ELISA for assessing the burden of Neutrophil Extracellular Traps (NETs) in clinical serum samples.

kathryn.hally [1,2,3]

[1]Department of Surgery and Anaesthesia, The University of Otago, Wellington, New Zealand;
[2]School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand;
[3]Wellington Cardiovascular Research Group, Wellington, New Zealand.

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The University of Otago, Wellington, New Zealand

kathryn.hally

ABSTRACT

This protocol steps through the ELISA procedure for detecting three NET-specific biomarkers: 1) myeloperoxidase-DNA (MPO-DNA) complexes, 2) neutrophil elastase-DNA (NE-DNA) complexes, and 3) citrullinated histone H3 (H3(cit)).

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Preparing ELISA standards. 6h

1 6h

Supernatant from PMA-stimulated neutrophils will be used as the standard for the MPO-DNA and H3(cit) ELISAs.

Carry out the following to obtain NET-rich supernatant.

Isolating granulocytes:

- From a healthy volunteer, draw **10 mL blood** into EDTA blood collection tubes.
- To two 15 mL centrifuge tubes, add **5 mL Polymorphprep** .

Polymorphprep **Axis-Shield Catalog #1114683**

- Carefully layer **5 mL undiluted blood** on top of each Polymorphprep layer.
- Centrifuge these tubes **650 x g, 25°C, 00:30:00 , with soft start and brakeless deceleration** .
- Following centrifugation, two cell layers will have formed which are distinct from the packed red blood cell fraction. Aspirate of and discard the first cell layer (PBMCs).
- Carefully aspirate off the remaining granulocyte layer and pool in a 50 mL centrifuge tube and add 1x PBS to **25 mL** .
- Centrifuge at **100 x g, 4°C, 00:20:00** .
- Resuspend the granulocyte pellet in **1 mL ice-cold ddH2O** **On ice** . This will lyse any contaminating red blood cells.
- After **00:00:30** , add **24 mL 1x PBS** .
- Centrifuge at **100 x g, 4°C, 00:20:00** .
- Resuspend in **1 mL cell culture media** , count and adjust cell count to 2 x 10^6 cells/mL. Cell culture media used here: 10% FCS, 2 mM L-glutamate, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.01M HEPES buffer, 0.1% β-mercaptoethanol, 0.01 nM non-essential amino acids.

Diluting phorbol 12-myristate 13-acetate (PMA):

- PMA is provided as a lyophilized powder that is reconstituted in DMSO. Dilute PMA to a final concentration of **200 Nanomolar (nM)** using cell culture media. Ensure that the concentration of DMSO does not exceed 0.1% in this solution.

Phorbol 12-myristate 13-acetate (PMA) **Sigma Aldrich Catalog #P1585**

PMA stimulation:

- Add isolated granulocytes with diluted PMA in a 1:1 ratio (50 µL: 50 µL) into wells within a 96-well non-tissue culture treated plate.
- Incubate for **04:00:00** at **37 °C and 5% CO2** .
- After incubation, centrifuge at **400 x g, 25°C, 00:20:00** .
- Aspirate off the NET-rich supernatant, aliquot and store at **-80 °C** until required. Be careful not to disturb the neutrophil monolayer, only collect cell-free supernatant.

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Repeat:

- Repeat this protocol for a total of 10 times. Each time, draw blood from a new healthy donor.

2 3d

For the NE-DNA ELISA, a number of unknown clinical serum samples should be screened to determine a number of samples (preferably > 5) that show the highest level of endogenous NE-DNA complexes.

The NE-DNA ELISA procedure described below can be used to determine these samples.

3 30m

Preparation and storage of NET standards:

- *For MPO-DNA and H3(cit) ELISAs.*

For each of 10 healthy volunteers, NET-rich supernatant from PMA-stimulated neutrophils has been aliquoted and stored. Before proceeding with NET ELISAs, thaw an aliquot from each healthy volunteer and pool.

Aliquot out pooled NET-rich supernatant in **150 µl aliquots** and store at **-80 °C** until required.

