LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric)

Catalog Number KA1603

48 assays

Version: 02

Intended for research use only

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Introduction

Intended Use

The LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric) is suitable for measuring LSD1 activity/inhibition using nuclear extracts or purified enzymes from a broad range of species such as mammals, plants, fungi, and bacteria, in a variety of forms including cultured cells and fresh tissues. Nuclear extracts can be prepared by using your own successful method. Nuclear extracts can be used immediately or stored at -80°C for future use. Purified enzymes can be active LSD1 from recombinant proteins or isolated from cell/tissues.

Background

Lysine histone methylation is one of the most robust epigenetic marks and is essential for the regulation of multiple cellular processes. The methylation of H3-K4 seems to be of particular significance, as it is associated with active regions of the genome. H3-K4 methylation was considered irreversible until the identification of a large number of histone demethylases indicated that demethylation events play an important role in histone modification dynamics. So far at least 2 classes of H3-K4 specific histone demethylase, LSD1 (BHC110, KDM1) and JARIDs have been identified. LSD1 can remove di- and mono-methylation from H3-K4 by using an amine oxidase reaction. LSD1 is associated with complexes that function as both transcriptional inactivators and activators. It demethylates mono-/di-methyl H3-K4 when associated with the Co-REST complex at neuronal genes, or mono-/di-methyl H3-K9 when associated with the androgen receptor.

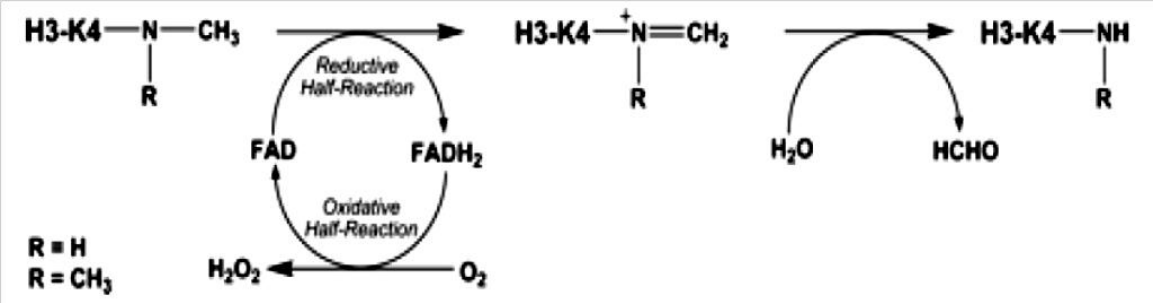


Fig 1. Histone H3-K4 demethylation reaction catalyzed by LSD1.

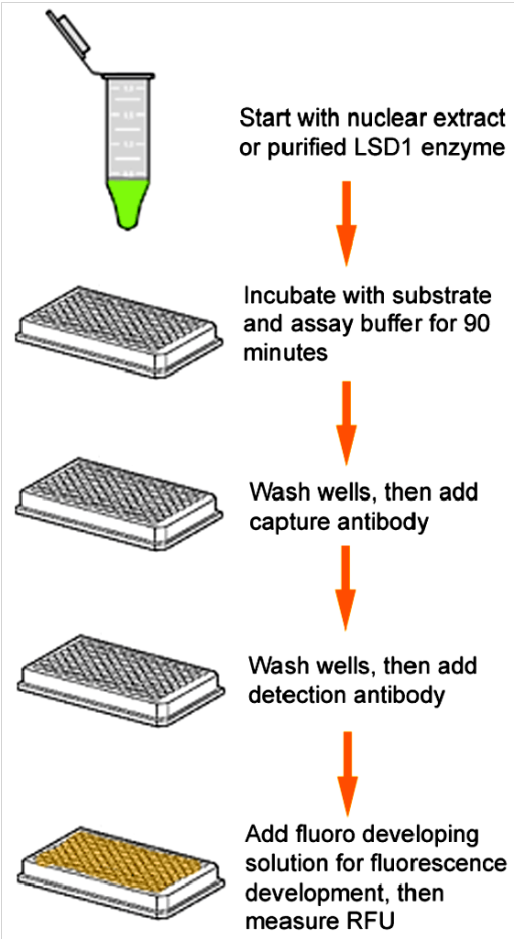
LSD1 is found to be pivotal in development and differentiation. For example, this enzyme is required to induce skeletal muscle differentiation, and mutation of drosophila LSD1 results in tissue-specific defect in development through disrupting H3-K4 methylation. LSD1 is also shown to participate in regulation of chromatin remodeling, cell death and global DNA methylation. More importantly, LSD1 is found to be involved in some pathological processes such as cancer and inflammatory diseases. For example, expression of LSD1 is observed in cancer and LSD1 triggers MYC and E2F-mediated transcription in cancer cells. Detection of activity and inhibition of LSD1 would be important in elucidating mechanisms of epigenetic regulation of gene activation and silencing and benefiting cancer diagnostics and therapeutics.

