User Guide

MILLIPLEX® Multi-Species Hormone Magnetic Bead Panel

96-Well Plate Assay

MSHMAG-21K

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Introduction

Steroid hormones and thyroid hormones are small molecule endocrine hormones secreted from adrenal gland (cortisol), ovaries (estradiol and progesterone), and thyroid (T3 and T4). These steroid and thyroid hormones play key roles in the regulation of energy balance, mental and physical development, sex organ development and pregnancy. We have developed a multiplex assay panel for simultaneous measurement of 5 of these hormones. The multiplex assay provides biomedical researchers with quality tools for the study of the biological functions of these hormones and patho-physiology of many diseases such as thyroid-related diseases (Grave's disease and hyper/hypothyroidism), steroid-related diseases (Cushing's syndrome), and stress related diseases.

To identify specific levels of steroid and thyroid hormones, it might be necessary to screen panels containing several hormones, often requiring some level of automation and/or high throughput.

The MILLIPLEX® portfolio offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the verification process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (for example, detectability and stability).

Each MILLIPLEX® panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition, each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The MILLIPLEX® Multi-Species Hormone Magnetic Bead Panel thus enables you to focus on the therapeutic potential of endocrine hormones cortisol, estradiol, progesterone, T3 and T4. Coupled with the Luminex® xMAP® platform in a magnetic bead format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously, which can dramatically improve productivity.

The MILLIPLEX® Multi-Species Hormone Magnetic Bead Panel is part of the most versatile system available for multi-species metabolism/endocrine research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically verify and build the most comprehensive library available for protein detection and quantitation.

MILLIPLEX® products offer you:

- The ability to choose any combination of analytes from our panel of 5 analytes to design a custom kit that better meets your needs.
- A convenient “all-in-one” box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

The MILLIPLEX® Multi-Species Hormone Magnetic Bead Panel is a 5-plex kit to be used for the simultaneous quantification of any or all of the following 5 analytes: Cortisol, Estradiol, Progesterone, T3 and T4.

This multi-species kit may be used to detect these analytes in human, dog, rat, mouse, cynomolgous monkey, rhesus monkey, horse, rabbit, cat, guinea pig, pig and hamster. However, the kit was validated with human and canine samples only and the amount of cross-reactivity to other species tested, has not been determined.

All analytes in this kit are run in a competitive assay format.

For research use only. Not for use in diagnostic procedures.
Please read entire protocol before use.
It is important to use same assay incubation conditions throughout your study.

Principle

MILLIPLEX® products are based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life-sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex®-C microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500-5.6 µm polystyrene microspheres or 80-6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The following Luminex® instruments can be used to acquire and analyze data using two detection methods:
    - The Luminex® analyzers, Luminex® 200™, FLEXMAP 3D® and xMAP® INTELLIFLEX, are flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex® analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.

- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. We combine the streamlined data acquisition power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.
- xMAP® INTELLIFLEX runs on INTELLIFLEX software for instrument control, run setup and generating high quality data with flexible output options. Data can be exported in xPONENT® style CSV files for compatibility with many existing analytical applications, or in the new, customizable INTELLIFLEX file format. The INTELLIFLEX file format is intended for flexibility and simplicity, allowing the user to freely select which data points to include and to reduce the time to analysis.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

Storage Conditions Upon Receipt

- Recommended storage for kit components is 2–8 °C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20 °C. Avoid multiple (> 2) freeze thaw cycles.
- **DO NOT FREEZE** Antibody-Immobilized Beads, HRP Conjugate, Detection Antibody and Streptavidin-Phycoerythrin.

Reagents Supplied

Store all reagents at 2–8 °C.

