LUMINOS®
INTERACTIVE ASSAY SOLUTIONS

Measure LSD1 & *Jumonji* HDM activity directly! Perfect for HTS

DetectX™

Universal Histone Demethylase Fluorescent Detection Kit

Catalog Number K010-F1

Sample Types Validated:

LSD1-and *Jumonji*-type Demethylases

Patent Pending

Licensed exclusively to Luminos LLC

Please read this insert completely prior to using the product.

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROTOCOLS

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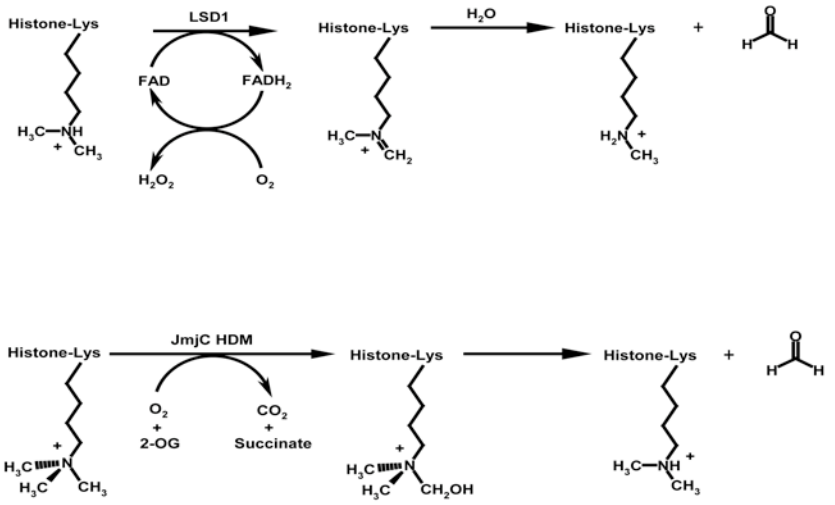
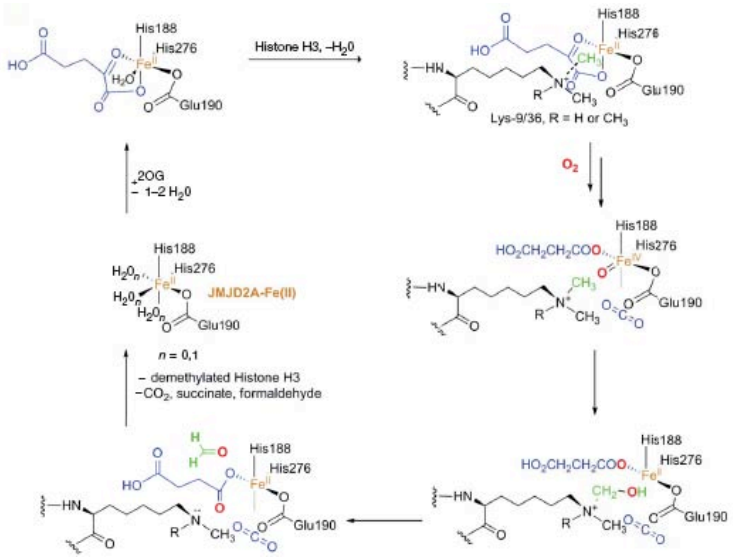
INTRODUCTION

Formaldehyde is a common byproduct formed in the oxidative demethylation of proteins, nucleic acids, and biological small molecules. Examples of formaldehyde-producing enzymes include DNA demethylases, histone demethylases (HDMs), and cytochrome P450 enzymes that demethylate drugs and other xenobiotic compounds[1-6]. HDMs catalyze the site-specific demethylation of methyl-lysine residues in histones to dynamically regulate chromatin structure, gene expression, and potentially other genomic functions. Lysine-specific HDMs were first discovered in 2004 and are currently among the most actively studied formaldehyde-producing enzymes[7]. At present, there are two known classes of HDMs: the flavin adenine dinucleotide (FAD)-dependent Lysine Specific Demethylase 1 (LSD1) family and the Fe(II)-dependent Jumonji C (JmjC) family. Although the LSD1 and JmjC HDMs employ different cofactors and catalytic mechanisms (Figure 1), both produce formaldehyde as a byproduct of the demethylation reaction. Despite their biological importance, HDMs have proven difficult to quantitatively assay owing to their relatively low turnover numbers, hindering our understanding of their kinetic properties, substrate specificities, and reaction mechanisms.