- *For NE-DNA ELISA.*

Having identified a number of clinical serum samples with high endogenous levels of NE-DNA complexes, thaw an aliquot from each sample and pool.

Aliquot out pooled serum in **150 µl aliquots** and store at **-80 °C** until required.

Solutions to be prepared for ELISAs. 1h 10m

4 10m

For Day 1:

- Coating buffer: **100 mL 1x PBS**

Phosphate buffered saline **Sigma Aldrich Catalog #P4417-50TAB**

5 30m

For Day 2:

- Wash buffer (0.1% Tween 20 in 1X PBS): **1.5 mL Tween 20** + **1498.5 mL 1X PBS** , adjust to **pH 7.4**

Tween 20 **Sigma Aldrich Catalog #P1379**

- Assay diluent (1% BSA in 1X PBS): **0.8 g BSA powder** + **80 mL 1x PBS** , filter before use

- Blocking buffer (5% BSA in 1X PBS): **4.0 g BSA powder** + **80 mL 1x PBS** , filter before use

Bovine serum albumin **MP Biomedicals Catalog #0219989680**

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6 30m

For Day 3:

- Stop solution (1.8M H2SO4): **5.5 mL sulfuric acid (H2SO4)** + **44.5 mL 1x PBS**

Sulfuric acid (H2SO4) **Sigma**
Aldrich Catalog #258105

- Substrate buffer (0.11M acetate buffer): **1.5 g sodium acetate** + **100 mL ddH2O** , adjust to **pH 5.5** with 100% acetic acid. Can be stored for up to two weeks refrigerated.

Sodium acetate trihydrate **Sigma**
Aldrich Catalog #S8625

- 6 mg/mL TMB solution: **9 mg TMB** + **1.5 mL DMSO** . Protect against light exposure. Can be stored for up to one month at room temperature.

3355-Tetramethylbenzidine (TMB) powder **Sigma**
Aldrich Catalog #860336

- 3% hydrogen peroxide: **10 µl 30% hydrogen peroxide** + **90 µl ddH2O** . Can be stored for up to one week refrigerated.

- TMB substrate solution: **42 mL substrate buffer** + **700 µl TMB solution** + **42 µl 3% hydrogen peroxide** . Protect against light exposure.

ELISA day 1. 25m

7 15m

- Label three 96-well ELISA plates as follows: MPO-DNA, NE-DNA, H3(cit).

Nunc MaxiSorp™ flat-bottom **Thermo**
Fisher Catalog #44-2404-21

- Add **200 µl coating buffer** to each well and incubate for **00:01:00** .

- Following this, flick off coating buffer and bang each plate face down on paper towels.

8 10m

- Dilute each capture antibody using coating buffer, following the table below:

Type of ELISA	Capture antibody	Dilution	Antibody amount	Coating buffer amount	Final volume
MPO-DNA	MPO	1:300	35 µL	10,465 µL	10,500 µL
NE-DNA	Elastase	1:300	35 µL	10,465 µL	10,500 µL
H3(cit)	Histone	1:100	105 µL	10,395 µL	10,500 µL

[Anti-histone antibody (clone H11-4) is supplied as a part of the Cell Death Detection ELISA]

Mouse anti-human myeloperoxidase (MPO) clone 4A4 **Bio-rad**
Laboratories Catalog #0400-0002

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Mouse anti-human neutrophil elastase clone ELA10-101.5 **Life Technologies Catalog #MA1-10608**

Cell Death Detection ELISA **Roche Catalog #11544675001**

- Add **100 µl appropriate diluted capture antibody** to each well in the ELISA plate.
- Seal each plate with a cover slip and incubate **Overnight** at **4 °C** .

ELISA day 2. 4h 20m

9 3h 20m

- Thoroughly remove the capture antibody by flicking off the solution and banging each plate face down on paper towels.
- Add **200 µl wash buffer** to each well and incubate for **00:01:00** . Flick and bang again.
- Repeat another four times (five washes in total).
- Add **200 µl blocking buffer** to each well, cover and incubate for **03:00:00** at **Room temperature** .

