ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES

MOST SENSITIVE

DetectX®

Levonorgestrel (LNG)
Enzyme Immunoassay Kit

1 Plate Kit Catalog Number K058-H1
5 Plate Kit Catalog Number K058-H5

Species Independent

Sample Types Validated:

**Serum, EDTA and Heparin Plasma,
Saliva, Urine, Dried Fecal Extracts, Milk,
Water and Tissue Culture Media**

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

www.ArborAssays.com

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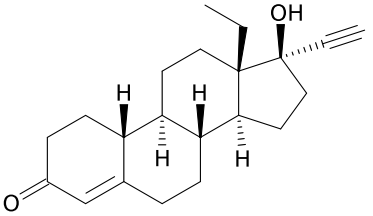
BACKGROUND

Levonorgestrel ($C_{21}H_{28}O_2$, 4-Estren-17α-Ethynyl-18-homo-17β-ol-3-one, LNG) is a synthetic steroid analogue used in a number of birth control methods. Levonorgestrel was first made in the 1960s and its use as a method of birth control began in the 1980s. It is on the World Health Organization's List of Essential Medicines, the most important medication needed in a basic health system. It is available as a generic medication. In pill form, sold under the brand name "Plan B" among others, it is useful within 120 hours as emergency birth control. It becomes less effective the longer after sex and only works before pregnancy has occurred. It is also combined with an estrogen to make a combined oral birth control pill. As an IUD, sold as Mirena among others, it is effective for long term prevention of pregnancy. An implantable form of levonorgestrel, Inplanon, is also available in some countries.

Levonorgestrel (LNG) uses include contraception, treatment of heavy menstrual bleeding and dysmenorrhea, and endometrial protection during estrogen replacement therapy in postmenopausal women. LNG has been shown to be an effective treatment modality for a great variety of gynecologic conditions: idiopathic, myoma- or adenomyosis-related heavy menstral bleeding, endometriosis- or adenomyosis-related pelvic pain, as well as endometrial hyperplasia and early-stage endometrial cancer.



Common side effects include nausea, breast tenderness, headaches, and increased, decreased, or irregular menstrual bleeding. If used as a form of emergency contraception during pregnancy there is no evidence it affects the baby. It is safe to use during breastfeeding. It works by decreasing ovulation, changing the mucus in the cervix to prevent the passage of sperm, and altering the uterine lining.

ASSAY PRINCIPLE

The DetectX® Levonorgestrel (LNG) Immunoassay kit is designed to quantitatively measure Levonorgestrel present in serum, plasma, saliva, urine, dried fecal samples, milk, and tissue culture media samples. Please read the complete kit insert before performing this assay. This kit measures total LNG in extracted serum, plasma, milk and fecal samples.

A levonorgestrel stock solution is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with an antibody to capture rabbit antibodies. A levonorgestrel-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a rabbit polyclonal antibody to levonorgestrel to each well. After an hour incubation the plate is washed and substrate is added. The substrate reacts with the bound levonorgestrel-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The concentration of the levonorgestrel in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

RELATED PRODUCTS

Kits	Catalog No.
Urinary Creatinine Detection Kits	K002-H1/H5
17-Hydroxyprogesterone EIA kits	K053-H1/H5
Aldosterone EIA & CLIA Kits	K052-H1/H5, -C1/C5
Allopregnanolone EIA & CLIA Kits	K044-H1/H5, -C1/C5
Ceruloplasmin Colorimetric Activity Kit	K035-H1
Dehydroepiandrosterone Sulfate (DHEA-S) EIA Kits	K054-H1/H5
Estradiol Non-Invasive & Serum EIA Kits	K030-H1/H5, KB30-H1/H5
Estrone-3-Glucuronide (E1G) EIA Kits	K036-H1/H5
Oxytocin EIA & CLIA Kits	K048-H1/H5, -C1/C5
PGFM (13,14-Dihydro-15-keto-Prostaglandin $F_{1\alpha}$) EIA Kits	K022-H1/H5
Pregnandiol-3-Glucuronide (PDG) EIA Kits	K037-H1/H5
Progesterone EIA Kits	K025-H1/H5
Prolactin EIA Kit	K040-H1
Testosterone EIA Kits	K032-H1/H5

SUPPLIED COMPONENTS

Coated Clear 96 Well Plates
A clear plastic microtiter plate(s) coated with goat anti-rabbit IgG.