Figure 1



Proposed mechanism for JMJD2A (from reference 8)



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THE LUMINOS DetectX™ DEMETHYLASE ACTIVITY

FLUORESCENT DETECTION KIT

Assay Principle

The DetectX™ Demethylase Activity kit is designed to quantitatively measure the enzymatic activity of formaldehyde-producing enzymes such as Cytochrome P450, DNA and Histone Demethylases. The kit is unique in that the product of these enzymatic demethylation reactions, formaldehyde, is quantitated directly by a fluorescent product. No separation or washing is required. The kit has been validated for both LSD1 and JMJD2A histone Demethylases (HDMs) and should work with any biological system that is producing formaldehyde as a product, such as DNA demethylases, Cyp3A4 and other demethylating P450 enzymes.

The kit provides optimized buffers for the HDMs, LSD1 and JMJD2a, a stable formaldehyde standard, the Formaldehyde Detection Reagent (FDR) and two 96 well plates for detecting the generated fluorescent signal. The end user will have to provide the demethylase system and any cofactors, etc. necessary for activity, along with any test inhibitors or activators. The demethylase reaction should be carried out in our supplied buffers using each demethylase reaction optimized conditions for the demethylation.

Following the formaldehyde generating reaction, the reaction can be stopped by addition of a suitable inhibitor. The FDR is then added to all the wells. If calibration to formaldehyde is needed (for cross lab comparisons) then a formaldehyde standard curve generated from the supplied standard should be run.

After a short incubation at 37°C for 30 minutes, the fluorescent product is read at 510 nm in a fluorescent plate reader with excitation at 450 nm. The demethylase activity is determined based upon formaldehyde production, after making a suitable correction for any dilution of the sample, using software available with most fluorescence plate readers. We have provided two 96 well plates for measurement but this assay is adaptable for higher density plate formats. The end user should ensure that their HTS black plate is suitable for use with these reagents prior to running samples.

Demethylase Reaction Conditions

We have ensured that the DetectX™ Demethylase Assay detects the activity of the 2 types of demethylase systems, the LSD1-type and the *Jumonji*-type. Below we have listed the conditions we used in validating this fluorescent detection system, its compatibility with the LSD1 and JMJD2A reactions and the ability to quantitate the formaldehyde produced by both enzymatic reactions.

Typical LSD1 Enzyme Reaction

To duplicate wells add 50 µL of either formaldehyde standards, LSD1 enzyme or a blank all made using the LSD1-type Assay Buffer, 50 µL of a specific 1-21 sequence of Histone H3 with the dimethylated lysine at amino acid 4, (H3K4-Me2) dissolved in LSD1-type Assay Buffer was added. The plate was sealed and incubated at 30°C for one hour. At the end of the incubation, 25µL of the FDR was added to each well, the plate was resealed and incubated at 37°C for 30 minutes.

Typical JMJD2A Enzyme Reaction

To duplicate wells add 50 µL of 2mM ascorbate, 100mM $FeSO_4$, 25 µL of either formaldehyde standards, JMJD2A enzyme or a blank all made using the *Jumonji*-type Assay Buffer was added, followed by 25 µL of a specific 1-24 sequence of Histone H3 with the trimethylated lysine at amino acid 9, (H3K9-Me3) containing 2mM alpha-ketoglutarate dissolved in *Jumonji*-type Assay Buffer. The plate was sealed and incubated at 30°C for 30 minutes. 5µL of 4 mM deferoxamine in *Jumonji*-type Assay Buffer was added. 25µL of the FDR was then added to each well, the plate was resealed and incubated at 37°C for 30 minutes.

