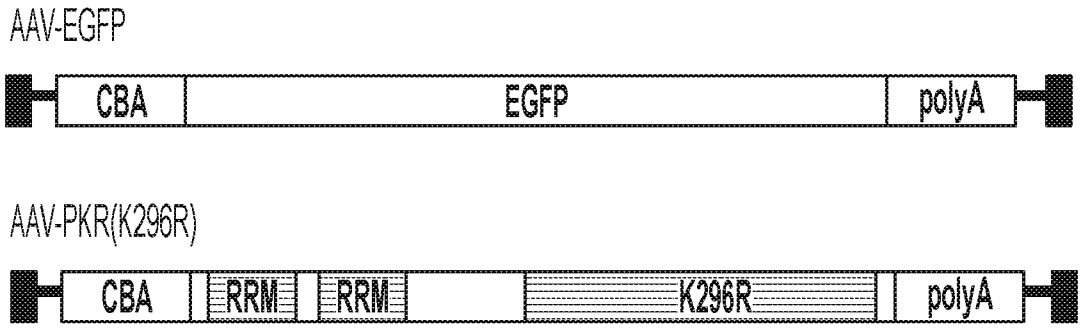


Figure 3A

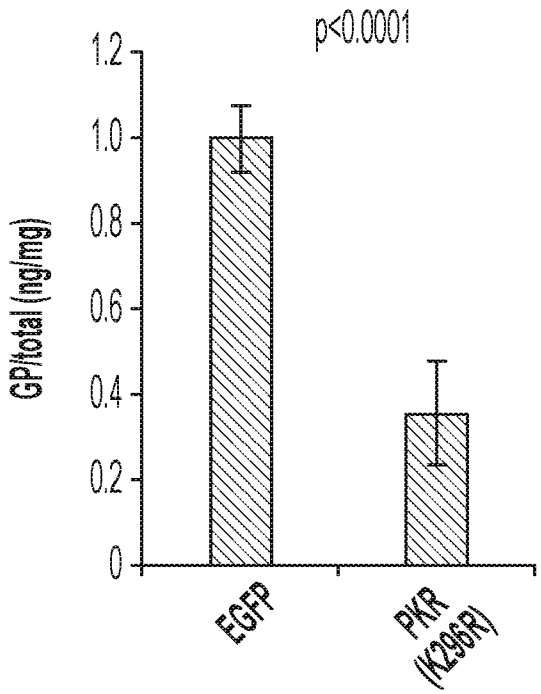


Figure 3B

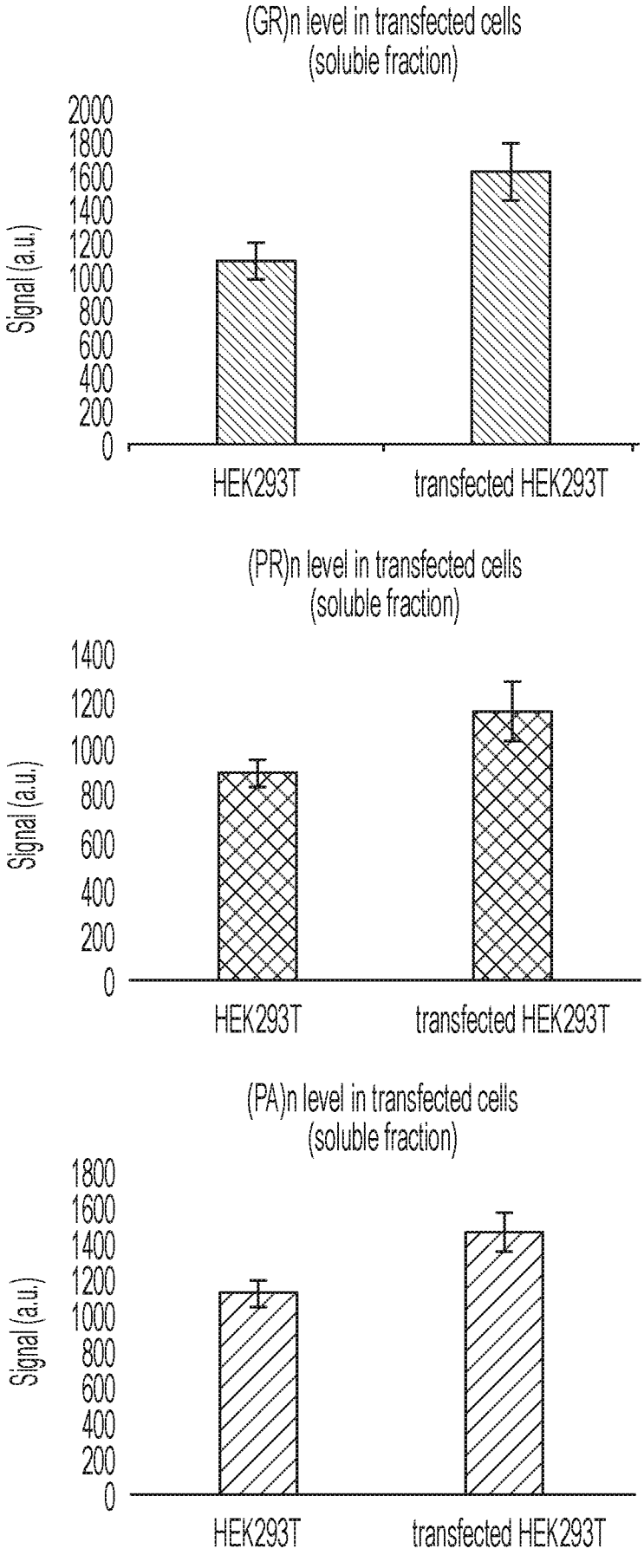

(GR)n level in transfected cells
(soluble fraction)
Signal (a.u.)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
HEK293T
transfected HEK293T
(PR)n level in transfected cells
(soluble fraction)
Signal (a.u.)
1400
1200
1000
800
600
400
200
0
HEK293T
transfected HEK293T
(PA)n level in transfected cells
(soluble fraction)
Signal (a.u.)
1800
1600
1400
1200
1000
800
600
400
200
0
HEK293T
transfected HEK293T

