NEUROMICS

NF-H ELISA Kit User's Manual

Catalog Number:	EL22000	**Host:**	Chicken IgY and rabbit IgG
Product Type:	Affinity Purified	**Species Reactivity:**	Human, horse, cow, pig, chicken, rat,, mouse
Immunogen Sequence:	Phosphorylated axonal NF-h (pNF-H)	**MW:**	110 kDa

Applications: ELISA of blood, CSF and tissue extracts.
Dilutions listed as a recommendation. Optimal dilution should be determined by investigator.

Storage: Shipped on ice. Store at 4°C.

References:

Buki A and Povlishock JT. All roads lead to disconnection? – Traumatic axonal injury revisited. Acta Neurochir (Wien). 148:181-93 (2005).

Matsushige T, Ichiyama T, Anlar B, Tohyama J, Nomura K, Yamashita Y, Furukawa S. CSF neurofilament and soluble TNF receptor 1 levels in subacute sclerosing panencephalitis. J Neuroimmunology [Epub ahead of print] (2008).

Boylan, K., Yang, C., Crook, J., Overstreet, K., Heckman, M., Wang, Y., Borchelt, D. and Shaw, G. Immunoreactivity of the phosphorylated axonal neurofilament H subunit (pNF-H) in blood of ALS model rodents and ALS patients: Evaluation of blood pNF-H as a potential ALS biomarker. J. Neurochem 111:1182-1191 (2009).

Introduction:

This is an enzyme linked immunosorbent assay (ELISA) for the sensitive detection of pNF-H, one of the major proteins of the axon. When neurological disease or injury occurs, pNF-H leaks from damaged axons into the cerebrospinal fluid and blood, and can be detected by ELISAs such as this one. The level of pNF-H detected can give information about the degree of injury, the progression and recovery state and response of an individual to therapies. The kit can be used to quantify levels of this protein in tissue extracts, in cells in tissue culture in cerebrospinal fluid (CSF) and blood. It has been shown to detect pNF-H, released from damaged or degenerating axons in a variety of animal models of human disease and also in several human disease states. The kit has been shown to work in human, pig, rat, mouse, cat and dog, and is expected to work in many other species also.

The intermediate or 10nm filament protein/gene superfamily which also includes the keratins, the major structural proteins of skin. Neurofilaments are generally regarded as being composed of three major protein subunits referred to as NF-L, NF-M and NF-H. NF-L is the "low" or "light" subunit, NF-M is the "medium" or "middle" subunit and NF-H is the "heavy" or "high" subunit. This nomenclature is based on the apparent size of these proteins on SDS-PAGE gels, on which NF-L is typically ~68 kDa, NF-M is ~150 kDa and NF-H is ~200 kDa. The three proteins were discovered in the 1970s as a result of studies of axonally transported proteins (Hoffman and Lasek 1975). These three proteins are referred to as the neurofilament triplet. A fourth protein internexin, a.k.a NF66, which was discovered a little later as a protein which copurifies with the neurofilament triplet proteins (Pachter and Liem 1985). A fifth protein, peripherin, is found in some neurofilaments along with the other four proteins, mostly in the peripheral nervous system (Portier et al. 1983).

Neurofilaments are major proteins of neurons and are particularly concentrated in axons. The protein NF-H has some very unusual properties. Firstly, it contain 50 back to back hexa, hepta or octapeptide repeats each

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containing the sequence Lysine- Serine-Proline (KSP). The serine residues in these peptide repeats are in vivo phosphorylation sites, and, in axonal neurofilaments, these sites are heavily phosphorylated. The phosphorylated forms of NF-H are also quite resistant to proteases, which suggests that on being released from damaged and diseased axons, this very abundant protein might be particularly resistant to proteases. This means that detection of this protein in cerebrospinal fluid (CSF) and blood would provide information about the degree of axonal injury which has occurred (reviewed in Petzold 2005).