REACTION OVERVIEW

1. **Carry out demethylating enzyme reaction.**
2. **Stop the reaction (optimal), add FDR.**
3. **Incubate at 37°C for 30 minutes, read signal.**
4. **Calibrate to Formaldehyde generated.**

Supplied Components

Black Half Area Microtiter Plates 2 plates Catalog Number X037-2EA

LSD1-type Assay Buffer 60 mL Catalog Number X038-60ML
A phosphate buffer containing detergents and stabilizers.

JMJD2A-type Assay Buffer 60 mL Catalog Number X039-60ML
A HEPES buffer containing stabilizers.

Formaldehyde Standard 0.5 mL Catalog Number C001-500UL
2,000 μM formaldehyde solution in deionized water. Outer container has formaldehyde absorbing pad. The standard is stable if kept tightly sealed.
KEEP TIGHTLY SEALED

DetectX™ Formaldehyde Reagent 5 mL Catalog Number C002-5ML
Special formulation of reagents to detect formaldehyde in solution.
Contains ≤0.09% sodium azide as a preservative.

Plate Sealers 2 Each Catalog Number X002-2EA

Storage Instructions

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

Other Materials Required

Incubators. Incubators capable of accurately maintaining 30°C and 37°C.

Demethylase enzyme samples. A source of LSD1-type or Jumonji-type demethylase, along with any cofactors, enzyme substrates, inhibitors, and/or activators.

Fluorescence 96 well plate reader capable of reading fluorescent emission at 510 nm, with excitation at 450 nm.

Software for converting raw relative fluorescent unit (FLU) 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.

Some of the components of this kit contain sodium azide as a preservative, which may react with lead or copper plumbing to form potentially explosive complexes. When disposing of reagents always flush with large volumes of water to prevent azide build-up.

Sample Types

Histone demethylases diluted in the assay buffers provided are compatible with this assay.

Formaldehyde Standard Preparation

Label seven glass test tubes as #1 through #7. Pipet 450 µL of Assay Buffer containing all cofactors and additives into tube #1 and 250 µL into tubes #2-#7. Add 50 µL of the Formaldehyde stock solution to tube #1 and vortex completely. Add 250 µL of tube #1 to tube #2 and vortex completely. Repeat this for tubes #3 through #7. The concentration of formaldehyde in tubes 1 through 7 will be 200, 100, 50, 25, 12.5, 6.25 and 3.125 µM.

Sufficient 2,000 µM formaldehyde stock is provided to allow the end user to fit the standard curve to the expected formaldehyde generated by each enzyme system.

Use all Standards within 2 hours of preparation.

Assay Protocol

Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine activity. Ensure that all samples have reached room temperature prior and have been diluted as appropriate prior to running them in the kit. Demethylase reaction volume should be no more than 100 µL in each well including all cofactors, inhibitors and activators.

Demethylase Reaction

1. Set up the appropriate demethylase reaction in one of the supplied buffers.

2. Pipet 100 µL standards or samples plus all cofactors and inhibitors into wells in the black plate.

3. Carry out demethylation reaction and preferably stop reaction at an appropriate time.

Formaldehyde Detection

4. Add 25 µL of the DetectX™ Formaldehyde Detection Reagent to each well using a repeater or multichannel pipet.

5. Gently tap the sides of the plate to ensure adequate mixing of the reagents.

6. Incubate at 37°C for 30 minutes. Room temperature incubation will yield approximately 75% of the fluorescent signal generated with 37°C incubation.

7. Read the fluorescent signal from each well in a plate reader capable of reading the fluorescent signal at 510 nm with excitation at 450 nm. Please contact your plate reader manufacturer for suitable filter sets.