There are only a couple of methods used for detecting LSD1 activity/inhibition. These methods are based on the measurement of H_2O_2 or formaldehyde release, a by-product of LSD1 enzymatic reaction and have significant weaknesses including: (1) Large amount (at µg: level) of substrate and enzyme are required; (2) Nuclear extracts from cell/tissues can not be used; (3) Redox-sensitive LSD1 inhibitiors are not suitable for testing with these methods; (4) Highly interfered by DMSO and thiol-containing chemicals, which are often contained in enzyme solution or tested compound solvents; and (5) Less accuracy than direct measurement of LSD1-converted demethylated product. These problems were averted with our LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric). This latest method retains the simplicity, rapidness, high throughput, and non-radioactivity, and has the following advantages:

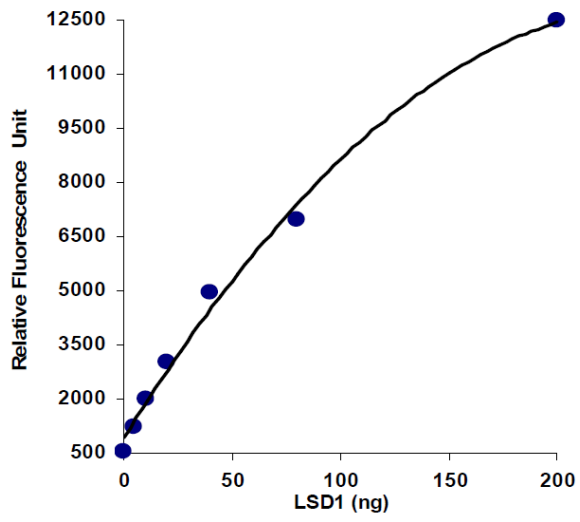
- Strip-well microplate format makes the assay flexible and quick: manual or high throughput analysis that can be completed within 3 hours.
- Enhanced kit composition enables background signals to be extremely low, which allows the assay to be more accurate, sensitive, reliable, and consistent.
- Innovative fluorometric assay directly measures LSD1 activity by a straightforward detection of LSD1-converted demethylated product, rather than by-products. Thus it eliminates assay interferences caused by thiol-containing chemicals such as DTT, GSH, and 2-mercaptoethanol.
- Both cell/tissue extracts and purified LSD1 can be used, which allows for the detection of inhibitory effects of LSD1 inhibitor *in vivo* and *in vitro*.
- Novel assay principle allows high sensitivity to be achieved. The activity can be detected from as low as 2 ng of purified LSD1 enzyme, which is about 50 fold higher than that obtained by H_2O_2/formaldehyde release-based LSD1 assays.
- Demethylated H3-K4 standard is included, which allows the specific activity of LSD1 to be quantified.