10 30m

- During this incubation, dilute all unknown clinical serum samples with assay diluent as follows:

A	B	C	D
ELISA	**Dilution (% of final volume)**	**Amount of serum**	**Amount of assay diluent**
MPO-DNA	5%	6 µL	114 µL
NE-DNA	40%	48 µL	72 µL
H3(cit)	20%	24 µL	96 µL

- Also during this incubation, dilute NET standards with assay diluent as follows:

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A	B	C	D	E	F	G
ELISA	**Type of standard**	**Dilution of Standard 1 (% of final volume)**	**Amount of standard**	**Amount of assay diluent**	**Serial dilution**	**Concentration of standards**
MPO-DNA	Supernatant from PMA-stimulated neutrophils	5%	12 µL	228 µL	1:1	5.00, 2.50, 1.25, 0.63, 0.31, 0.16
NE-DNA	Pooled clinical serum samples with high endogenous levels of target antigen	50%	120 µL	120 µL	1:1	50.00, 25.00, 12.50, 6.25, 3.13, 1.56, 0.78
H3(cit)	Supernatant from PMA-stimulated neutrophils	40%	96 µL	144 µL	1:1	40.00, 20.00, 10.00, 5.00, 2.50, 1.25

All sample dilutions should be empirically established according to spike-and-recovery and parallelism experiments. Likewise, the concentrations for the standard should be empirically established to ensure saturation and acceptable recovery. We provide these dilutions as a starting point.

11 30m

- After three hours of blocking, wash each plate five times with wash buffer as described above.
- Add **50 µl standard** or **50 µl diluted serum** to each well, and ensure that each standard/serum sample is run in duplicate.
- Cover and incubate **Overnight** at **4 °C** .

ELISA Day 3. 6h 40m

12 2h 30m

- Dilute each detection antibody using assay diluent, following the table below:

Type of ELISA	Detection antibody	Dilution	Antibody amount	Coating buffer amount	Final volume
MPO-DNA and NE-DNA	DNA-Peroxidase	1:200	105 µL	20,895 µL	21,000 µL
H3(cit)	H3(cit)	1:4000	2.5 µL	9,997.5 µL	10,000 µL

[Anti-DNA-peroxidase (clone MCA-33) is supplied as a part of the Cell Death Detection ELISA]

All antibody dilutions should be empirically established to ensure maximal signal-to-noise ratio, but we provide these dilutions as a starting point.

Cell Death Detection

ELISA **Roche Catalog #11544675001**

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Anti-histone H3 (citrulline R2 R8 R17) antibody **Abcam Catalog #ab5103**

- Wash each plate five times with wash buffer and add **100 µl diluted detection antibody** to each well in the ELISA plate.
- Cover and incubate for **02:00:00** at **Room temperature** in the dark.

13 30m

- During this incubation, make up the second detection antibody for the H3(cit) ELISA using assay diluent, as follows:

A	B	C	D	E	F
Type of ELISA	**Detection antibody**	**Dilution**	**Antibody amount**	**Coating buffer amount**	**Final volume**
H3(cit)	Goat anti-rabbit IgG-HRP	1:2500	4.2 µL	10,495.8 µL	10,500 µL

All antibody dilutions should be empirically established to ensure maximal signal-to-noise ratio, but we provide these dilutions as a starting point.

Goat anti-rabbit IgG HRP **Bio-rad Laboratories Catalog #1706515**

- Wash each plate five times with wash buffer.

14 1h 10m

FOR MPO-DNA and NE-DNA ELISA plates:

- Add **100 µl TMB substrate solution** to each well, cover and incubate for **00:45:00** at **Room temperature** in the dark.
- Stop the colour change reaction by adding **100 µl stop solution** .
- Read the optical density at 450 nm.

15 2h 30m

For the H3(cit) ELISA plate:

- Add **100 µl second detection antibody** to each well, cover and incubate for **01:00:00** at **Room temperature** in the dark.
- Wash the plate five times with wash buffer.
- Add **100 µl TMB substrate solution** to each well, cover and incubate for **00:45:00** at **Room temperature** in the dark.
- Stop the colour change reaction by adding **100 µl stop solution** .

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- Read the optical density at 450 nm.

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