8. Use the plate reader's built-in 4PLC software capabilities to calculate formaldehyde concentrations for each sample.

Typical Data - LSD1 and JMJD2A Type Reactions

LSD1 Assay

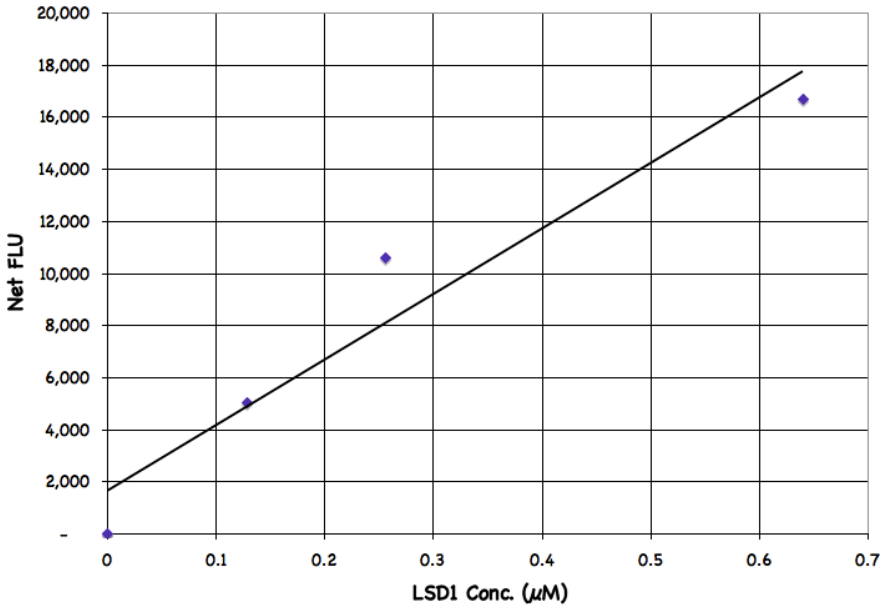
LSD1 Conc. (μM)	Mean FLU	Mean Net FLU
Zero	1,827	0
0.64	18,522	16,695
0.256	12,410	10,584
0.128	6,849	5,022

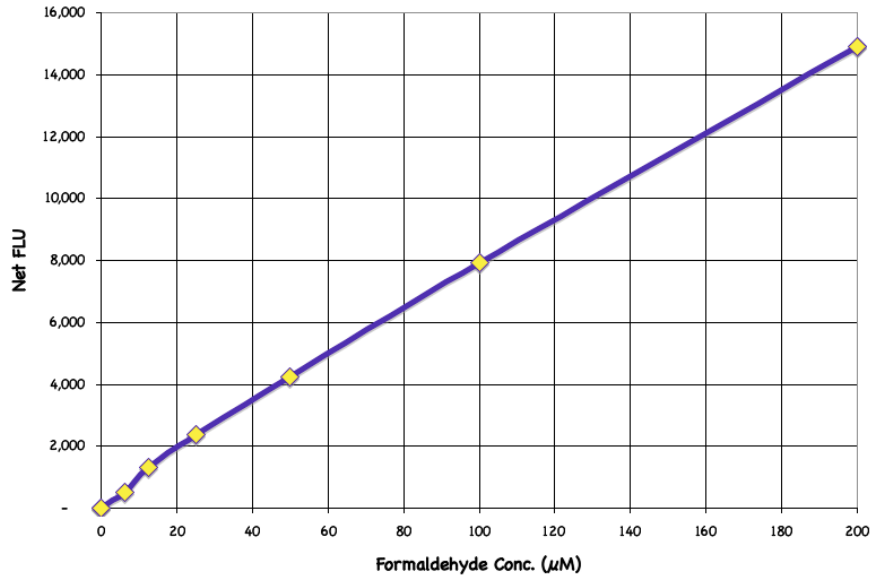
JMJD2A Assay

JMJD2A Conc. (μM)	Mean FLU	Mean Net FLU
Zero	3,028	0
10	17,973	14,945
5	10,719	7,691
2.5	4,528	1,500

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

Typical LDS1 and Formaldehyde Standard Curves





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

LSD1 Interference Study

The following additives were added to the LSD1 enzyme reaction with its 1-21 methylated peptide substrate to test for interference with the signal generation.

Organic Solvents

5% of the organic solvents methanol, N,N-dimethylformamide and dimethylsulfoxide had no negative effect on the generation of fluorescence in the presence of 0.256 μM LSD1.