Kit K058-H1 **or** -H5	1 **or** 5 Each	Catalog Number X016-1EA

(-)-Levonorgestrel Standard
(-)-Levonorgestrel at 4,000 pg/mL in a special stabilizing solution.

Kit K058-H1 **or** -H5	70 µL **or** 350 µL	Catalog Number C219-70UL **or** -350UL

DetectX® Levonorgestrel Antibody
A rabbit polyclonal antibody specific for Levonorgestrel.

Kit K058-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C217-3ML **or** -13ML

DetectX® Levonorgestrel Conjugate
A Levonorgestrel-peroxidase conjugate in a special stabilizing solution.

Kit K058-H1 **or** -H5	3 mL **or** 13 mL	Catalog Number C218-3ML **or** -13ML

Assay Buffer Concentrate
A 5X concentrate that must be diluted with deionized or distilled water.

Kit K058-H1 **or** -H5	28 mL **or** 55 mL	Catalog Number X053-28ML **or** -55ML

Wash Buffer Concentrate
A 20X concentrate that should be diluted with deionized or distilled water.

Kit K058-H1 **or** -H5	30 mL **or** 125 mL	Catalog Number X007-30ML **or** -125ML

TMB Substrate

Kit K058-H1 **or** -H5	11 mL **or** 55 mL	Catalog Number X019-11ML **or** -55ML

Stop Solution
A 1M solution of hydrochloric acid. **CAUSTIC**.

Kit K058-H1 **or** -H5	5 mL **or** 25 mL	Catalog Number X020-5ML **or** -25ML

Plate Sealer

Kit K058-H1 **or** -H5	1 **or** 5 Each	Catalog Number X002-1EA

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C until the expiration date of the kit.

The DetectX® Levonorgestrel Conjugate will lose about 50% of its signal when stored at 4°C for the life of the kit. Samples will read correctly throughout the shelf life of the kit.

To retain full signal store the conjugate at -20°C.

OTHER MATERIALS REQUIRED

Distilled or deionized water.

Repeater pipet with disposable tips capable of dispensing 25, 50, and 100 µL.

Plate shaker.

Colorimetric 96 well microplate reader capable of reading optical density at 450 nm.

Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The antibody coated plate needs to be stored desiccated. The silica gel pack included in the foil ziploc bag will keep the plate dry. The silica gel pack will turn from blue to pink if the ziploc has not been closed properly.

This kit utilizes a peroxidase-based readout system. Buffers, including other manufacturers Wash Buffers, containing sodium azide will inhibit color production from the enzyme. Make sure **all** buffers used for samples are **azide free**. Ensure that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer as prepared on Page 8.

Laboratory temperature is important. Please make sure that the kit incubates at a temperature between 22°C and 24°C.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

SAMPLE TYPES

This assay has been validated for extracted serum, EDTA and heparin plasma, and milk as well as for diluted saliva and urine samples and for tissue culture samples. It has also been validated for dried fecal extract samples. Samples containing visible particulate should be centrifuged prior to using. Moderate to severely hemolyzed samples should not be used in this kit.

Levonorgestrel is identical across all species and we expect this kit may measure levonorgestrel in sources other than human. The end user should evaluate recoveries of levonorgestrel in other samples being tested.

SAMPLE PREPARATION

LNG can be assayed in other sample types by using one of the extraction protocols available on our website at: www.arborassays.com/resources/#protocols

Saliva Samples

Saliva samples should be diluted ≥ 1:2 with the diluted Assay Buffer prior to running in the assay.

Urine Samples
Urine samples should be diluted ≥ 1:4 with the diluted Assay Buffer prior to running in the assay. Please see our Urinary Creatinine Detection kits, K002-H1 and K002-H5, for assays to measure urine creatinine which can be used to allow normalization of levonorgestrel in a random urine specimen.

Serum and Plasma Samples
Serum and plasma samples need to be extracted. We would recommend the following protocol for serum and plasma.

1. Add ethyl acetate to serum or plasma samples at a 5:1 (v/v) solvent:sample ratio. Mix solutions by vortexing for 2 minutes. Allow layers to separate for 5 minutes.