Figure 4

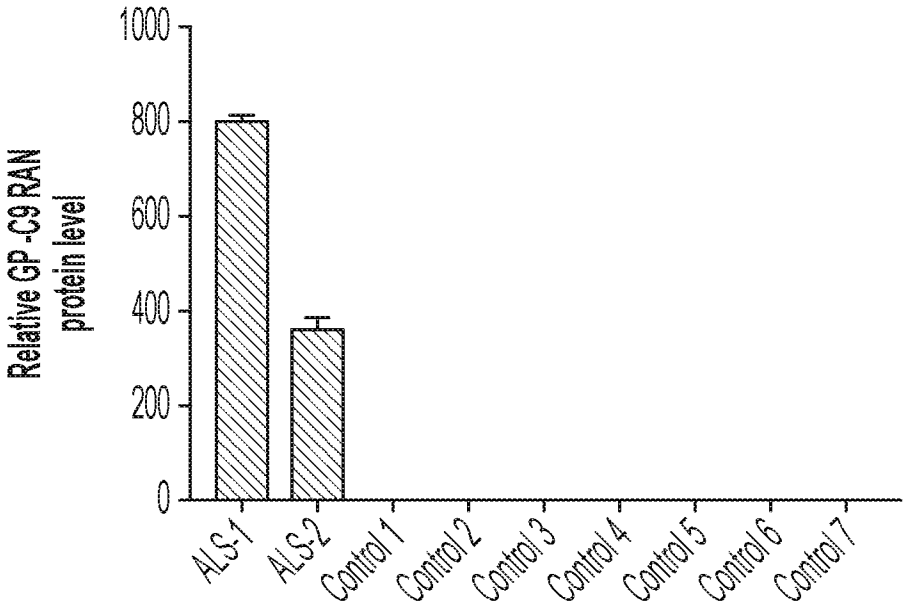


Figure 5

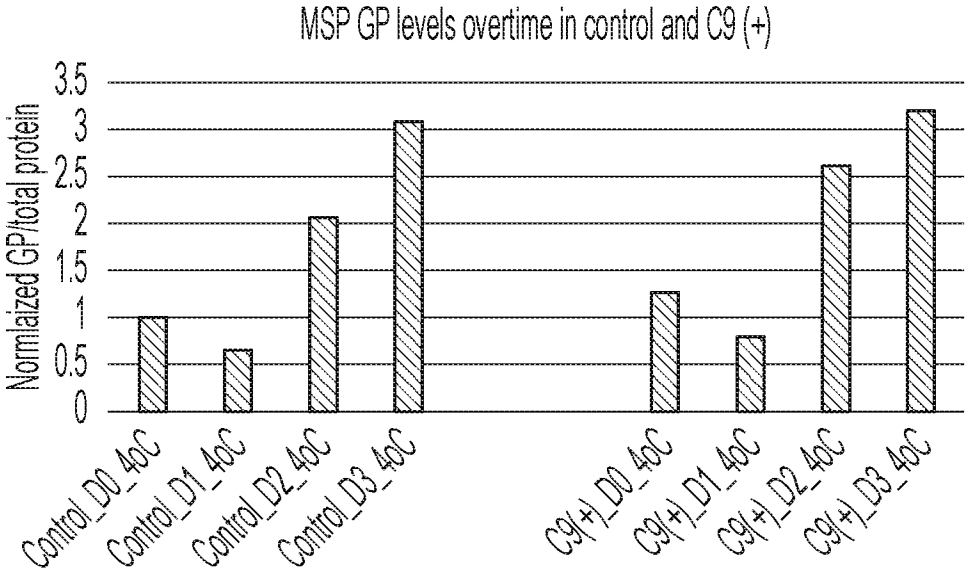


Figure 6A

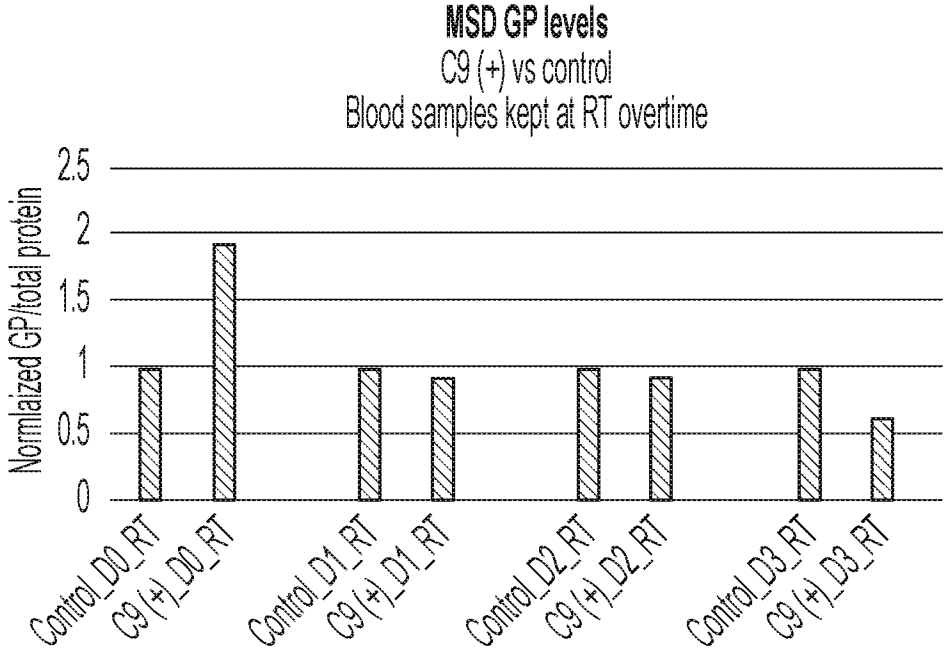
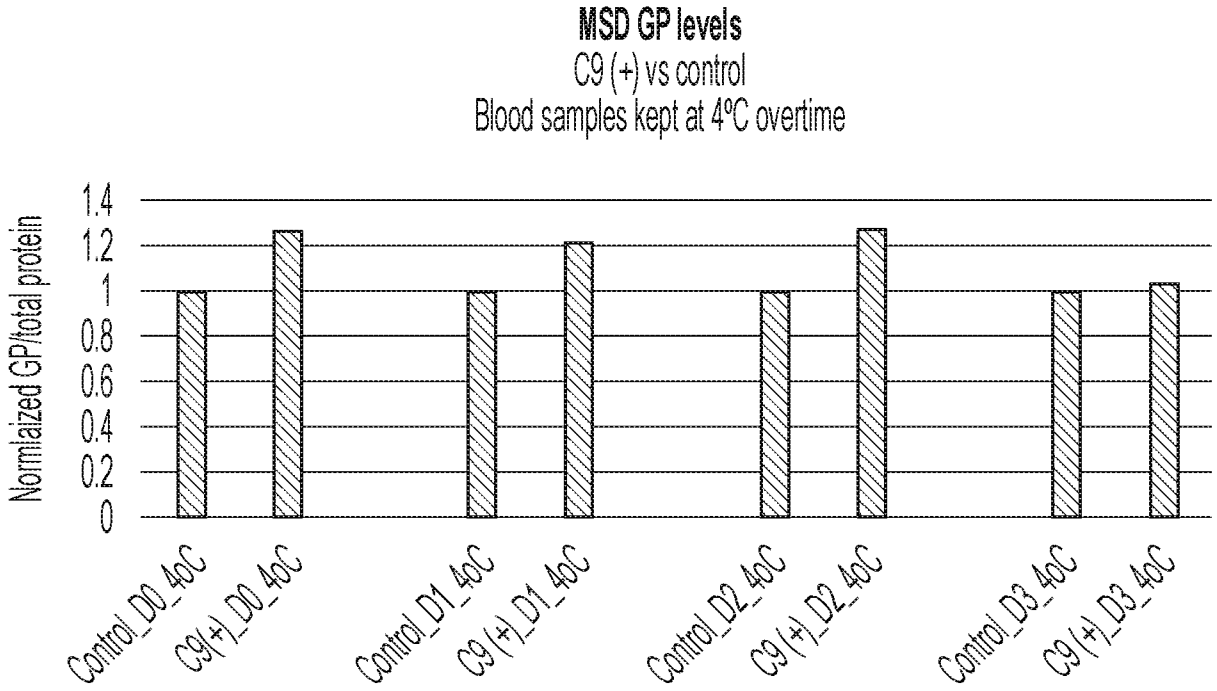

MSD GP levels
C9 (+) vs control
Blood samples kept at RT overtime
Normlaized GP/total protein
2.5
2
1.5
1
0.5
0
Control_D0_RT
C9 (+)_D0_RT
Control_D1_RT
C9 (+)_D1_RT
Control_D2_RT
C9 (+)_D2_RT
Control_D3_RT
C9 (+)_D3_RT

Figure 6B


MSD GP levels
C9 (+) vs control
Blood samples kept at 4ºC overtime
Normlaized GP/total protein
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control_D0_4oC
C9(+)_D0_4oC
Control_D1_4oC
C9 (+)_D1_4oC
Control_D2_4oC
C9 (+)_D2_4oC
Control_D3_4oC
C9 (+)_D3_4oC

