REF 5FU-RGT

5-Fluorouracil (My5-FU™) Assay

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Key to Symbols Used

IVD	*in vitro* Diagnostic Device		Consult Instructions for Use
REF	Catalog Number		Use By
LOT	Batch Code		Temperature Limitation
	Manufacturer	(N) x	Gently invert reagents (R1 and R2) N number of times prior to use
R1	Reagent 1	R2	Reagent 2
CH REP	Authorized Representative in Switzerland		
EC REP	Authorized Representative in the European Community		

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Intended Use

The Saladax 5-Fluorouracil (My5-FU) Assay is an *in vitro* diagnostic medical device intended for the quantitative determination of 5-FU in human plasma using automated clinical chemistry analyzers as an aid in the management of 5-FU therapy.

Summary and Explanation of the Test

5-FU (Adrucil, among others) is a chemotherapeutic agent used in the treatment of several solid tumor cancers, especially in colorectal, stomach, breast, pancreatic and head and neck cancers.[1-2] Since its development in 1957, it has been the mainstay of colorectal cancer treatment.[3] The metabolic pathways of 5-FU have been extensively investigated. Several studies have reported high inter-individual variability of 5-FU metabolism. Blood concentrations of 5-FU can vary by over 10-fold despite equal dose administration by Body Surface Area (BSA).[4-8]

5-FU is routinely administered by infusion or orally as a prodrug. In plasma it has a half-life of approximately ten to fifteen minutes and achieves steady state concentrations in two hours with continuous infusion. Severe side effects,

including gastrointestinal, neurological, hematological and mucosal toxicity, have been encountered during conventional 5-FU treatment.[9] Studies have shown that over 30% of treated patients exhibit dose-limiting toxicity. Recent studies also reported severe toxicity (grade 3/4) and death following treatment with 5-FU. Research has suggested that only 40% to 50% of patients with severe toxicity display partial or profound dihydropyrimidine dehydrogenase (DPD) enzyme deficiency, demonstrating that there are other factors besides a genetic DPD defect that can affect 5-FU levels.[10-15]

Clinical studies have consistently reported that the majority of patients are not in the target therapeutic range when dosed by BSA and indeed most of those are underdosed.[16-23] Clinical data also demonstrate that pharmacokinetically (PK) guided dose adjustment of blood levels of 5-FU can result in lowered toxicity, improved overall response, and increased survival.[4-8,16,17,19,20,22,24-38]

In combination with other clinical information, monitoring 5-FU levels provides physicians with an effective tool to aid in dose management in order to achieve optimal therapeutic effect while avoiding sub-therapeutic or toxic drug levels.[4-8,16,17,19,20,22,24-39] With reduced toxicity, patients are able to maintain treatment for longer periods of time. Today, PK assessment of 5-FU in patients can only be performed by physical analytical methods such as high performance liquid chromatography (HPLC)[40-42] or liquid chromatography-tandem mass spectroscopy (LC-MS/MS).[34,42] These physical techniques are time-consuming, expensive, involve sample preparation and require full-time technicians, all of which hinder widespread adoption of a PK-guided dose management approach.

By contrast, a homogenous immunoassay for determining levels of 5-FU provides rapid quality results correlated to physical methods utilizing routine clinical analyzers, comparable to other therapeutic drug monitoring determinations that have been routinely used for more than thirty years. The My5-FU Assay provides a convenient, cost-effective, and timely tool to aid oncologists in 5-FU dose management.

Principles of the Procedure

The My5-FU Assay (US Patent No. 7,205,116) is a homogeneous two-reagent nanoparticle agglutination immunoassay used for detection of 5-FU in human plasma. It is based upon the principle of measuring changes in scattered light or absorbance which result when nanoparticles aggregate. This aggregation is measured at wavelengths between 400 and 650 nm using automated clinical chemistry or immunoassay analyzers. Multivalent drug-conjugates serve as a binding partner to antibodies selective for 5-FU which are covalently attached to the surface of nanoparticles. In the absence of free 5-FU, this reaction creates large aggregates, resulting in a solution that scatters incident light and leads to an increase in the observed absorption of the solution. When a sample containing 5-FU is introduced, the agglutination reaction is partially inhibited. Antibody bound to sample drug is no longer available to promote nanoparticle aggregation, resulting in less scattering of incident light and lower observed absorption of the solution. Thus, a classic inhibition curve with respect to 5-FU concentration is obtained with the maximum absorption occurring with low levels of drug and minimum absorption occurring with high levels of drug. Monitoring the changes in scattered light or absorbance as a function of drug levels results in a concentration-dependent curve.[43-44]