2. Freeze samples in a dry ice/ethanol bath and pipet off the solvent solution from the top of the sample into a clean tube. Repeat steps 1-3 for maximum extraction efficiency, combining the solvent solutions. Dry pooled solvent extracts down in a speedvac for 2-3 hrs. If samples need to be stored they should be kept at -20°C, desiccated.

3. Redissolve samples at room temperature in diluted Assay Buffer. A minimum of 250 µL of the Assay Buffer is recommended for reconstitution to allow for duplicate sample measurement.

Dried Fecal Samples
We have a detailed Steroid Solid Extraction Protocol available on our website at: www.arborassays.com/resources/#protocols. The ethanol concentration in the final diluted Assay Buffer dilution added to the well should be ≤ 2.5%.

Milk Samples
Milk samples must be extracted with 80% ethyl acetate/ 20% hexane. It is recommended to also perform extraction efficiency with each sample, due to the varying lipid composition in samples. Run each sample with and without a known amount of LNG added (spiked) and an Assay Buffer control (similarly spiked) for comparison.

1. Heat samples to 45˚C for 15 minutes. Vortex thoroughly to redistribute lipid content. Add 80% ethyl acetate/20% hexane to milk samples at a 5:1 (v/v) solvent:sample ratio. Extract a minimum of 60 µL milk to allow for duplicate sample measurement in the assay.

2. Mix solutions by vortexing thoroughly 1-2 minutes. Allow layers to separate for 5 minutes and freeze samples in a dry ice/ethanol bath or at -80˚C. Pipet off the solvent solution from the top of each sample into clean tubes. Repeat steps 1 and 2 two times, combining extracts.

3. Dry the pooled solvent extracts in a centrifugal evaporator. Samples can be stored at -20˚C desiccated. Redissolve samples at room temperature in diluted Assay Buffer at 4x original sample volume. Vortex, allow to sit 5 minutes and vortex again. This 1:4 dilution can be further diluted with Assay Buffer if needed.

Tissue Culture Media
For measuring levonorgestrel in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM. We have validated the assay using RPMI-1640.

Use all Samples within 2 hours of preparation, or stored at ≤ -20°C until assaying.

REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Assay Buffer

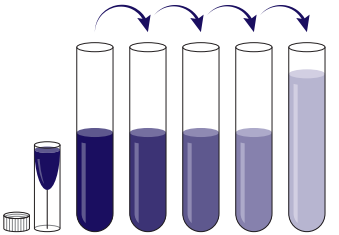
Dilute Assay Buffer Concentrate 1:5 by adding one part of the concentrate to four parts of deionized water. Once diluted this is stable for 3 months at 4°C.

Wash Buffer

Dilute Wash Buffer Concentrate 1:20 by adding one part of the concentrate to nineteen parts of deionized water. Once diluted this is stable for 3 months at room temperature.

Standard Preparation

Label test tubes as #1 through #6. Pipet 475 µL of Assay Buffer into tube #1 and 250 µL into tubes #2 to #6. **The Levonorgestrel stock solution contains an organic solvent. Prerinse the pipet tip several times to ensure accurate delivery.** Carefully add 25 µL of the Levonorgestrel stock solution to tube #1 and vortex completely. Take 250 µL of the Levonorgestrel solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #6. The concentration of Levonorgestrel in tubes 1 through 6 will be 200, 100, 50, 25, 12.5, and 6.25 pg/mL.



Use all Standards within 2 hour of preparation.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Assay Buffer (µL)	**475**	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 5	Std 6
Vol of Addition (µL)	**25**	250	250	250	250	250
Final Conc (pg/mL)	200	100	50	25	12.5	6.25

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine Levonorgestrel concentrations.

1. Use the plate layout sheet on the back page to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to the foil pouch with desiccant. Seal the ziploc plate bag and store at 4˚C.
2. Pipet 100 µL of samples or standards into wells in the plate.
3. Pipet 125 µL of Assay Buffer into the non-specific binding (NSB) wells.
4. Pipet 100 µL of Assay Buffer into the maximum binding (B0 or Zero standard) wells.
5. Add 25 µL of the DetectX® Levonorgestrel Conjugate to each well using a repeater pipet.
6. Add 25 µL of the DetectX® Levonorgestrel Antibody to each well, **except the NSB wells**, using a repeater pipet.
7. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour. We recommend shaking at around 700–900 rpm. If the plate is not shaken, signals bound will be approximately 15-20% lower.
8. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
9. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
10. Incubate the plate at room temperature for 30 minutes without shaking.
11. Add 50 µL of the Stop Solution to each well, using a repeater or a multichannel pipet.
12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.
13. Use the plate reader's built-in 4PLC software capabilities to calculate levonorgestrel concentration for each sample.