The pNF-H protein can be detected in quite large amounts following experimental spinal cord and brain injury in rats (Shaw et al. 2005). Levels of greater than 100 ng/mL of pNF-H were detectable in blood following serious spinal cord injury and lower but still easily detectable levels were seen in blood of animals given experimental brain injury. More recent studies have revealed considerable amounts of this protein in the blood of mice transgenic for mutations of human copper/zinc superoxide dismutase 1 which are associated with amyotrophic lateral sclerosis (ALS, Boylan et al. 2008). These mice develop an axonal degeneration pathology similar to that seen in humans with ALS, and blood pNF-H levels can be used to monitor this. Interestingly, pNF-H is detectable before the onset of obvious disease symptoms. Other studies show that pNF-H can be detected in tissue homogenates. Other experiments show that pNF-H can be detected in the plasma of humans suffering from optic neuritis (Petzold et al 2004) and in elevated levels in the cerebrospinal fluid (CSF) of individuals suffering from brain tumors and stroke (Petzold and Shaw 2007). More recent work has shown that the original pNFH ELISA can detect informative levels of pNF-H in the blood and CSF of a variety of CNS damage and disease states, such patients suffering from aneurysmal subarachnoid hemorrhage (Lewis et al. 2008), animal models of Multiple Sclerosis (Gresle et al. 2008), animal models of traumatic brain injury (Anderson et al. 2008), patients with Leber's hereditary optic neuropathy (Guy et al. 2008) and both animal models and patients with ALS (Boylan et al. 2009). The Boylan et al. study described a novel pNF-H ELISA that was developed making use of two monoclonal antibodies to pNF-H.

Protocol:

1) How the kit work: ELISA provides a convenient method for measuring the level of protein targets within a single, small-volume sample. This a 2-site sandwich enzyme linked immunoassay. We provide a plate pre-coated with capture chicken polyclonal antibody against pNF-H. the user incubates the samples on the plate and any pNF-H protein in the sample binds to the capture antibody. The user then incubates the plate with a solution containing rabbit polyclonal pNF-H detection antibody, which bines to the complex of antigen and captured Ab. The detection antibody is then itself bond by goat anti-rabbit-Horse Radish Peroxidase (HRP) conjugated antibodies. The user adds a substrate TMB solution which produces a blue colored product in wells containing HRP. The intensity of the color corresponds to the amount of pNF-H in the sample. Finally the user stops the reaction by adding H2SO4. The color changes from blue to yello and can be quantified by measurement on a suitable ELISA plate reader using an absorbance wave length of 450 nm
2) Kit Contents:
    a) **Plate**: One 96-well plate coated with purified anti-pNF-H capture antibody. The plate is ready to use, and can be split into 8 rows of 12 wells each if required. Each well is shipped with 50 µL of TBS with 5 mM NaN3 (see MSDS). Store at 4^{O}C.
    b) **pNF Standard**: Bonine pNF-H protein, lyophilized, 2 vials. Store at 4°C
    c) **Rabbit pNF-H:** Detection, rabbit polyclonal pNF-H antibody, 1 x 20 ul. Store at -20°C
    d) **HRP conjugated antibody**: Goat anti-rabbit IgG, HRP conjugated antibody, 1 x 200 ul. Store at -20°C
    e) **TBS**: 1xTBS, ready to use to rinse plate solution. 1 x 12 ml. Store at 4°C
    f) **TBST**: 1xTBS – Tween concentration. 1 x 12 ml. Store at 4°C
    g) **TBM**: 1 x TMB (Tetramethylbenzidine) substrate ready to use solution. 1 x 12 ml. Store at 4°C

3) Additional Materials and Equipment:
    a) Microplate reader (450nm)
    b) Microplate shaker
    c) Automated plate washer
    d) Multichannel pipette
    e) Appropriately-sized tubes for reagent preparation

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f) Polypropylene microcentrifuge tubes for preparing dilutions
g) Stop Solution (2 N H_2SO_4)
h) Wash butter (1x TBST)