Reagents

My5-FU Assay **REF** 5FU-RGT	Quantity x Volume
Reagent 1 **R1** Reaction buffer that contains drug-conjugate, protein and buffer	1 x 10.0 mL
Reagent 2 **R2** Nanoparticle reagent that contains monoclonal antibody bound to nanoparticles in a buffered solution	1 x 10.0 mL

Precautions and Warnings

For In Vitro Diagnostic Use Only.

Exercise the normal precautions required for handling all laboratory reagents.

Follow reagent handling instructions. Improper mixing of reagents can affect assay performance.

Materials of human origin were tested for HIV1 and HIV2, Hepatitis B and Hepatitis C by FDA-approved methods and the findings were negative. However, as no test method can rule out the potential risk of infection with absolute certainty, the material must be handled just as carefully as a patient sample. In the event of exposure, the directives of the responsible health authorities should be followed.

All components of the My5-FU Assay contain less than 0.1% sodium azide. For specific listing, refer to the reagent section of this package insert. Avoid contact with skin and mucous membranes. Flush affected areas with copious amounts of water. Seek immediate medical attention if reagents are ingested or come into contact with eyes. When disposing of such reagents, always flush with large amounts of water to prevent accumulation of azide.

Handling and Storage Instructions

Store reagents, calibrators and controls refrigerated at 2-8°C. Do not freeze.

Mix the reagents (R1 and R2) by gently inverting five times, avoiding the formation of bubbles then place them on the analyser.

Mix the reagents (R1 and R2) before pouring them into any analyser-specific (secondary) reagent carrier. Before placing analyser-specific (secondary) reagent carriers on the analyser, mix the reagents (R1 and R2) by gently inverting five times, avoiding the formation of bubbles.

Indications of Stability

Reagents are stable until the expiration date when stored and handled as directed.

Sample Collection and Handling

Plasma (EDTA or heparin) specimens may be used with the My5-FU Assay. Draw the sample towards the end of the infusion, preferably 2 – 4 hours before the end, but ensure that the pump still contains solution during the sample draw. For 46-hour infusions draw the sample at least 18^{18} hours after the start of the infusion. Start time of continuous infusion and actual sampling time should be recorded.

Collect a minimum of 2 mL of blood into an EDTA or heparin tube. Collect the blood sample by venipuncture or through a peripheral IV line. This is to avoid contamination by the infusing drug. DO NOT collect the blood sample from the infusional IV line.

Sample Stabilizer

Inject collection tube with the My5-FU sample stabilizer immediately after draw and gently invert 3 times. Do not place sample on ice and do not refrigerate when using the stabilizer. Centrifuge sample within 24 hours of draw. Draw off plasma from the top of the tube, avoiding the cell layer and transfer to a capped secondary tube. Plasma must be free of cells. Store the plasma sample at 2-8°C or room temperature for up to one week or freeze (at ≤ -20°C) if storing the sample longer. Samples may be shipped at ambient temperature. Provide the laboratory with the times of infusion start and finish. Refer to the My5-FU sample stabilizer package insert for complete instructions for use of the sample stabilizer.

No Sample Stabilizer

Use of the sample stabilizer is strongly recommended. If the sample stabilizer is not used, samples should be centrifuged within twenty minutes of collection to isolate the plasma. Alternatively, place sample on ice immediately upon collection and centrifuge within one hour of collection. Draw off plasma from the top of the tube, avoiding the cell layer (contamination of plasma with blood cells can cause 5-FU degradation), and transfer to a capped secondary tube. Store the sample at 2-8°C for up to one week or freeze (≤ -20°C) if storing for a longer period. If the sample will be shipped to an outside testing laboratory, freeze (≤ -20°C) plasma sample for a minimum of 16 hours before shipment to decrease sample degradation. Samples may be then shipped to the laboratory at ambient temperature.