Detergents

0.005% SDS inhibited the enzymatic generation of formaldehyde by 89%. 0.1% or less Triton X-100 and 0.01% or less Tween 20 had no effect on signal generation.

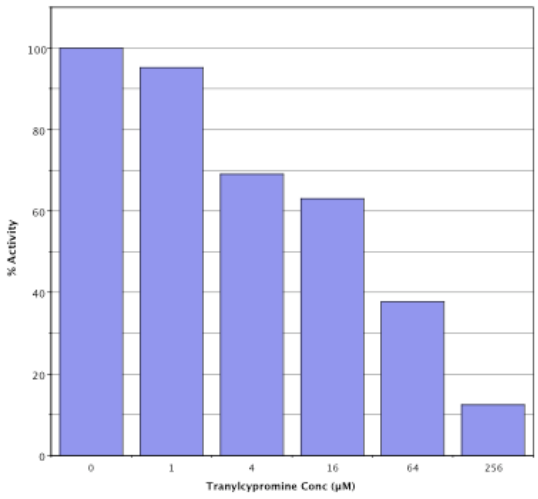
Preservatives

0.09% sodium azide in the assay buffer reduced the signal by 20%. 0.09% Kathon inhibited the signal by 91.3%, 0.005% gentamicin had no negative effect on the signal generation.

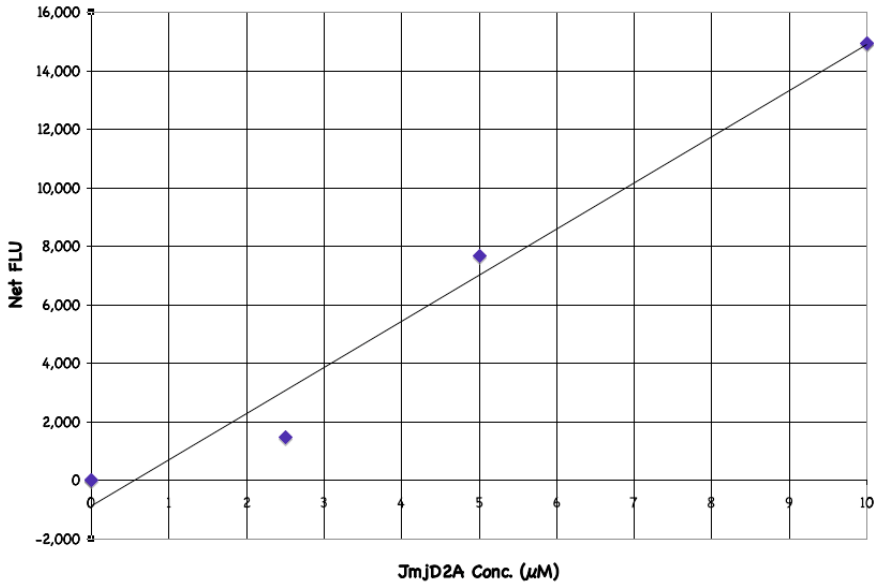
Inhibition of LSD1 Activity

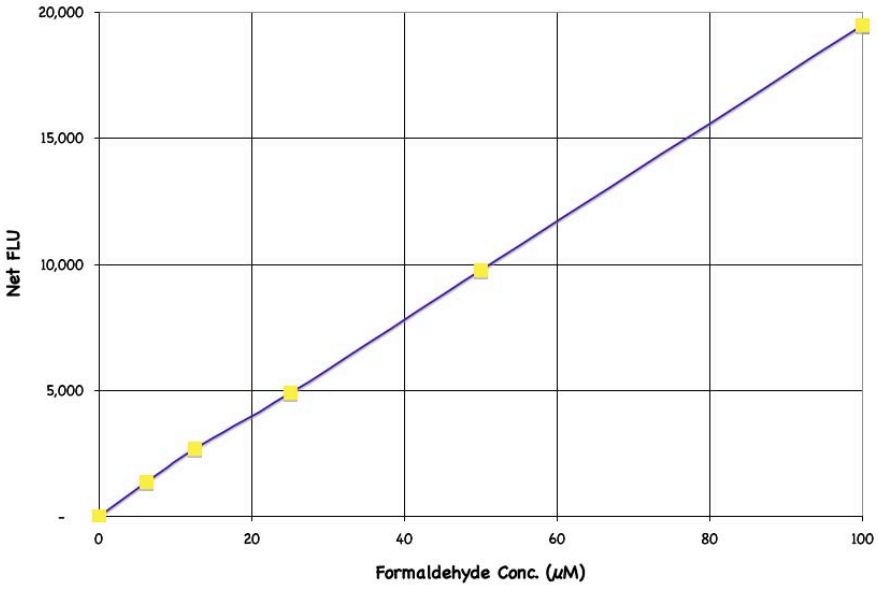
The LSD1 inhibitor, Tranylcypromine, was added to LSD1 enzyme reaction with its 1-21 methylated peptide substrate at concentrations ranging from 256 μM to 1 μM.