NOTE: If you are using only part of a strip well plate, at the end of the assay throw away the used wells and retain the plate frame for use with the remaining unused wells.

CALCULATION OF RESULTS

Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/B0 curve, should be multiplied by the dilution factor to obtain neat sample values.

Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-levonorgestrel-eia-kit.assay

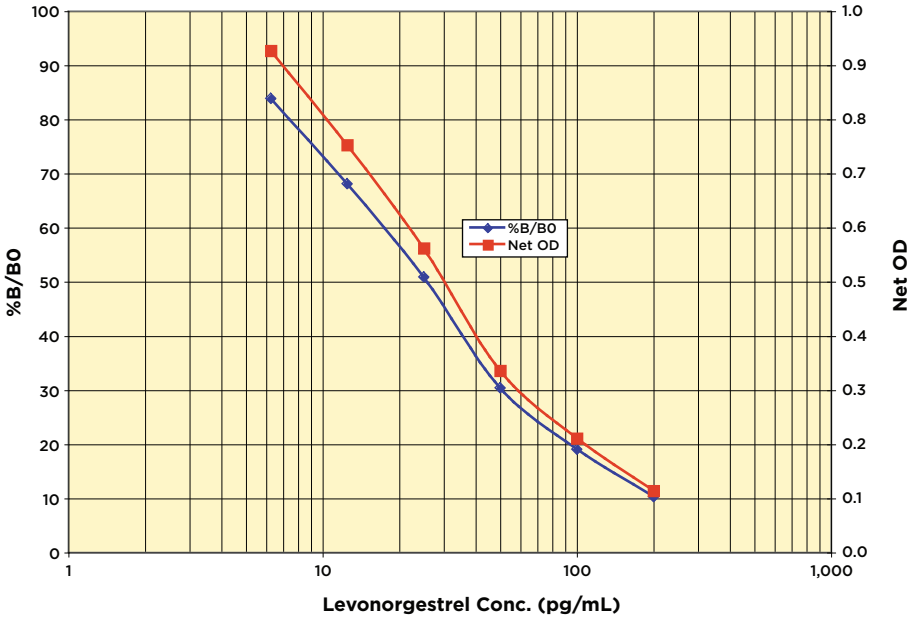
TYPICAL DATA

Sample	Mean OD	Net OD	% B/B0	Levonorgestrel Conc. (pg/mL)
NSB	0.080	0.000	-	-
Standard 1	0.194	0.114	10.3	200
Standard 2	0.291	0.211	19.1	100
Standard 3	0.416	0.336	30.4	50
Standard 4	0.642	0.562	50.9	25
Standard 5	0.833	0.753	68.1	12.5
Standard 6	1.007	0.927	83.9	6.25
B0	1.185	1.105	100	0
Sample 1	0.400	0.320	29.0	55.88
Sample 2	0.605	0.525	47.5	27.24

Always run your own standard curve for calculation of results. Do not use this data.

Conversion Factor: 100 pg/mL of Levonorgestrel is equivalent to 320.1 pM.

Typical Normal Range Standard Curves



Always run your own standard curves for calculation of results. Do not use this data.

VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the OD's for twenty wells run for each of the B0 and standard #6. The detection limit was determined at two (2) standard deviations from the B0 along the standard curve. **Sensitivity was determined as 2.20 pg/mL.**