Procedure

Materials Provided:

REF 5FU-RGT – My5FU Assay

Additional Materials Required But Not Provided:

REF 5FU-CAL – My5-FU Calibrator Kit

REF 5FU-CON – My5-FU Control Kit

REF 5FU-STB – My5-FU Sample Stabilizer Packs (20 kits) OR REF 5FU-STB-V– My5-FU Sample Stabilizer Vial

Instruments

Reagents may need to be transferred to analyser-specific reagent containers.

The performance of applications not validated by Saladax Biomedical, Inc. is not warranted and must be user defined.

Assay

To run the assay, see the instrument specific application sheet and appropriate analyser operator's manual.

Specimen Dilution Procedure

Samples containing 5-FU in concentrations greater than 1,800 ng/mL can be diluted 1:5 to give an upper range of 9,000 ng/mL. Refer to the instrument specific operation manual for an automatic dilution protocol (by cuvette only) of 5-FU samples with water. Alternatively, specimens out of range can be manually diluted 1:10 or 1:100 with deionized water or 1:5 in the 0 ng/mL Calibrator and placed in the sample rack for analysis.

Calibration

The My5-FU Assay produces a calibration curve with a 0 to 1,800 ng/mL range using the My5-FU Calibrator Kit. The minimum detectable concentration of 5-FU in plasma for the My5-FU Assay is 52 ng/mL.

Validate the assay calibration by testing My5-FU Controls.

Calibration Frequency

Calibration is recommended:

- After a reagent kit lot change,
- After performance of monthly instrument maintenance,
- As required following quality control procedures.

Quality Control

The My5-FU Control Kit contains three levels of controls at low, medium and high concentrations of 5-FU.

Each laboratory should establish its own control ranges and frequency. Good laboratory practice suggests that at least two concentrations of quality control be tested each day patient samples are assayed and each time calibration is performed. Reassess control targets and ranges following a change of reagent (kit) or control lot.

Results and Expected Values

The instrument software calculates a best fit non-linear curve equation that is used to generate a calibration curve that ranges from 0 to 1,800 ng/mL of 5-FU concentration. This curve is stored on the analyzer and concentrations of drug in the unknown samples are calculated from this curve using absorbance values generated for each sample.

Limitations of the Procedure

As with all analyte determinations, the My5-FU Assay should be used in conjunction with information available from clinical evaluation and other diagnostic procedures.

Performance characteristics for the My5-FU Assay have not been established for body fluids other than human plasma containing EDTA or heparin.

No significant interferences were observed from samples with the following conditions:

Interferent	Level	
Rheumatoid Factor	500 IU/mL	
Total Protein Matrix Effect	12 g/dL	120 g/L
Icteric Interference	95 mg/dL	1624 µmol/L
Lipemic Interference	1700 mg/dL	19 mmol/L
Hemolysate	1000 mg/dL	

5-FU is not stable in whole blood. Hemolysis should be avoided.

Theophylline, when tested at 10,000 ng/mL, has a 4.6% cross reactivity in the My5-FU Assay. Elevated 5-FU levels may be seen in samples from patients taking theophylline. Theobromine, when tested at 20,000 ng/mL has a cross-reactivity of 2.2%. 5-FU levels may be elevated in samples from patients who have eaten chocolate during or shortly before their infusion.

As with any assay utilizing mouse antibodies, the possibility exists for interference by human anti-mouse antibodies (HAMA) in the sample. Samples containing such antibodies can potentially produce erroneous 5-FU results, which are inconsistent with the patient's clinical profile. If you suspect this occurrence, please contact Saladax Technical Service for assistance.

