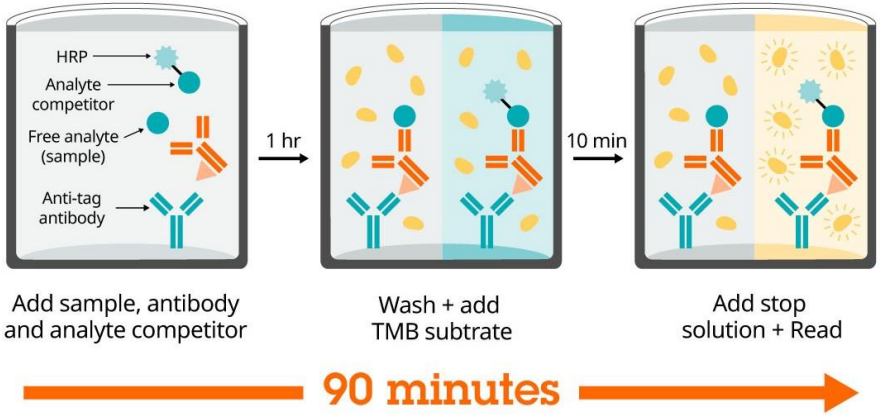
Ab315264 – 3-Nitrotyrosine SimpleStep ELISA® Kit - Extracellular

For the quantitative measurement of 3-Nitrotyrosine in serum, plasma (citrate), plasma (EDTA), plasma (heparin), and urine.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/Ab315264



Storage and Stability: Store kit at 2-8°C immediately upon receipt. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Standard Preparation and Reagent preparation sections.

Materials Supplied

Item	Quantity 1 x 96 tests	Quantity 10 x 96 tests	Storage Condition
3-Nitrotyrosine CaptSure™ Conjugate 50X	80 µL	10 x 80 µL	+4°C
3-Nitrotyrosine HRP Conjugate 50X	80 µL	10 x 80 µL	+4°C
3-Nitrotyrosine Lyophilized Standard	2 Vials	10 x 2 Vials	+4°C
Antibody Diluent 4BI	8 mL	10 x 8 mL	+4°C
Sample Diluent NS	12 mL	2 x 50 mL	+4°C
Wash Buffer PT 10X	20 mL	200 mL	+4°C
TMB Development Solution	12 mL	120 mL	+4°C
Stop Solution	12 mL	120 mL	+4°C
SimpleStep Pre-Coated 96-Well Microplate	96 wells	10 x 96 wells	+4°C
Plate Seal	1	10	+4°C

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

Microplate reader capable of measuring absorbance at 450 or 600 nm.
Deionized water.
Multi- and single-channel pipettes.
Tubes for standard dilution.
Plate shaker for all incubation steps.
Optional: Method for determining total protein concentration in sample (BCA assay recommended).
Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.

Prepare only as much reagent as is needed on the day of the experiment. The CaptSure and HRP Conjugates have only been tested for stability in the provided 50X formulation.

1X Wash Buffer PT: Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

CaptSure Conjugate Solution: Prepare CaptSure Conjugate Solution by diluting the 50X CaptSure Conjugate in Antibody Diluent 4BI. To make 2 mL of the Capture Conjugate Solution combine 40 µL 50X CaptSure Conjugate with 1.96 mL Antibody Diluent 4BI. Mix thoroughly and gently.

HRP Conjugate Solution: Prepare HRP Conjugate Solution by diluting the 50X HRP Conjugate in Antibody Diluent 4BI. To make 2 mL of the HRP Conjugate Solution combine 40 µL 50X HRP Conjugate with 1.96 mL Antibody Diluent 4BI. Mix thoroughly and gently.

Standard Preparation

Always prepare a fresh set of standards for every use. Discard working standard dilutions after use as they do not store well. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. Reconstitute 3-Nitrotyrosine standard sample by adding the volume of Sample Diluent NS indicated on the standard vial label. Hold at room temperature for 10 minutes. Mix thoroughly and gently. This is the 20,000 ng/mL **Stock Standard** Solution.
2. Label eight tubes, Standards 1– 8.
3. Add 288 µL of Sample Diluent NS into tube number 1 and 180 µL of Sample Diluent NS into numbers 2-8.
4. Use the **Stock Standard** to prepare the following dilution series. Standard #8 contains no standard and is the zero standard control:

Standard #	Dilution Sample	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	**Stock Standard**	72	288	20,000	4,000
2	Standard#1	180	180	4,000	2,000
3	Standard#2	180	180	2,000	1,000
4	Standard#3	180	180	1,000	500
5	Standard#4	180	180	500	250
6	Standard#5	180	180	250	125
7	Standard#6	180	180	125	62.5
8	Zero Standard (B_0)	0	180	N/A	N/A

Sample Preparation

Typical Sample Dynamic Range	
Sample Type	**Range**
Serum	6.25 – 50%
Plasma - Citrate	3.125 – 50%
Plasma - EDTA	3.125 – 50%
Plasma - Heparin	3.125 – 50%
Urine	3.125 – 25%

Serum Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.
Plasma Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples at least 1:2 into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
Urine Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Dilute samples at least 1:2 in Sample Diluent NS and assay. Store un-diluted urine samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Plate Preparation

The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
For each assay performed, a minimum of two wells must be used as the zero control.
For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
Differences in well absorbance or "edge effects" have not been observed with this assay.