Reagents	Volume	Quantity	Cat. No.
Multi-Species Hormone Standard	Lyophilized	1 vial	MSPHM-8021
Multi-Species Hormone Quality Controls 1 and 2	Lyophilized	1 vial each	MSPHM-6021
Set of one 96-Well Plates with 2 sealers	-	1 set	-
Assay Buffer	30 mL	2 bottles	L-AB
10X Wash Buffer*	60 mL	1 bottle	L-WB
Multi-Species Hormone HRP Conjugate	3.2 mL	1 bottle	MSPHM-HRP

Multi-Species Hormone Detection Antibody	3.2 mL	1 bottle	MSPHM-1021
Streptavidin-Phycoerythrin	3.2 mL	1 bottle	MC-SAPE2
Bead Diluent	3.5 mL	1 bottle	LA-BD
Mixing Bottle	-	1 bottle	-

* Contains 0.05% Proclin

Multi-Species Hormone Panel Antibody-Immobilized Magnetic Beads

		Customizable Beads (20X Concentration, 200 µL)	
Bead/Analyte Name	**Luminex® Bead Region**	**Available**	**Cat. No.**
Anti-Cortisol Bead	12	✓	C0RT-MAG
Anti-Estradiol Bead	14	✓	ESTRDL-MAG
Anti-Progesterone Bead	22	✓	PRGST-MAG
Anti-T3 Bead	35	✓	T3-MAG
Anti-T4 Bead	37	✓	T4-MAG

Materials Required (not included)

Reagents

- MAGPIX® Drive Fluid PLUS (Cat. No. 40-50030), xMAP® Sheath Fluid PLUS (Cat. No. 40-50021), or xMAP® Sheath Concentrate PLUS (Cat. No. 40-50023)
- Acetonitrile
- Trifluoroacetic Acid (optional)

Instrumentation/Materials

- Adjustable pipettes with tips capable of delivering 25 µL to 1000 µL
- Multichannel pipettes capable of delivering 5 µL to 50 µL, or 25 µL to 200 µL
- Reagent reservoirs
- Polypropylene microfuge tubes
- Rubber bands
- Aluminum foil
- Absorbent pads

- Laboratory vortex mixer
- Sonicator (Branson Ultrasonic Cleaner Model B200 or equivalent)
- Titer plate shaker (VWR® Microplate Shaker Cat. No. 12620-926 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instrument with xPONENT® software, or xMAP® INTELLIFLEX instrument with INTELLIFLEX software by Luminex® Corporation
- Automatic plate washer for magnetic beads (BioTek® 405 LS and 405 TS, Cat. No. 40-094, 40-095, 40-096, 40-097 or equivalent) or Handheld Magnetic Separation Block (Cat. No. 40-285 or equivalent).
- Extraction plate (Waters Oasis HLB 60 μM (60 mg) Extraction Plate Cat. No. 186000679) (optional)
- Extraction Plate Manifold (Waters) (optional)
- Speed vacuum

Note: If a magnetic plate washer or handheld magnetic block for magnetic beads is not available, a microtiter filter plate cannot be used to run the assay.

Safety Precautions

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide or Proclin™ has been added to some reagents as a preservative. Although the concentrations are low, sodium azide and Proclin™ may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Symbol Definitions

Ingredient	Cat. No.	Label	
10X Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.
Multi-Species Hormone Panel Standard	MSPHM-8021		**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.

Multi-Species Hormone Panel Quality Control 1 & 2	MSPHM-6021		**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Multi-Species Hormone Detection Antibody	MSPHM-1021		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Streptavidin-Phycoerythrin	MC-SAPE2		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Technical Guidelines

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -20 °C for 1 month and at ≤ -80 °C for greater than one month.

- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8 °C for up to one month.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8 °C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on the Luminex® 200™ instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate. When reading the assay on the MAGPIX® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.
- For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated.
- For xMAP® INTELLIFLEX, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
- For cell culture supernatants or tissue extraction, use 25 µL of the culture or extraction medium as a matrix solution in background, standard curve and control wells in place of 25 µL of the assay buffer. If samples are diluted in assay buffer, use the assay buffer as instructed
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

Sample Collection and Storage

Preparation of Serum Samples

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C.

- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

Preparation of Plasma Samples

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000 x g within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

Preparation of Tissue Culture Supernatant

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. Tissue/cell extracts should be done in neutral buffers containing reagents and conditions that do not interfere with assay performance. Excess concentrations of detergent, salt, denaturants, high or low pH, etc. will negatively affect the assay. Organic solvents should be avoided. The tissue/cell extract samples should be free of particles such as cells or tissue debris.

NOTE:

- A maximum of 25 µL per well of **extracted** serum or plasma can be used. Tissue culture or other media may also be used.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

Extraction of Serum and Plasma Human Samples

Use one of the following protocols:

OPTION 1. Waters 96-well HLB Extraction Plate

- Allow 1 mL acetonitrile to pass through the extraction wells using gravity.
- Equilibrate with 2 x 1 mL 0.1% Trifluoracetic acid (TFA) in water (Solvent A).
- Acidify 250 µL serum or plasma samples by adding 250 µL 1% TFA in water. Mix well.

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- Load the acidified sample in the wells and pull through at a vacuum setting of Hg 2-5.
- Wash each well 3 times with 1 mL Solvent A by vacuum at setting of Hg 2-5.
- Elute the samples in 96-well collecting plate with 0.5 mL acetonitrile/water/TFA (60%/40%/0.1%, v/v/v) by vacuum at setting of Hg 2-5.
- Dry the samples by using Speed Vac at highest vacuum setting. Dried samples can be covered and stored at ≤ -20 ºC.
- Reconstitute samples with 250 µL Assay Buffer. Shake for 10 minutes. Assay immediately or store at ≤ -20 ºC.

OPTION 2. Acetonitrile Precipitation

- Put 250 µL sample into a microfuge tube.
- Add 375 µL acetonitrile to the sample, vortex 5 seconds, and let sit for 10 minutes at room temperature.
- Vortex again for 5 seconds, then centrifuge at 17,000 x g for 5 minutes.
- Carefully remove 500 µL of supernatant into a 96-well collecting plate or microfuge tubes.
- Dry the supernatant samples by using Speed Vac at highest vacuum setting. Dried samples can be covered and stored at ≤ -20 ºC.
- Reconstitute the dried samples with 200 µL Assay Buffer. Shake for 10 minutes. Assay immediately or store at ≤ -20 ºC.

Store extracted samples at -20 °C.

Note: Smaller sample volumes may be used with either extraction method for smaller animal such as rats and mice. The sample size may be reduced to 100 µL.

For example: If 150 µL sample is used in Option 1, the samples are acidified with 150 µL of 1% TFA, loaded, washed and eluted as above. Reconstitute dried samples in 150 µL Assay Buffer. If 150 µL sample is used in Option 2, add 225 µL acetonitrile, vortex and centrifuge as above, remove 300 µL supernatant. Reconstitute dried samples in 120 µL Assay Buffer.

Preparation of Reagents for Immunoassay

Preparation of Antibody-Immobilized Beads

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. Vortex the mixed beads well. Unused portion may be stored at 2-8 °C for up to one month.
(**Note:** Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

Example 1: When using 5 antibody-immobilized beads, add 150 µL from each of the 5 bead sets to the Mixing Bottle. Then add 2.25 mL Bead Diluent.

Example 2: When using 4 antibody-immobilized beads, add 150 µL from each of the 4 bead sets to the Mixing Bottle. Then add 2.4 mL Bead Diluent.

Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes and then invert the vial several times to mix. Transfer the reconstituted Quality Control 1 and Quality Control 2 into two polypropylene microfuge tubes. Unused portion may be stored at ≤ -20 °C for up to one month.

Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer with a total of 540 mL deionized water. Store unused portion at 2-8 °C for up to one month.

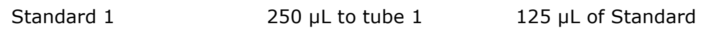
Preparation of Multi-Species Hormone Standard

1. Prior to use, reconstitute the Multi-Species Standard with 250 µL deionized water. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. Transfer the reconstituted standard to a polypropylene microfuge tube. This will be used as Standard 7; the unused portion may be stored at ≤ -20 °C for up to one month.