Expected Values

No precise relationship between 5-FU plasma levels and antineoplastic efficacy has been established. Recent clinical studies in colorectal cancer have used target 5-FU AUC ranges from 20 to 24 or to 30 mg·h/L.[18-21,23,38] AUC levels greater than 25 mg·h/L have been associated with increased risk of toxicity in the form of diarrhea, hand-foot syndrome, mucositis, stomatitis, and leucopenia.[4,17,24-37]

Use of 5-FU plasma area under the curve (AUC) calculations in which AUC is determined from the concentration of 5-FU at steady state (Css) multiplied by the duration of the 5-FU infusion cycle has been shown to be useful in determining optimal individual doses of 5-FU. The AUC can be calculated by multiplying the Css by the time (hours) of infusion:

$$\text{Css} \times \text{Time of infusion} = \text{AUC}$$

5-FU drug concentrations should not be the only means of therapeutic drug management. The assay should be used in conjunction with information available from clinical evaluations and other diagnostic procedures. The patient's current and past medical condition, the complexity of the clinical state, individual differences in sensitivity to 5-FU and toxic effects of 5-FU, co-administration of other drugs, and a number of other factors may result in different optimal blood concentrations of 5-FU for any individual. Each patient should undergo comprehensive clinical assessment prior to modification of the treatment plan and clinicians should carefully monitor patients during therapy initiation and dose adjustments. Given the heterogeneity of the patient's clinical state, clinicians should establish a desired therapeutic management range based on their own experience as well as each patient's clinical requirements. 5-FU concentrations for individual patients should be determined using a single, consistent method to minimize confounding effects associated with cross-reactivity and recognition of metabolites.

Specific Performance Characteristics

Typical performance data for the My5-FU Assay obtained on a Beckman (formerly Olympus) AU400 are shown below. Results obtained in individual laboratories may differ from these data.

Precision

Precision was determined as described in CLSI Guideline EP5-A2.

Low, medium and high 5-FU controls and 4 patient sample pools containing varying 5-FU levels were assayed in duplicate twice a day for twenty days at two sites using 2 assay lots. The means were determined and within run, total SD and % CVs were calculated.

The following are representative results from one assay lot at one site.

Sample Type	Assigned Value (ng/mL)	N	Mean (ng/mL)	Within Run		Total	
				SD	%CV	SD	%CV
Controls	225	80	223	5.5	2.5	11.5	5.2
	450	80	450	6.5	1.4	9.7	2.1
	900	80	910	10.4	1.1	14.7	1.6
Human Plasma	240	80	238	11.6	4.9	13.2	5.5
	470	80	475	8.5	1.8	12.1	2.6
	700	80	705	13.2	1.9	15.1	2.1
	1300	80	1341	18.6	1.4	27.0	2.0

Lower Limit of Quantitation (LoQ)

This is defined as the lowest drug concentration that can be measured with acceptable accuracy and precision. It is considered the measured drug concentration at which the assay coefficient of variation is not greater than 15%, and the recovery is 90 to 110%. To determine the LoQ, 3 negative plasma samples were spiked with a known amount of 5-FU and the amount of drug was quantified by the assay using two lots of reagents over five runs (n = 5/run). The LoQ was determined to be 85 ng/mL.

Limit of Detection (LoD)

This is defined as the lowest drug concentration that can be detected with 95% confidence. To determine the LoD, 3 negative plasma samples were spiked with a known amount of 5-FU and the amount of drug was quantified by the assay using two lots of reagents over five runs (n = 5/run). The LoD was determined to be 52 ng/mL.

Specificity

<u>5-FU metabolites and Structurally Related Compounds</u>

Unless otherwise indicated, 10,000 ng/mL of each of the following 5-FU metabolites or structurally related compounds were added to 5-FU free plasma and were assayed using the My5-FU Assay. The % cross-reactivity in the assay for each compound is given below.