Principle of the Assay

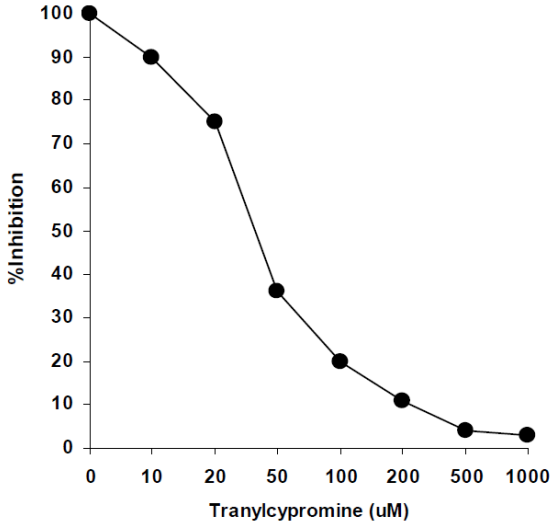
The LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric) contains all reagents necessary for the measurement of LSD1 activity/inhibition. In this assay, di-methylated histone H3-K4 LSD1 substrate is stably coated onto the strip wells. Active LSD1 binds to the substrate and removes methyl groups from the substrate. The LSD1-demethylated products can be recognized with a specific antibody. The ratio or amount of demethylated products, which is proportional to enzyme activity, can then be fluorometrically measured by reading the fluorescence microplate reader. The activity of LSD1 enzyme is proportional to the fluorescent density intensity measured.



Schematic procedure of the LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric)



Demonstration of high sensitivity of LSD1 activity assay achieved by using recombinant LSD1 with the LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric).



Demonstration of inhibitory effect of LSD1 inhibitor detected by the LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric). [LSD1 concentration: 200 ng/well]

General Information

Materials Supplied

List of component

Component	Amount
LF1 (10X Wash Buffer)	14 mL
LF2 (LSD1 Assay Buffer)	4 mL
LF3 (LSD1 Substrate, 50 µg/mL)*	60 µL
LF4 (LSD1 Assay Standard, 50 µg/mL)*	10 µL
LF5 (Capture Antibody, 1000 µg/mL*)	5 µL
LF6 (Detection Antibody, 400 µg/mL)*	6 µL
LF7 (LSD1 Inhibitor Tranylcypromine, 1 mM)*	20 µL
LF8 (Fluoro Developer)	10 µL
LF9 (Fluoro Enhancer)	10 µL
LF10 (Fluoro Diluter)	4 mL
8-Well Assay Strips (With Frame)	6 strips
Adhesive Covering Film	1 slice

* Spin the solution down to the bottom prior to use.

Storage Instruction

- Upon receipt: (1) Store LF3, LF4, LF6 and LF8 at –20°C away from light; (2) Store LF1, LF5, LF7, LF9, and 8-Well Assay Strips at 4°C away from light; (3) Store remaining components (LF2, LF10, and Adhesive Covering Film) at room temperature away from light.
- All components of the kit are stable for 6 months from the date of shipment, when stored properly.

Note: (1) Check if LF1 (10X Wash Buffer) contains salt precipitates before use. If so, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved.

Materials Required but Not Supplied

- ✓ Adjustable pipette or multiple-channel pipette
- ✓ Multiple-channel pipette reservoirs
- ✓ Aerosol resistant pipette tips
- ✓ Fluorescence microplate reader capable of reading fluorescence at $530_{ex}/590_{em}$ nm
- ✓ 1.5 mL microcentrifuge tubes
- ✓ Incubator for 37°C incubation
- ✓ Distilled water
- ✓ Nuclear extract or purified enzymes