Assay Procedure

Equilibrate all materials and prepared reagents to room temperature prior to use.
We recommend that you assay all standards, controls and samples in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
3. Add 60 µL of all sample or standard to appropriate wells.
4. Add 30 µL of the CaptSure Conjugate Solution to each well.
    a. Optional – for non-specific binding wells, add 30 µL of Antibody Diluent 4BI in lieu of CaptSure Conjugate Solution.
5. Add 30 µL of the HRP Conjugate Solution to each well.
6. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
7. Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.
8. Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
   Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
9. Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.
10. Alternative to 8 – 9: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode	Kinetic
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm.

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:
www.abcam.com/protocols/the-complete-elisa-guide
For technical support contact information, visit: www.abcam.com/contactus

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Additional information

ASSAY SPECIFICITY

This kit is designed for the quantification of 3-Nitrotyrosine.

The standard in this kit is nitrated Bovine Serum Albumin.

Native signal was detected in serum, plasma (heparin), plasma (EDTA), plasma (citrate), and urine.

Spiked protein experiments were used to validate serum, plasma (heparin), plasma (EDTA), plasma (citrate) and urine.

Saliva, milk, CSF, tissue extract samples have not been tested with this kit.

This kit is incompatible with cell culture supernatant.

For the measurement of 3-Nitrotyrosine in cell and tissue extract sample types, use 3-Nitrotyrosine ELISA kit ab316905.

CROSS REACTIVITY

4,000 ng/mL of free non-nitrated tyrosine, phenylalanine, and 4-nitrophenylalanine were tested for cross reactivity. No cross reactivity was observed.

SPECIES REACTIVITY

Validated in Human samples, reactivity is species-independent.

CALCULATION

- Optional: Non-specific binding (NSB) well subtracted values can be calculated by averaging the absorbance values for the NSB wells and subtracting the average NSB absorbance value from all other absorbance values.
- Create a standard curve by plotting the average absorbance value for each standard concentration (y-axis) against the target concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.
- Note: Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4 parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- Determine the concentration of the target analyte in the sample by interpolating absorbance values against the standard curve. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target analyte in the sample.
- Samples generating absorbance values lower than that of the highest concentration standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance value greater than that of the lowest concentration standard should be retested in a less dilute form.
- Optional: The binding percentage, B/B_0, can be calculated by dividing the average absorbance value for each standard or sample by the average absorbance of the zero standard (B_0).

TYPICAL DATA

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Standard Curve Measurements				
Concentration (ng/mL)	O.D 450 nm		Mean O.D	B/B_0 (%)
	1	2		
NSB	0.037	0.043	0.040	-
0 (B_0)	1.534	1.558	1.546	100%
62.5	1.366	1.318	1.342	87%
125	1.183	1.159	1.171	76%
250	0.914	0.963	0.939	61%
500	0.681	0.696	0.688	45%
1,000	0.448	0.457	0.453	29%
2,000	0.272	0.286	0.279	18%
4,000	0.185	0.178	0.181	12%

Table 1. Example of 3-Nitrotyrosine standard curve in Sample Diluent NS. The 3-Nitrotyrosine standard curve was prepared as described in the Standard Preparation section. The table shows raw data values.

TYPICAL SAMPLE VALUES

Sensitivity:

The calculated minimal detectable dose (MDD) is 41 ng/mL. The MDD was determined by calculating the mean of zero standard replicates (n=8) and subtracting 2 standard deviations, then extrapolating the corresponding concentration.

Recovery

3 concentrations of 3-Nitrotyrosine were spiked, in duplicate, to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
50% Human Serum	84	80 – 89
50% Human Plasma – Citrate	99	86 – 111
50% Human Plasma – EDTA	91	81 – 100
50% Human Plasma - Heparin	98	88 – 112
50% Human Urine	101	85 – 125

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

3-Nitrotyrosine was spiked in in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	50% Human Serum	50% Human Plasma (Citrate)	50% Human Plasma (EDTA)	50% Human Plasma (Heparin)	25% Human Urine
Undiluted	ng/mL	1,983	1,842	2,024	2,360	2,198
	% Expected value	100	100	100	100	100
2	ng/mL	1,013	874	988	1,126	1,124
	% Expected value	102	95	98	95	102
4	ng/mL	469	421	501	590	560
	% Expected value	95	92	99	100	102
8	ng/mL	255	239	221	263	262
	% Expected value	103	104	87	89	94

Native 3-Nitrotyrosine was measured in urine in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	50% Human Urine
Undiluted	ng/mL	673
	% Expected value	100
2	ng/mL	292
	% Expected value	88
4	ng/mL	131
	% Expected value	77

Precision

Mean coefficient of variations of interpolated values of 3-Nitrotyrosine from two concentrations of human serum within the working range of the assay.

	Intra-assay	Inter-assay
N=	8	3
CV (%)	9.5	9.4

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:
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