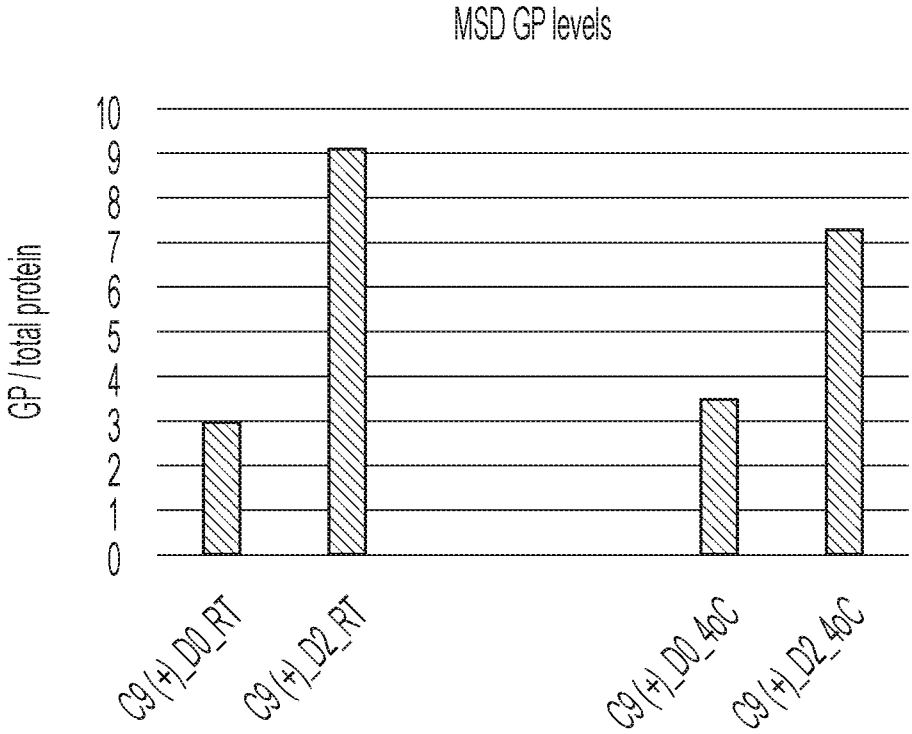

MSD GP levels
GP / total protein
10
9
8
7
6
5
4
3
2
1
0
C9 (+)_D0_RT
C9 (+)_D2_RT
C9 (+)_D0_4oC
C9 (+)_D2_4oC

Figure 6C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/52745

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-18, 25
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/52745

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 14/47, 16/18; C12Q 1/68; G01N 33/483, 33/487, 33/50, 33/53, 33/68 (2018.01)

CPC - C07K 14/47, 16/18; C12Q 1/6804, 1/6809, 1/6883; G01N 33/4833, 33/487, 33/5005, 33/5091, 33/5308, 33/6896

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2016/0025747 A1 (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.) 28 January 2016; paragraphs [0004], [0005], [0021], [0040], [0054], [0059], [0062], [0078], [0084], [0127], [0148], [0157], [0182], [0191], [0192]; Figure 29	1-2, 3/1-2, 4/3/1-2, 5/3/1-2, 19-20, 21/19-20 --- 22-23, 24/22-23
Y	US 2009/0312395 A1 (El-Tanani, MK et al.) 17 December 2009; abstract; paragraph [0080], [0147]	22-23, 24/22-23
A	(ZU, T et al.) RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. Proceedings of the National Academy of Sciences of the U.S.A. 17 December 2013, Epub 18 November 2013, Vol. 110, No. 51; pages E4968-E4977; DOI: 10.1073/pnas.1315438110	1-2, 3/1-2, 4/3/1-2, 5/3/1-2, 19-20, 21/19-20, 22-23, 24/22-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 November 2018 (01.11.2018)	06 DEC 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774