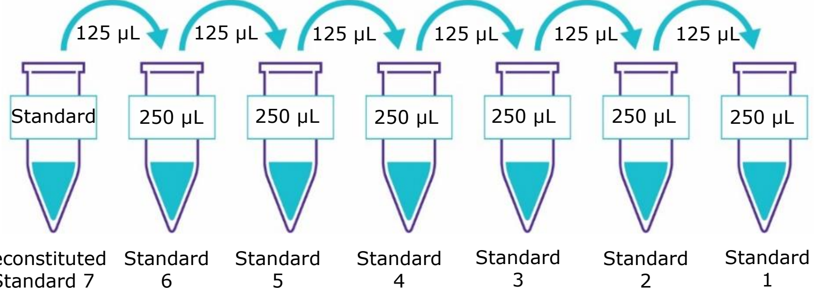
2. Preparation of Working Standards
   Label six polypropylene microfuge tubes 1, 2, 3, 4, 5 and 6. Add 250 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 125 µL of the reconstituted standard 7 to the Standard 6 tube, mix well and transfer 125 µL of Standard 6 to the Standard 5 tube, mix well and transfer 125 µL of Standard 5 to the Standard 4 tube, mix well and transfer 125 µL of Standard 4 to the Standard 3 tube, mix well and transfer 125 µL of Standard 3 to the Standard 2 tube, mix well and transfer 125 µL of Standard 2 to the Standard 1 tube and mix well. The 0 ng/mL standard (Background) will be Assay Buffer.

Standard Tube No.	Add Deionized Water (volume)	Add Standard (volume)
Reconstituted Standard 7	250 µL	0

Standard Tube No.	Add Assay Buffer (volume)	Add Standard (volume)
Standard 6	250 µL to tube 6	125 µL of Standard 7
Standard 5	250 µL to tube 5	125 µL of Standard 6
Standard 4	250 µL to tube 4	125 µL of Standard 5
Standard 3	250 µL to tube 3	125 µL of Standard 4
Standard 2	250 µL to tube 2	125 µL of Standard 3



Preparation of Standards



After Dilution, each tube has the following concentrations for each analyte:

Standard	Cortisol (ng/mL)	Estradiol (ng/mL)	Progesterone (ng/mL)	T3 (ng/mL)	T4 (ng/mL)
Standard 1	0.27	0.014	0.14	0.07	0.34
Standard 2	0.82	0.04	0.41	0.21	1.03
Standard 3	2.47	0.12	1.23	0.62	3.09
Standard 4	7.41	0.37	3.70	1.85	9.26
Standard 5	22.22	1.11	11.11	5.56	27.78
Standard 6	66.67	3.33	33.33	16.67	83.33
Standard 7	200	10	100	50	250

Immunoassay Procedure

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
- Diagram the placement of Standards, Background, Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration.
  (**Note:** Most instruments will only read the 96-well plate vertically by default.)
  It is recommended to run the assay in duplicate.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25 °C).

3. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.

4. Add 25 µL of Assay Buffer to all wells. No matrix is used since samples must be extracted. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.

5. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 ng/mL standard (Background).

6. Add 25 µL of Samples into the appropriate wells.

7. Add 25 µL of HRP Conjugate to all wells.

8. Vortex Prepared Bead Bottle and add 25 µL of Prepared Beads to each well. (**Note:** During addition of Beads, shake bead bottle intermittently to avoid settling.)

9. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker 16-20 hours at 4 °C.