- ✓ Parafilm M or aluminum foil

Precautions for Use

- ✓ Input Material: Input materials can be nuclear extracts or purified LSD1 enzymes. The amount of nuclear extracts for each assay can be 0.5 µg to 20 µg with an optimal range of 5-10 µg. The amount of purified enzymes can be 2 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.
- ✓ Internal Control: The LSD1 assay standard (demethylated hsitone H3-K4) is provided in this kit for quantification of LSD1 enzyme activity. Because LSD1 activity can vary from tissue to tissue, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated is validated.
- ✓ Quality Control: Each lot of the LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric) is tested against predetermined specifications to ensure consistent product quality. Abnova guarantees the performance of all products in the manner described in our product instructions.
- ✓ Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
- ✓ Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.
- ✓ Usage Limitation: The LSD1 Demethylase Activity/Inhibition Assay Kit (Fluorometric) is for research use only and is not intended for diagnostic or therapeutic application.
- ✓ Protocol: For the best results, please read the protocol in its entirety prior to starting your experiment.

Assay Protocol

Reagent Preparation

- ✓ Prepare Diluted LF1 1X Wash Buffer: Add 13 mL of LF1 10X Wash Buffer to 117 mL of distilled water and adjust pH to 7.2-7.5. This Diluted LF1 1X Wash Buffer can now be stored at 4°C for up to six months.
- ✓ Prepare Diluted LF5 Capture Antibody Solution: Dilute LF5 Capture Antibody with Diluted LF1 1X Wash Buffer at a ratio of 1:1000 (add 1 µL of LF5 to 1000 µL of Diluted LF1 1X Wash Buffer). 50 µL of Diluted LF5 will be required for each assay well.
- ✓ Prepare Diluted LF6 Detection Antibody Solution: Dilute LF6 Detection Antibody with Diluted LF1 1X Wash Buffer at a ratio of 1:2000 (add 1 µL of LF6 Detection Antibody to 2000 µL of Diluted LF1 1X Wash Buffer). 50 µL of Diluted LF6 will be required for each assay well.
- ✓ Prepare Diluted LF4 Assay Standard Solution: Suggested Standard Curve Preparation: First, dilute LF4 with LF2 to 5 ng/µL by adding 1 µL of LF4 to 9 µL of LF2. Then, further prepare five concentrations by combining the 5 ng/µL diluted LF4 with LF2 into final concentrations of 0.2, 0.5, 1, 2, and 5 ng/µL according to the following dilution chart:

Tube	LF4 (5 ng/µL)	LF2	Resulting LF4 Concentration
1	1.0 µL	25.0 µL	0.2 ng/µL
2	1.0 µL	9.0 µL	0.5 ng/µL
3	1.0 µL	4.0 µL	1.0 ng/µL
4	2.0 µL	3.0 µL	2.0 ng/µL
5	4.0 µL	0.0 µL	5.0 ng/µL

- ✓ Prepare Fluorescence Development Solution: Add 1 µL of LF8 Fluoro Developer and 1 µL of LF9 Fluoro Enhancer to every 500 µL of LF10 Fluoro Diluter.

Note: Keep each of diluted solutions except Diluted LD1 1X Wash Buffer on ice until use. Any remaining diluted solutions other than Diluted LD1 should be discarded if not used within the same day.

- Suggested Buffer and solution Setup

Table 1. Approximate amount of required buffers and solutions for defined assay wells based on the protocol.

Reagents	1 well	1 strip (8 wells)	2 strips (16 wells)	6 strips (48 wells)
Diluted LF1	2.5 mL	20 mL	40 mL	120 mL
LF2	50 µL	400 µL	800 µL	2400 µL
LF3	1 µL	8 µL	16 µL	50 µL
LF4	NA	NA	1 µL (optional)	2 µL
Diluted LF5	50 µL	400 µL	800 µL	2400 µL
Diluted LF6	50 µL	400 µL	800 µL	2400 µL
Fluorescence Development Solution	0.05 mL	0.4 mL	0.8 mL	2.4 mL