4) Best Practices & Technical Hints:
   a) Do not mix or substitute reagents from different sources or different kits
   b) Dilute samples in polypropylene microcentrifuge tubes; use a fresh pipette tip for each dilution. Two common pipetting methods used for ELISA are standard (forward) and reverse. Use standard (forward) pipetting for the preparation for sample dilutions, and rverse pipetting for the addition of diluted samples, controls and reagents.
5) Procedure:
   a) Unpack the ELISA plate and make sure there are no problems with it. The plate can be broken down to 8 strips of 12 wells per strip, and one or more strips can be run at a time if desired.
   b) Rehydrate the plate with 1 x TBS. Add 100 ul of the solution and aspirate it.
   c) Designate two or three columns of the plate for the standard curve. Perform standard curve
   d) Add 50 µl of diluted samples per well or dilute them directly in the wells. Incubate the plate for at least 2 hours at room temperature
   e) Wash the plate 2X3 times with 300 µl of TBST using an automated plate washer, roate the plate 180 degrees between wash steps
   f) Add 100 µl of dection antibody solution (rabbit pNF-H, 1:1000 dilution or 12 mL of DB + 12 µL of rabbit pNF-H) to each well. Incubate the plate for at least 2 hours at room temperature or overnight at 4°C with gentle shaking
   g) Wash the plate 2X3 times with 300µl of TBST using an automated plate washer, roate the plate 180 degrees between wash steps
   h) Add 100 µl of a-Rb-HRP (1:100 dilution or 12 mL of DB + 120µl of a-Rb-HRP) to each well. Incubate the plate for 1-1.5 hours at room temperature with gentle shaking.
   i) Bring TMB solution to room temperature
   j) Wash the plate 2X3 times with 300µl of TBST using an automated plate washer, rotate the plate 180 degrees between wash steps.
   k) Add 100 µl to TMB (the solution should be at RT developing solution. Incubate the plate until you will reach a desired color change; the highest standard should be dark blue, while Blank should stay colorless. Time of incubation varies from 5 to 20 minutes.
   l) Stop reaction with 50µl of sotp solution; 2N H2SO4. Note, that the color changes from blue to yellow. No incubation in stop solution is required before reading plate
   m) Read the plate on the plate reader spectrophotometer at 450nm. Follow the manufacture's instruction for detail of operation.
6) Standard Curve:
   a) The highest standard point (10ng/mL) is obtained by reconstituting one vial of lyophilized pNF-H stand with 400µL of the dilution buffer. This would be tube #1
   b) Label 6 more microtubes. One for each standard point: 5, 2.5, 0.625, 0.313 and 0.156 nng/ml (tubes #2-7).
   c) Add 200µl of dilution buffer into each tubes starting form the second one
   d) Preform serial 1:2 dilutions from the standard – 10 ng/ml by transferring 200µl for the preceding sample to 200 µl of DB in the next tube
   e) Vortex well before the next transfer. And use a new pipette tip during each transfer.
   f) Add 50µl of each standards to the desired wells on the plate
   g) Add 50 µl od dilution buffer along to the wells designated fro the Blank
7) Tissue Samples: Tissues should be excised, carefully weighed and then homogenized in 4 M urea, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF in 10 mM Tris-HCl at pH7.2. We used 10 mg of wet weight of tissue per mL of buffer. After homogenization the material was centrifuged in microcentrifuge tubes for 5 minutes at top speed (10-14,000 rpm). 1 µL or less of CNS tissue will give a strong signal in the ELISA.
8) CSF Samples: Samples of CSF from control rodents have undetectable levels of pNF-H, while those with CNS injuries frequently have levels in 5 ng/mL range. Samples of 10 µL, made up to 50 µL with dilution buffer, are probably a good starting point.
9) Blood Samples: Blood can be assayed directly, but for most assay purposes is either allowed to clot or is centrifuged down to pellet out red and white cells. Clotting requires several hours at room temperature,

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and the clear yellow liquid, the serum, can be used for pNF-H analysis. More rapid and convenient approach is to spin the blood down at top speed in a centrifuge and take the clear yellow liquid, the plasma, for analysis (serum is therefore basically the same as plasma, except that the blood clotting factors are absent). We have obtained workable results with blood, serum and plasma, but have standardized on plasma for reproducibility. We take a series of blood samples and freeze each one at 20^{O}C or lower. Then, when we have a complete series, we thaw them all out and pellet out the red and white cells in microcentrifuge tubes at top speed for 5 minutes in a centrifuge. The plasma is then run in the ELISA.

We have found that rats with serious spinal cord injuries will give strong signals with as little as 1 µL of plasma. Rats with brain injuries needed 10 µL or more. Mice generally appear to give stronger signals, and we have seen very strong signals in the plasma for mice transgenic for mutant forms of human copper/zinc superoxide dismutase 1 (SOD1). Humans with these mutations develop a serious form of amyotrophic lateral sclerosis (ALS) and the transgenic mice develop a progressive ALS-like phenotype. The pNF-H ELISA can therefore be used to measure axonal degeneration in these and presumably many other transgenic models of human disease.