Compound	% Cross-Reactivity
Dihydro-5-fluorouracil	<0.1
Dihydrouracil*	0.4
Eniluracil	0.9
Uracil	11.1
Thymidine	<0.1
5-Fluorouridine	<0.1
Uridine	<0.1
Pseudouridine*	<0.01
Tegafur	<0.1

Compound	% Cross-Reactivity
Capecitabine*	<0.01
5′-deoxy-5-fluorouridine	<0.1
5′-deoxy-5-fluorocytidine	<0.1

*100,000 ng/mL concentration tested

Common Co-Administered Drugs

Unless otherwise indicated, 100,000 ng/mL of each compound was spiked into 5-FU free plasma or plasma spiked with 1,000 ng/mL 5-FU. With the exception of theophylline, with 4.6% cross-reactivity in the assay and theobromine, with 2.2% cross-reactivity, all compounds cross-reacted ≤1% in the assay.

Acetaminophen	Irinotecan*	Prednisone
N-Acetylprocainamide	Kanamycin A	Procainamide
Allopurinol	Kanamycin B	Prochlorperazine
Amikacin	Leucovorin*	Quinidine Sulfate
Ampicillin	Lidocaine	Rifampicin
Azathioprine	Methotrexate	Salicylic Acid
Caffeine	Methylprednisolone	Spectinomycin
Carbamazepine	Morphine sulfate*	Streptomycin Sulfate
Ceftriaxone	Oxaliplatin*	Theobromine**
Cephalosporin	Paraxanthine**	Theophylline*
Erythromycin	Penicillin G	Tobramycin
Gemcitabine	Phenobarbital	Valproic Acid
Gentamycin	Phenytoin	Vancomycin
Hydrocortisol	Prednisolone	Xanthine

*10,000 ng/mL concentration tested

**20,000 ng/mL concentration tested

Recovery

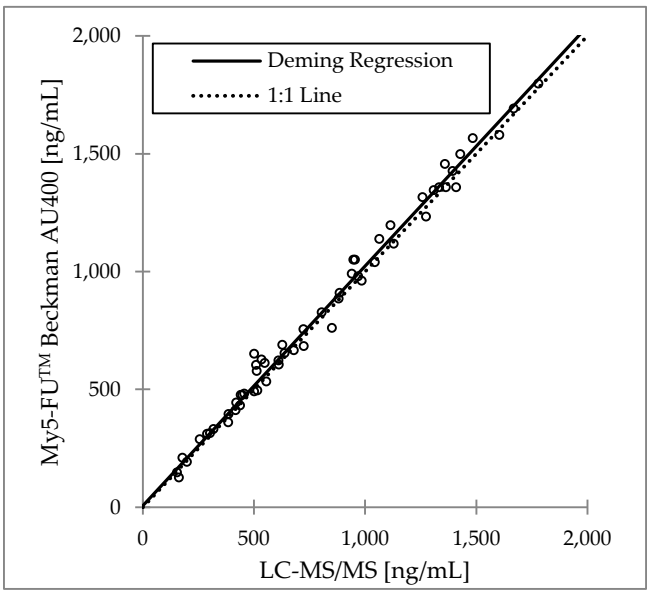
To assess recovery, 5-FU was spiked into normal 5-FU free plasma samples and into patient samples containing a known concentration of 5-FU. The percent recovery was determined by dividing the observed concentration of each sample by the expected concentration of added 5-FU plus original 5-FU present in the sample. The percent recovery ranged from 96 to 108%.

Linearity by Sample Dilution

To assess assay linearity, 11 samples with 5-FU concentrations covering the range of the assay were prepared by using the dilution scheme in CLSI Approved Guideline EP6-A vol. 23 No. 16 and were tested (n = 10) with 2 assay lots. Linearity at specific dilutions was considered acceptable if the percent deviation was within ± 10% of expected value for concentrations ≥ 150 ng/mL or ± 15% for concentrations < 150 ng/mL. The assay was found to be linear over the reportable range of the assay.

Method Comparison

A comparison between the Saladax My5-FU Assay and LC-MS/MS was performed using 57 human plasma samples obtained from patients receiving 5-FU therapy. The range of 5-FU concentration by the My5-FU Assay was 122-1,801 ng/mL with a mean of 816 ng/mL. The 5-FU concentration range for the validated LC-MS/MS reference method was 141-1,810 ng/mL with a mean of 796 ng/mL. Results of the Deming regression analysis are below.



Slope = 1.017
y-intercept = 6.78
Correlation Coefficient (R) = 0.9925

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