Tranycypromine Inhibition Curve



Typical JMJD2A and Formaldehyde Standard Curves





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

JMJD2A Interference Study

The following additives were added to the JMJD2A enzyme reaction with its 1-24 methylated peptide substrate to test for interference with the signal generation.

Organic Solvents

5% of the organic solvents methanol, N,N-dimethylformamide and dimethylsulfoxide had no negative effect on the generation of fluorescence in the presence of JMJD2A.

Detergents

0.005% SDS inhibited the enzymatic generation of formaldehyde by 7.1%. 0.1% Triton X-100 inhibited the signal by 4.2%. 0.01% or less Tween 20 had no effect on signal generation.

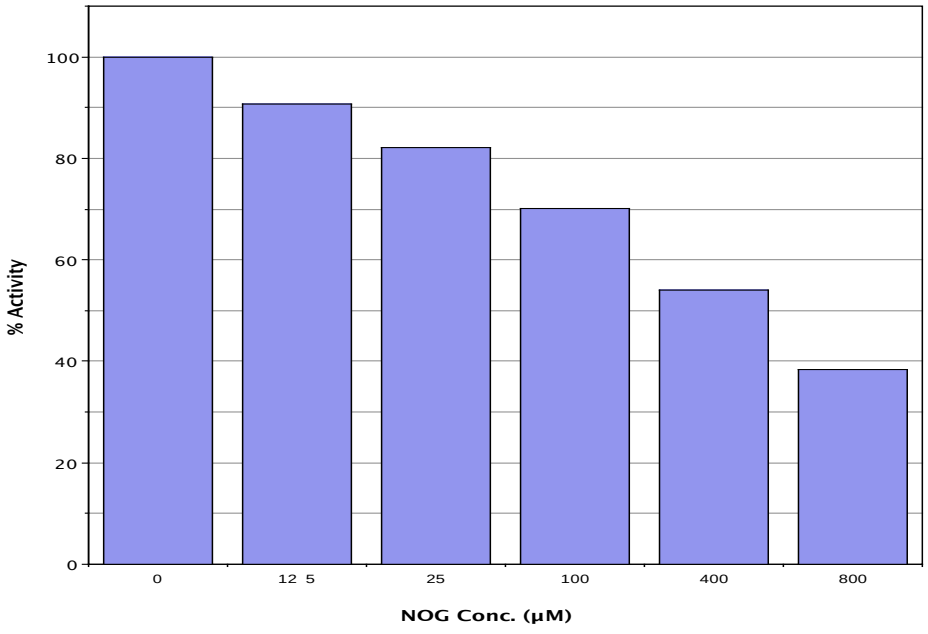
Preservatives

0.09% sodium azide in the assay buffer reduced the signal by 35.3%. 0.09% Kathon inhibited the signal by 56.9, 0.005% gentamicin had no negative effect on the signal generation.

Inhibition of JMJD2A Activity

The JMJD2A inhibitor, N-Oxalylglycine, was added to the JMJD2A enzyme reaction with its 1-24 methylated peptide substrate at concentrations ranging from 800 μM to 12.5 μM.

NOG Inhibition Curve



LIMITED WARRANTY

Luminos LLC 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.

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PLATE LAYOUT

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