HUMAN NEUTROPHIL ELASTASE SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: NEL-SP-20

FOR RESEARCH USE ONLY

INTRODUCTION
Neutrophil elastase (NE) is a serine protease that is secreted by neutrophils. Increased serum levels have been associated with colorectal cancer (ref 1). Increased sputum levels have been found in COPD patients with bacterial infection (ref 2) and in patients with bronchiectasis (ref 3).

PRINCIPLE OF THE ASSAY
The human NE SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 4) assay uses NE antibodies that were developed at Life Diagnostics, Inc. One is conjugated to horseradish peroxidase (HRP), the other to acridan, a chemiluminescent substrate. When the HRP and acridan conjugates bind to NE they are brought into proximity. With the addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of chemiluminescence. Acridan conjugated antibodies distant from HRP (not bound to NE) produce no signal. This principle allows the development of a homogeneous assay that allows rapid measurement of NE.

Standards and diluted samples are mixed with HRP and acridan-conjugates in the wells of the 96-well SPARCL™ plate[2] provided with the kit. After incubation for 30 minutes on a shaker at 25°C and 150 rpm, the plate is placed into a luminometer. Trigger solution containing hydrogen peroxide is injected into each well and luminescence is immediately measured. The concentration of NE is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS
Materials provided with the kit:
- Anti-NE HRP conjugate **Store ≤ -70°C**
- Anti-NE acridan conjugate **Store ≤ -70°C**
- NE stock (lyophilized), 2 vials **Store ≤ -70°C**
- Diluent; CSD50-1, 2 x 50 ml
- Trigger solution; TS7-1, 7 ml
- White SPARCL™ plate (12 x 8-well)
- Clear untreated 96-well plate
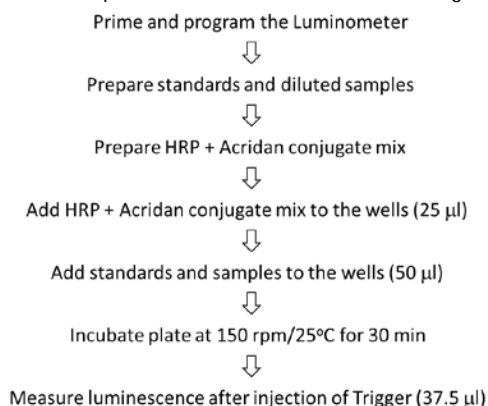
Materials required but not provided:
- Precision pipettes and tips
- Polypropylene microcentrifuge tubes
- Vortex mixer
- Plate incubator/shaker
- Luminometer capable of simultaneous injection/measurement
- Curve fitting software

STORAGE
Store the HRP conjugate, acridan conjugate and NE stock at -70°C (they may be stored at -20°C for one week). The remainder of the kit should be stored at 2-8°C. The SPARCL™ plate should be kept in a sealed bag with desiccant and antioxidant. The kit will remain stable for at least six months from the date of purchase, provided that the components are stored as described.

GENERAL INSTRUCTIONS
1. This kit is for research purposes only. Under no circumstances should it be used for diagnostic purposes.
2. Please take the time to completely read all instructions before starting your assay. Contact us if you need clarification.
3. All reagents used in the assay should be allowed to reach room temperature (25°C) before use.
4. It is important that standards and samples be added to the SPARCL™ plate quickly. If testing large numbers of samples, rather than pipetting standards and samples directly into the white SPARCL™ plate using a single channel pipettor, we recommend the following. First, pipette an excess volume of standards and samples into appropriate wells of the clear 96-well plate. Then use an 8- or 12-channel multipipettor to quickly and efficiently transfer 50 μl aliquots to the appropriate wells of the white SPARCL™ plate. The wells of the clear plate have a maximum volume of 300 μl.
5. Follow the sequence of events below when running the assay.

Prime and program the Luminometer
⇩
Prepare standards and diluted samples
⇩
Prepare HRP + Acridan conjugate mix
⇩
Add HRP + Acridan conjugate mix to the wells (25 μl)
⇩
Add standards and samples to the wells (50 μl)
⇩
Incubate plate at 150 rpm/25°C for 30 min
⇩
Measure luminescence after injection of Trigger (37.5 μl)

STANDARD PREPARATION
The NE stock is comprised of pure human NE lyophilized in a stabilizing carrier protein matrix.
1. Label 5 polypropylene tubes as 10, 5, 2.5, 1.25, and 0.625 ng/ml and dispense 150 μl of diluent into each tube.
2. Reconstitute the lyophilized NE stock as directed on the vial label. Mix gently. This provides the 20 ng/ml standard.
3. Pipette 150 μl of the 20 ng/ml NE standard into the tube labeled 10 ng/ml and mix. This provides the 10 ng/ml NE standard.
4. Similarly prepare the remaining standards by two-fold serial dilution.