c) Incubate for 1 hour at room temperature with gentle shaking.
d) Wash plates extensively. We recommend a dedicated plate washer using TBST (see below for recipe). A frequent problem with commercial plate washers is that some of the nozzles may become partially or wholly blocked, leading to inconsistent washing. To get around this problem we carefully monitor our washing apparatus to make sure that each nozzle is washing correctly. As an extra precaution we typically wash each plate twice, flipping the plate through 180 degrees between washes. We program the washer to perform. 5 cycles of washing. **In our experience, careful and thorough washing is the single most important step in getting good quality data from ELISAs.**
c) Add 100 µL of HRP-conjugated detection antibody per well. This should be made by dilution of 5 µL antibody in the appropriate vial 1:2,000 in dilution buffer. Incubate for 1 hour at room temperature with gentle shaking.
d) Wash plates extensively, as step d) above.
e) Add 100 µL development reagent. This is equal volumes of development buffer A and B. A blue color should be visible in wells with the most concentrated standards after only a few minutes incubation. Incubate with mild shaking. The optical density at 600 nm absorbance can be monitored at any time. If the reaction in the samples is weak, development can be continued for up to one hour.
f) Reaction can be stopped at any time by addition of 50 µL per well of 2N H2SO4 producing a yellow solution which is monitored at 450 nm absorbance.
g) Plot out the data using software in your plate reader, or using Microsoft Excel or similar program (see below). Think about them!

12) Interpretation of results: The level of pNF-H detected in a particular situation should provide an unbiased quantification of the amount of axons present. Analysis of the levels in serum and CSF should provide information on the degree of axonal injury which has occurred. We typically use the top row of the plate for a standard curve which we generate by serially diluting standard 1:1 down the plate, leaving the last well without standard. It is important to leave the last well blank, which will give you some idea of the nonspecific background. The data below, shows, in the second column, the actual OD450 values obtained in a real experiment in which 5 µL of the pNF-H standard was serially diluted down the 12 wells of a row on a 96-well plate, with the last well with no standard.

To plot this data in Microsoft Excel select the Insert menu and then the Scatter plot, and choose the option which displays the data points and joins them with lines. The graph of this data (Fig. 1, top right) is a fairly typical one showing a linear range throughout the standard curve.

The lower range of this standard curve (Fig. 1, bottom right) is used for quantitation as it more closely resembles values typically found in humans.

The point at which the line intercepts the Y-axis gives the background of the assay, and this value can be subtracted from all the experimental data. For this data set that value is 0.071 OD450 units. This factor and slope of the line give the relationship of the OD450 units to ng/mL values. This can be calculated by measurement or by using a graphing software.

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In Excel (PC), you can use the right mouse click on the plot and select the "Add Trendline" option, followed by Linear regression.

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c (ng/mL)	OD450
12.500	3.75
6.250	1.88
3.125	0.99
1.563	0.503
0.781	0.276
0.391	0.17
0.195	0.108
0.098	0.086
0.049	0.076
0.024	0.073
0.012	0.071
0	0.071

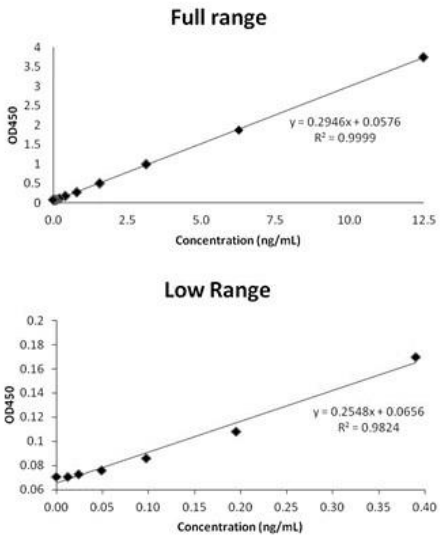

Figure 1. Left: Absorbance at OD450 obtained with the standard. Top right: graph of the total range of the standard curve. Bottom right: plot of low range of the standard curve.