10. Gently remove well contents and wash plate 4 times following instructions listed in the Plate Washing section.

Add 200 µL Assay Buffer per well

Shake 10 min, RT

Decant

- Add 25 µL Assay Buffer to all wells
- Add 25 µL Standard or Control to appropriate wells and Assay Buffer to the 0 ng/mL standard
- Add 25 µL Samples to sample wells
- Add 25 µL HRP Conjugate to all wells
- Add 25 µL Beads to each well

Incubate 16-20 hours at 4 °C with shaking

Remove well contents and wash 4X with 200 µL Wash Buffer

11. Add 25 µL of Detection Antibody into each well.
(**Note:** Allow the Detection Antibody to warm to room temperature prior to addition.)

12. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25 °C). **DO NOT ASPIRATE AFTER INCUBATION.**

13. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibody.

14. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25 °C).

15. Gently remove well contents and wash plate 2 times following instructions listed in the Plate Washing section.

16. Add 100 µL of Sheath Fluid PLUS (or Drive Fluid PLUS if using MAGPIX®) to all wells. Cover and re-suspend the beads on a plate shaker for 5 minutes.

17. Run plate on Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instrument with xPONENT® software or xMAP® INTELLIFLEX instrument with INTELLIFLEX software.

18. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating Multi-Species concentrations in samples.
(**Note:** For diluted samples, multiply the calculated concentration by the dilution factor.)

Add 25 µL Detection Antibody per well

Incubate 1 hour at RT

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

Remove well contents and wash 2X with 200 µL Wash Buffer

Add 100 µL Sheath Fluid PLUS or Drive Fluid PLUS per well

Read on Luminex® (50 µL, 50 beads per bead set)

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Plate Washing

Solid Plate

When using a solid plate, use either a handheld magnet or magnetic plate washer.

- Handheld magnet (Cat. No. 40-285)
  Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.
- Magnetic plate washer (Cat. No. 40-094, 40-095, 40-096 and 40-097)
  Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 µL of residual wash buffer in each well. This is expected when using the BioTek plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek® 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

Equipment Settings

Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® with xPONENT® software and xMAP® INTELLIFLEX with INTELLIFLEX software:

These specifications are for the above listed instruments and software. Luminex® instruments with other software (for example, MasterPlex®, StarStation, LiquiChip, Bio-Plex® Manager™, LABScan™100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex® magnetic beads.

For magnetic bead assays, each instrument must be calibrated and performance verified with the indicated calibration and verification kits.

Instrument	Calibration Kit	Verification Kit
Luminex® 200™ and HTS	xPONENT® 3.1 compatible Calibration Kit (Cat. No. LX2R-CAL-K25)	Performance Verification Kit (Cat. No. LX2R-PVER-K25)
FLEXMAP 3D®	FLEXMAP 3D® Calibrator Kit (Cat. No. F3D-CAL-K25)	FLEXMAP 3D® Performance Verification Kit (Cat. No. F3D-PVER-K25)
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (Cat. No. IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (Cat. No. IFX-PVER-K20)
MAGPIX®	MAGPIX® Calibration Kit (Cat. No. MPX-CAL-K25)	MAGPIX® Performance Verification Kit (Cat. No. MPX-PVER-K25)

NOTE: When setting up a Protocol using the xPONENT® software, you must select MagPlex® as the Bead Type in the Acquisition settings.

NOTE: These assays cannot be run on any instruments using Luminex® IS 2.3 or Luminex® 1.7 software.

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use Cat. No. MAG-PLATE, if additional plates are required for this purpose.

Events	50, per bead	
Sample Size	50 µL	
Gate Settings	8,000 to 15,000	
Time Out	60 seconds	
Bead Region	Cortisol	12
	Estradiol	14
	Progesterone	22
	T3	35
	T4	37

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at our website SigmaAldrich.com using the catalogue number as the keyword.

Assay Characteristics

Cross-Reactivity

Approximately 10% cross-reactivity to Cortisol is observed in the Progesterone assay and approximately 5% cross-reactivity to T3 is observed in the T4 assay. No other significant cross-reactivity is observed within the panel or to other similar analytes.