Assay Procedure

- ✓ *Input Amount: The amount of nuclear extracts for each assay can be 0.5 µg to 20 µg with optimized range of 5-10 µg. The amount of purified enzymes can be 2 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.*
- ✓ *Nuclear Extraction: You can use your method of choice for preparing nuclear extracts.*
- ✓ *Nuclear Extract or Purified LSD1 Storage: Nuclear extract or purified LSD1 enzyme should be stored in aliquots at –80°C until use.*

- Enzymatic Reaction

1. Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include blank and standard controls) to ensure that the signal generated is validated. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).
2. Blank Wells: Add 49 µL of LF2 and 1 µL of LF3 to each blank well.
3. Standard Wells: For a standard curve, add 49 µL of LF2 and 1 µL of Diluted LF4 standard solution to each standard well with a minimum of five wells, each at a different concentration between 0.2 to 5 ng/µL (based on the dilution chart in Reagent Preparation).
4. Sample Wells Without Inhibitor: Add 44 to 48 µL of LF2, 1 µL of LF3, and 1 to 5 µL of your nuclear extracts or 1 to 5 µL of purified LSD1 enzyme to each sample well without inhibitor. Total volume should be 50 µL per well.
5. Sample Well With Inhibitor: Add 40 to 43 µL of LF2, 1 µL of LF3, 1 to 4 µL of your nuclear extracts or 1 to 4 µL of purified LSD1 enzyme, and 5 µL of inhibitor solution. Total volume should be 50 µL per well.
   Note: (1) Follow the suggested well setup diagrams; (2) It is recommended to use 2 µg to 10 µg of nuclear extract per well or 10 ng to 100 ng of purified enzyme per well; (3) The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1 µM to 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with LF2 at a 1:10 ratio (e.g., add 0.5 µL of inhibitor to 4.5 µL of LF2), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less. The LSD1 inhibitor, Tranylcypromine (LF7) included in the kit can be used as a control inhibitor.
6. Tightly cover strip-well microplate with Adhesive Covering Film to avoid evaporation and incubate at 37°C for 60-120 min.
   Note: (1) The incubation time may depend on intrinsic LSD1 activity. However, in general, 60-90 min incubation is suitable for active purified LSD1 enzymes and 90-120 min incubation is required for nuclear extracts; (2) The Adhesive Covering Film can be cut to the required size to cover the strips based on the number of strips to be used.
7. Remove the reaction solution from each well. Wash each well with 150 µL of the Diluted LF1 1X Wash Buffer each time for three times.

- Antibody Binding and Signal Enhancing

1. Add 50 µL of the Diluted LF5 to each well, then cover and incubate at room temperature for 60 min.
2. Remove the Diluted LF5 solution from each well.
3. Wash each well with 150 µL of the Diluted LF1 each time for three times.
4. Add 50 µL of the Diluted LF6 to each well, then cover and incubate at room temperature for 30 min.
5. Remove the Diluted LF6 solution from each well.
6. Wash each well with 150 µL of the Diluted LF1 each time for four times.

Note: Ensure any residual wash buffer in the wells is removed as much as possible at each wash step.

- Signal Detection

1. Add 50 µL of Fluorescence Development Solution to each well and incubate at room temperature for 2 to 4 min away from direct light. The Fluorescence Development Solution will turn pink in the presence of sufficient methylated products.
2. Read the fluorescence on a fluorescence microplate reader with 2 to 10 min at $530_{ex}/590_{em}$ nm.

Note: If the strip-well microplate frame does not fit in the fluorescence microplate reader*, transfer the solution to a standard 96-well microplate.*

Data Analysis

Calculation of Results

LSD1 Activity Calculation

- ✓ Calculate the average duplicate readings for the sample wells and blank wells.
- ✓ Calculate LSD1 activity or inhibition using the following formulas:

- For simple calculation with a single standard control:

$$\text{LSD1 Activity(RFU/min/mg)} = \frac{(\text{Sample RFU} - \text{Blank RFU})}{(\text{Protein Amount(ug)} * \text{x min} * *)} \times 1000$$

* Protein amount (µg) added into the Enzymatic Reaction at step 4.