There is also an options menu from which you can select check boxes to "Display Equation on chart" and "Display R-squared value on chart". When you have done all this the plot will look as above. The equation y = 0.2548x+0.0656 allows you to translate OD450 values directly to ng/mL concentrations. The R2 value tells how close to a straight line the data actually is, and a value of 0.9824 is pretty good. You can try different regions of the linear end of the graph looking for the R2 value closest to 1. So, to translate the OD450 value in cell B2 of the ELISA well into ng/mL values, you can simply put the equation "=(B2-0.0656)/0.2548"into an appropriate cell in the spreadsheet and hit ENTER. If you copy this equation into the next 11 cells to the right Excel will automatically change the B1 to B2 etc. along the series. If you copy this entire row and paste into the 6 rows below, Excel will change B2 to C2 etc, so you can calculate values appropriate for the rest of the 96 well dataset. The data in each well is therefore transformed from OD450 to ng/mL. Of course, these values are ng/mL in the ELISA well, so if these samples were diluted by some factor, you need to multiply by that factor to get the true value.

13) Formulas for reagents Tris buffered saline- Tween (TBST): To make 1 L of 10X TBST solution weight out 12.1 g Tris base and 87.66 g NaCl, dissolve in 800 ml distilled water and adjust pH with concentrated HCl (be careful!) to 7.5. Store at room temperature. 1X TBS is 10 mM Tris/HCl, 150 mM NaCl, pH=7.5. To make TBST, add 1 g of Tween 20/L. (Tween 20 is also known as polyoxyethylene 20-sorbitan monolaurate, Fisher BP337-500).

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Reaction stopping solution: 2N H2SO4 - make up with care

Warranty: Data presented here were obtained using the protocol outlined here. Any modification or change in the protocol not suggested by Neuromics. may impact the results, in which event Neuromics disclaims all warranties.
Intended Use: This ELISA capture assay is intended for research use only. The total assay time is less than 5 hours. The kit measures the levels of phosphorylated NF-H in blood, cerebrospinal fluid, tissue extracts, and in cells in tissue culture. Quality control pNF-H purified from bovine spinal cord. No commercial use of this product is allowed.

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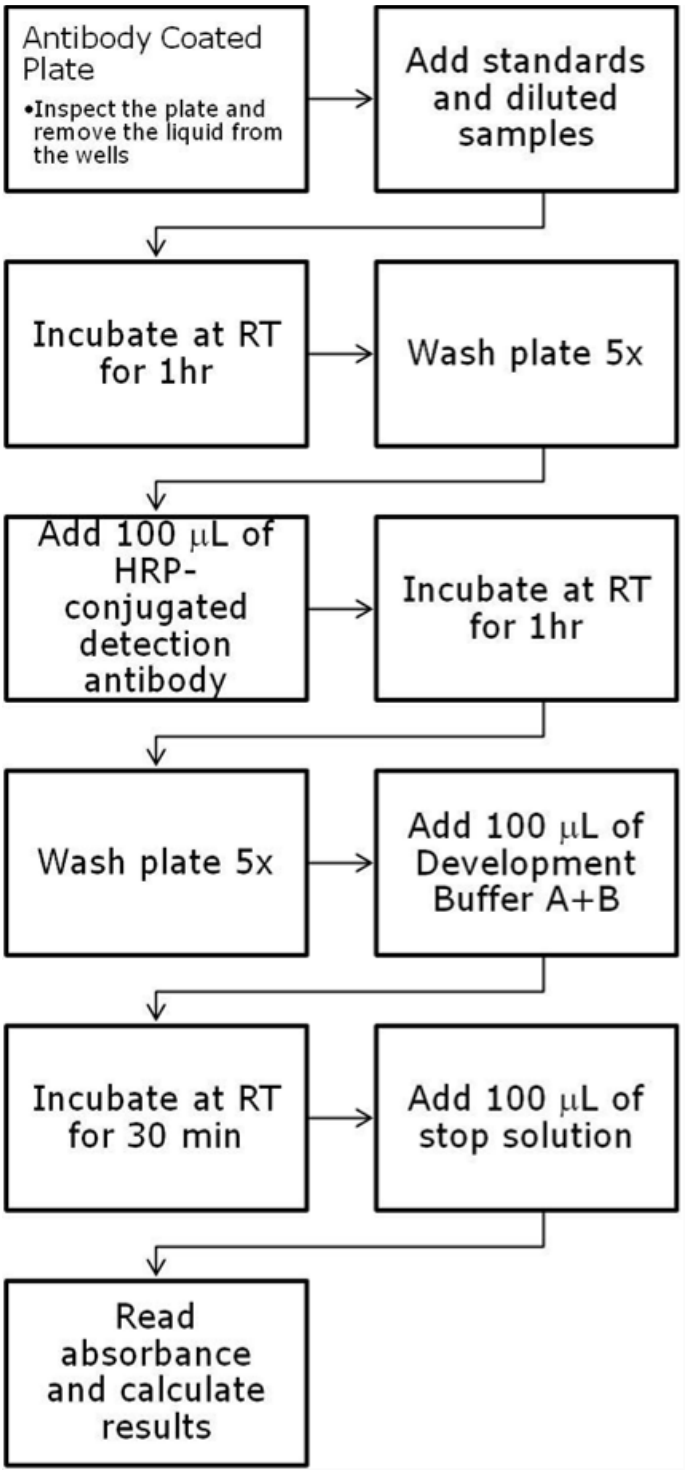