SAMPLE PREPARATION
We tested ascites fluid samples from cancer patients and found NE levels ranging from undetectable to >1000 ng/ml. Because of the wide range of possible values, we cannot recommend a single dilution that is appropriate for all samples. However, ascites fluid, serum and urine samples should be diluted at least 80-fold in order

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[1]The SPARCL technology was developed by Lumigen Corp.

[2]The plate provided with the kit has been treated with a reagent that reduces background chemiluminescence. Untreated plates cannot be used.

to eliminate matrix and prozone effects.[3] We **suggest** an initial dilution of 80-fold.

CONJUGATE MIX PREPARATION

Instructions for preparation of the conjugate mix are detailed on the box that contains the HRP and acridan conjugates. If necessary, after thawing, briefly centrifuge to ensure that the contents are at the bottom of the tubes. Prepare the mix shortly before use using the diluent provided with the kit.

LUMINOMETER SETUP

1. The luminometer must be capable of injection and simultaneous measurement of luminescence without any delay.
2. Prime the luminometer injection port with 1 ml of trigger solution.
3. Place the injection needle into the injection port as needed for BMG luminometers.
4. Program the luminometer to inject 37.5 μl of trigger solution per well and to measure from time zero for 1 second (50 x 0.02 second intervals).
5. Define the format of the assay using the luminometer software.
6. Because the white SPARCL™ plate is provided as a 12 x 8-well strips, allowing use of fewer than 96-wells, make sure that the luminometer is programmed to inject trigger solution only into the wells being used.
7. We use a BMG LUMIstar Omega set at a gain of 3600. Optimal gain should be determined by the end user.
8. There are a number of manufacturers of luminometers that are equipped to run a SPARCL™ assay. Please contact Life Diagnostics or Lumigen (www.lumigen.com) to discuss your luminometer.

PROCEDURE

1. Before starting the assay ensure that the luminometer is primed with trigger solution and that the injection needle is positioned in the injection port.
2. Secure the desired number of SPARCL™ 8-well strips in the holder. Immediately seal unused strips in the resealable bag with desiccant and antioxidant. Store unused strips at 2-8°C.
3. Aliquot 25.0 μl of conjugate mix into each well.
4. Dispense 50.0 μl of standards and diluted samples into the wells (we recommend that standards and samples be tested in duplicate).
5. Incubate on an orbital micro-plate shaker at 150 rpm 25°C for 30 minutes.
6. After the 30-minute incubation, place the plate in the luminometer and measure luminescence after injection of trigger solution (37.5 μl).
7. Remove the plate from the luminometer and discard the used strips. Keep the plate frame if future use is intended.
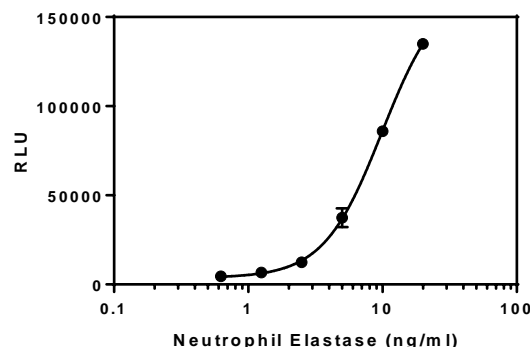
CALCULATION OF RESULTS

1. Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells. We routinely use the sum of RLU values from a 100-980 ms data collection window.
2. Determine the sum of RLU values within the data collection window for the standards and samples.

3. Using graphing software, construct a standard curve by plotting the sum of the RLU values for the standards versus $\log_{10}$ of the concentration and fit to a sigmoidal, 4PL model.
4. Derive the concentration of NE in the samples from the standard curve (remember to derive the concentration from the antilog).
5. Multiply the derived concentration by the dilution factor to determine the concentration of NE in the original sample.
6. If the sum of the RLU values of diluted samples fall outside the standard curve, samples should be appropriately diluted and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve is shown below. This curve is for illustration only and should not be used to calculate unknowns. A standard curve should be run with each experiment.

NE (ng/ml)	RLU
20	134944
10	85882
5	37463
2.5	12432
1.25	6651
0.625	4511

REFERENCES

1. Ho, Ai-Sheng, et. al. Neutrophil elastase as a diagnostic marker and therapeutic target in colorectal cancers. Oncotarget 5(2):127-131 (2014)
2. Thulborn S, et al. S45 Evaluating the sensitivity and specificity of active neutrophil elastase as a biomarker for bacterial infection in subjects with copd. Thorax 71:A28 (2016)
3. Gramegna A, et al. Neutrophil elastase in bronchiectasis. Respiratory Research 18:211 (2017)
4. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

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[3] SPARCL assays are homogeneous and therefore susceptible to a prozone effect. Samples with very high levels of NE may give false low values. We therefore recommend that samples be tested over a range of dilutions.