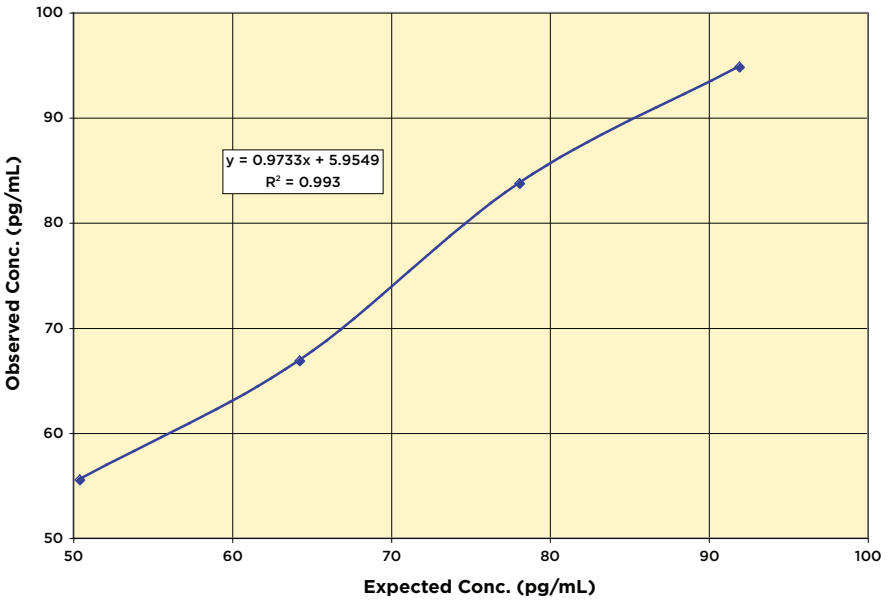
The Limit of Detection for the assay was determined in a similar manner by comparing the OD's for twenty runs for each of the zero standard and a low concentration human sample. **Limit of Detection was determined as 2.74 pg/mL**

Linearity

Linearity was determined by taking two urine samples, one with a low diluted Levonorgestrel level of 36.57 pg/mL and one with a higher diluted level of 105.8 pg/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

High Urine	Low Urine	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
80%	20%	91.94	94.82	103.1
60%	40%	78.10	83.72	107.2
40%	60%	64.25	66.86	104.1
20%	80%	50.41	55.53	110.2
			Mean Recovery	**106.3%**

Linearity



Intra Assay Precision

Three human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Levonorgestrel concentrations were:

Sample	Levonorgestrel Conc. (pg/mL)	%CV
1	63.5	5.3
2	26.7	7.1
3	10.6	14.6

Inter Assay Precision

Three human samples were diluted with Assay Buffer and run in duplicates in twenty-two assays run over multiple days by four operators. The mean and precision of the calculated Levonorgestrel concentrations were:

Sample	Levonorgestrel Conc. (pg/mL)	%CV
1	59.9	6.8
2	27.2	7.8
3	9.0	12.6

SAMPLE VALUES

Seven human serum samples from individuals using IUDs were tested in the assay. Neat sample values ranged from 95.4 to 200.7 pg/mL with an average of 149.3 pg/mL. The normal reference range for serum LNG concentrations for women using an Allergan plc LILETTA™ IUD is 84-375 pg/mL according to the Medical Information Department, Allergan plc.

Eight human saliva samples from individuals using IUDs were tested in the assay. Neat sample values ranged from 9.2 to 16.8 pg/mL with an average of 17.84 pg/mL.

Ten human urine samples from individuals using IUDs were tested in the assay. Neat sample values ranged from 114.6 to 848.2 pg/mL with an average of 406.2 pg/mL. In addition, 8 human urine samples from males or individuals not using IUDs were tested. Neat sample values ranged from 18.9 to 137.5 pg/mL with an average of 64.6 pg/mL.

Six human milk samples from individuals using IUDs were extracted and tested in the assay. Neat sample values ranged from 40.9 to 98.6 pg/mL with an average of 73.5 pg/mL

CROSS REACTIVITY

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Steroid	Cross Reactivity (%)
- Levonorgestrel	100%
Norgestrel (+/-)	60.10
Etonogestrel	3.53
19-Nortestosterone	0.12
Ethynylestradiol	0.08
Medroxyprogesterone	0.04
Diethylstilbestrol	< 0.02
Estradiol	< 0.02
Aldosterone	< 0.02
Cortisol (aka Hydrocortisone)	< 0.02
Pregnanolone	< 0.02
Progesterone	< 0.02
Testosterone	< 0.02

LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

For details concerning this kit or to order any of our products please contact us:

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with EIA kits for wildlife conservation research.

ASSAYS™
FOR SERIOUS SCIENCE

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