** Incubation time (minutes) at Enzymatic Reaction step 6.

Example calculation:

Average RFU of sample is 6800

Average RFU of blank is 800

Protein amount is 5 µg

Incubation time is 120 minutes (2 hours)

$$\text{LSD1 activity} = \frac{(6800 - 800)}{(5 \text{ x } 120)} \times 1000 = 10000 \text{ RFU/min/mg}$$

- For accurate or specific activity calculation:

1. Generate a standard curve and plot RFU value versus amount of LF4 at each concentration point.
2. Determine the slope as RFU/ng (you can use Microsoft Excel statistical functions for slope calculation), then calculate the amount of LSD1-converted demethylated product using the following formulas:

$$\text{Demethylated product (ng)} = \frac{(\text{Sample RFU} - \text{Blank RFU})}{\text{Slope}}$$

$$\text{LSD1 Activity(ng/min/mg)} = \frac{\text{Demethylated Product (ng)}}{(\text{Protein Amount(ug)} \text{ x min}*)} \times 1000$$

* Incubation time (minutes) at Enzymatic Reaction step 6.

- For inhibition calculation:

$$\text{Inhibition\%} = \left[1 - \frac{\text{Inhibitor Sample RFU} - \text{Blank RFU}}{\text{No Inhibitor Sample RFU} - \text{Blank RFU}}\right] \times 100\%$$

Resources

Troubleshooting

Problem	Possible Cause	Suggestion
No signal in both the positive control and sample wells	Reagents are added incorrectly.	Check if reagents are added in the proper order and if any steps in the protocol may have been omitted by mistake.
	The well is incorrectly washed before enzyme binding.	Ensure the well is not washed prior to adding the positive control and sample.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Incorrect fluorescence reading.	Check if appropriate fluorescence wavelength ($530_{ex}/590_{em}$ nm filter) is used.
	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperature and the cap is tightly capped after each opening or use.
No signal or weak signal in only the positive control wells	The standard amount is insufficiently added to the well in Enzymatic Reaction step 3	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance of this User Guide for storage of LF4 (LSD1 Assay Standard).
High background present in the negative control wells	Insufficient washing of wells.	Check if washing recommendations at each step is performed according to the protocol.
	Contaminated by sample or standard	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips.
	Incubation time with detection antibody is too long.	The incubation time at Antibody Binding and Signal Enhancing step 4 should not exceed 45 minutes.
	Over-development of fluorescence.	Decrease the development time in Signal Detection step 1
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for LSD1 protein extraction. Use fresh cells or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of purified enzymes or nuclear extracts is used as indicated in Enzymatic Reaction The sample can be titrated to determine

		the optimal amount to use in the assay.
	Sample was not stored properly or has been stored for too long.	Ensure sample is stored in aliquots at –80°C, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing.
	Little or no activity of LSD1 contained in the sample.	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared nuclear extracts or purified enzymes.
Uneven fluorescent development	Insufficient wash of the wells.	Ensure the wells are washed according to the protocol. Ensure any residues from the wash buffer are removed as much as possible.
	Delayed fluorescence development in the wells.	Ensure fluorescence development solution is added sequentially and consistent with the order you added the other reagents (e.g., from well A to well G or from well 1 to well 12).

Plate Layout

	1	2	3	4	5	6	7
A	Blank	Blank	Sample	Sample	Sample	Sample	Sample
B	LF4 0.2 ng	LF4 0.2 ng	Sample	Sample	Sample	Sample	Sample
C	LF4 0.5 ng	LF4 0.5 ng	Sample	Sample	Sample	Sample	Sample
D	LF4 1.0 ng	LF4 1.0 ng	Sample	Sample	Sample	Sample	Sample
E	LF4 2.0 ng	LF4 2.0 ng	Sample	Sample	Sample	Sample	Sample
F	LF4 5.0 ng	LF4 5.0 ng	Sample	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample	Sample