This multi species kit may be used to detect these five analytes in human, dog, rat, mouse, cynomolgous monkey, rhesus monkey, horse, rabbit, cat, guinea pig, pig and hamster. However, the kit was validated with human and canine samples only and the amount of cross-reactivity to other species tested, has not been determined.

Assay Sensitivities (minimum detectable concentrations, ng/mL)

Minimum Detectable Concentration (MinDC) is calculated by the MILLIPLEX® Analyst 5.1. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

	(n = 6 Assays)	
Analyte	**MinDC (ng/mL)**	**MinDC+2SD (ng/mL)**
Cortisol	0.17	0.33
Estradiol	0.01	0.01
Progesterone	0.14	0.25
T3	0.08	0.12
T4	0.24	0.51

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across 6 different assays.

Analyte	**Intra-Assay (%CV)**	**Inter-Assay (%CV)**
Cortisol	< 10	< 10
Estradiol	< 10	< 15
Progesterone	< 10	< 15
T3	< 10	< 15
T4	< 10	< 15

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in extracted serum samples.

Analyte	**Human Samples (n=5)**	**Canine Samples (n=4)**
Cortisol	135	117
Estradiol	80	80
Progesterone	85	67
T3	107	105
T4	97	87

Troubleshooting

Problem	Probable Cause	Solution
Insufficient Bead Count	Plate Washer aspirate height set too low	Adjust aspiration height according to manufacturers instructions.
	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with Alcohol flush, back flush and washes; or if needed probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on the Luminex® 200™ instrument, adjust probe height to the kit solid plate using 3 alignment discs. When reading the assay on the MAGPIX® instrument, adjust probe height to the kit solid plate using 2 alignment discs. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height to the kit solid plate using 1 alignment disc. For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated. When reading the assay on the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipetting with Multichannel pipets without touching reagent in plate
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross reacting components (for example, interleukin modified tissue culture medium)
	Insufficient washes	Increase number of washes

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Problem	Probable Cause	Solution
Beads not in region or gate	Luminex® not calibrated correctly or recently	Calibrate Luminex® based on Instrument Manufacturer's instructions, at least once a week or if temperature has changed by > 3 °C.
	Gate Settings not adjusted correctly	Some Luminex® instruments (for example, Bioplex®) require different gate settings than those described in the Kit protocol. Use Instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex® instrument 4 times to rid it of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been vacuumed out prior to adding Streptavidin-Phycoerythrin	May need to repeat assay if desired sensitivity not achieved
	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex® Instrument (for example, Bio-plex) Default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.

Problem	**Probable Cause**	**Solution**
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point.	Samples may require dilution and reanalysis for just that particular analyte
	Standard curve was saturated at higher end of curve.	See above
High Variation in samples and/or standards	Multichannel pipet may not be calibrated	Calibrate pipets
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without causing splashing
	Cross well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.

Product Ordering

Replacement Reagents	Cat. No.
Multi-Species Hormone Standard	MSPHM-8021
Multi-Species Hormone Quality Controls 1 and 2	MSPHM-6021
Multi-Species Hormone HRP Conjugate	MSPHM-HRP
Multi-Species Hormone Detection Antibody	MSPHM-1021
Bead Diluent	LA-BD
Streptavidin-Phycoerythrin	MC-SAPE2
Assay Buffer	L-AB
96-Well plates with 2 sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	Bead No.	Cat. No.
Cortisol	12	C0RT-MAG
Estradiol	14	ESTRDL-MAG
Progesterone	22	PRGST-MAG
T3	35	T3-MAG
T4	37	T4-MAG

Well Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK (Background)	Std 4	QC-1 Control									
B	BLANK (Background)	Std 4	QC-1 Control									
C	Std 1	Std 5	QC-2 Control									
D	Std 1	Std 5	QC-2 Control									
E	Std 2	Std 6	Sample 1									
F	Std 2	Std 6	Sample 1									
G	Std 3	Reconstituted Std 7	Sample 2									
H	Std 3	Reconstituted Std 7	Sample 2									

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