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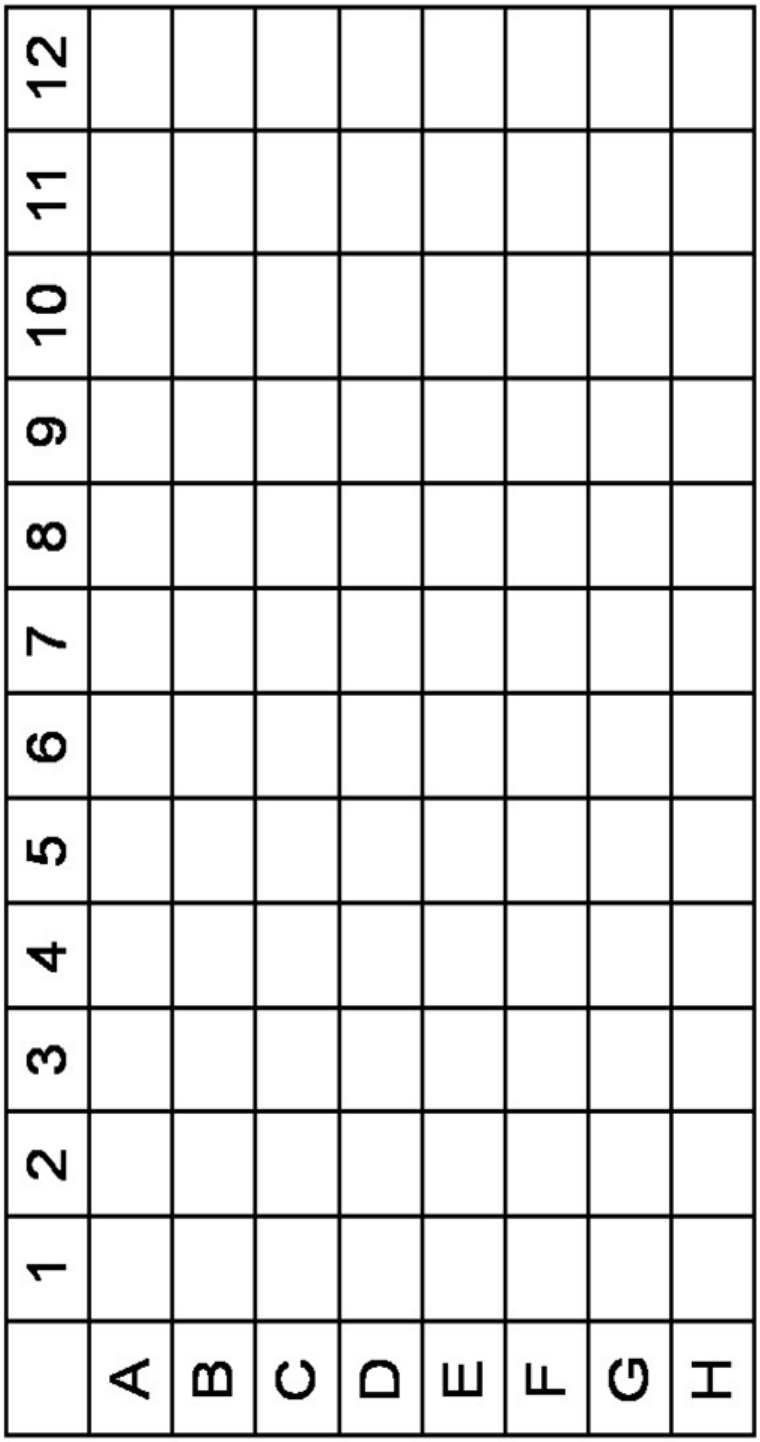

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