



# A Rapid Nanoparticle Immunoassay to Quantitate 5-Fluorouracil (5-FU) in Plasma

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## Abstract

**Background:** 5-Fluorouracil (5-FU) is the most widely used chemotherapy drug in the world and is prescribed primarily for treatment of gastrointestinal, head and neck, and breast cancers. Studies have demonstrated wide pharmacokinetic variability of this drug which can cause unexpected toxicity or ineffective treatment. Recent work has demonstrated that monitoring plasma 5-FU levels and adjusting the dose to target concentrations minimizes toxicity and improves outcome. Because routine quantitation of 5-FU with physical methods is time consuming, a nanoparticle-based immunoassay has been developed to provide oncologists with a rapid, cost-effective tool for determining 5-FU levels in plasma.

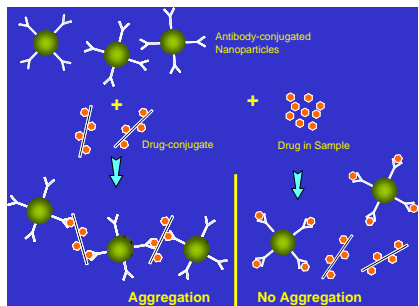
**Methods:** Novel, highly selective monoclonal antibodies for 5-FU were developed and bound to 200-nm particles, creating a homogeneous immunoassay to quantitate 5-FU in plasma. The degree of agglutination of the particles, which is inversely proportional to the concentration of 5-FU in the sample, was monitored at 600 nm. The assay was applied to an Olympus AU400™ Immuno Analyzer.

**Results:** The standard curve uses calibrators from 0 to 1800 ng/mL of 5-FU. With auto-dilution values up to 9000 ng/mL may be determined. On an Olympus AU400™, the time to first result was 11 minutes and 400 samples per hour could be quantitated from a stored standard curve. Cross-reactivity was <1% for dihydro-5-FU, 0.05% for capecitabine and 0.23% for Tegafur. The 97.5% confidence interval for 100 negative plasmas was <30 ng/mL and the coefficient of variation (CV) of spiked 5-FU plasma samples was <5%. Clinical samples from patients treated for colorectal and head and neck cancers were obtained and the immunoassay results correlated to results by LC-MS/MS or HPLC-UV quantitation (R<sup>2</sup> > 0.90) with a slope that ranged from 0.95 to 1.11 (p<0.01, range 13 to 775 ng/mL).

**Conclusions:** This novel immunoassay is suitable for determining 5-FU concentrations in plasma with advantages of speed, small sample size, minimal sample pre-treatment, and application on automated instrumentation. Automation and turn around time enable efficient routine monitoring of 5-FU concentrations in clinical practice for the purpose of therapeutic drug monitoring.

## Background

Individualized dose adjustment of 5-FU to a target plasma level instead of dosing based on body surface area (BSA) has been shown to decrease toxicity, increase overall response, and improve survival. Our objective was to develop a rapid automated immunoassay for 5-FU to provide an efficient method to accurately and precisely quantitate 5-FU in patient plasma for the purposes of therapeutic drug monitoring.



## Methods

5-FU is structurally related to a number of endogenous compounds, and has a structurally similar inactive metabolite. To provide the requisite performance, unique and novel, highly selective antibodies for 5-FU have been developed. Previously, there had been no known antibodies to 5-FU. Generating an appropriate monoclonal to 5-FU was challenging because of its low molecular weight and homology with endogenous compounds. A total of 25 monoclonals to 5-FU were eventually developed. For assay development, antibodies were chosen based on sensitivity, dynamic range, and selectivity, particularly for low cross-reactivity with the inactive metabolite 5-fluoro-dihydrouracil (5FDH). These antibodies were covalently bound to the surface of 200-nm particles and used in a homogeneous competitive immunoassay capable of quantitating 5-FU levels in plasma.

Patient plasma was pre-treated by centrifugation through a 100,000 MWCO filter. A 10 minute spin of 250 µL plasma generated sufficient sample filtrate for the instrument to pipette from a micro-cup. Seven microliters of sample were mixed with 95 µL of reagent 1 (R1), the 5-FU carrier conjugate, and then 95 µL of reagent 2 (R2) the anti-5-FU antibody coated nanoparticles.

The reaction of antibody coated nanoparticles was monitored at 600 nm and the degree of agglutination of the particles was inversely proportional to the amount of free drug in the sample. The assay was automated on the Olympus AU400™ analyzer.

5-FU control solution at three levels (240, 450 and 900 ng/mL) and a 5-FU spiked human plasma pool at two levels (511 (M) and 1046 (H) ng/mL) were analyzed in four laboratories on Olympus AU400™ analyzers for 20 days according to the standard Clinical and Laboratory Standards Institute (CLSI) protocol (N=2x/day for 20 days).

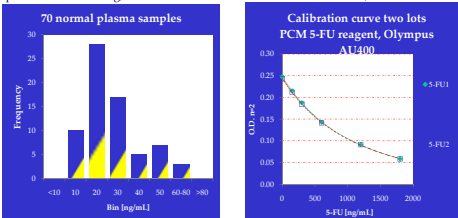
## Results

Table 1. % cross-reactivity of 5-FU monoclonals against analogues and pro-drugs

Antibody	Capca <sup>1</sup>	5FDRC <sup>2</sup>	5DFUR <sup>3</sup>	Thymine	5-FUridine	Tegafur	FUHV <sup>4</sup>	Uracil
7.1G4	<0.05%	<0.05%	<0.05%	2.7%	<0.05%	0.17%	<1%	11%
7.2A2	<0.05%	0.05%	0.06%	2.0%	<0.05%	0.26%	<1%	12%
7.4E10	0.1%	<0.05%	<0.05%	3.1%	<0.05%	0.23%	<1%	18%

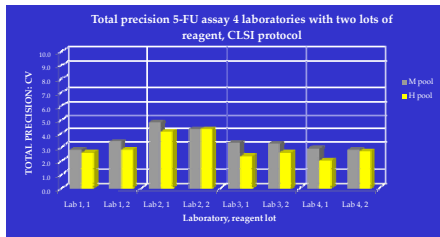
<sup>1</sup>capecitabine; <sup>2</sup>5-deoxy-5-fluorocytidine; <sup>3</sup>dihydro-5-fluoro-5,6-dihydrouracil

**Limit of Detection:** 70 normal plasma samples from six plasma calibrators was generated from a blood bank, showed no interference due to endogenous compounds. The limit of detection, calculated as 2SD from the mean was 52 ng/mL. The limit of quantitation was 86 ng/mL.



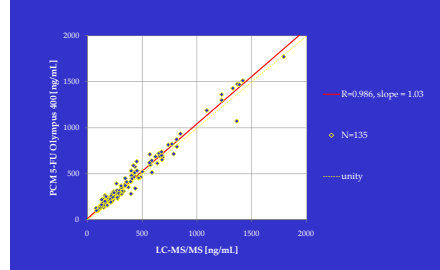
The Olympus AU400™ analyzer was able to analyze 400 samples per hour with a time to first result of less than 11 minutes. Stability studies (still ongoing) indicate that the standard curve is stable for at least 30 days (within 6% of starting value).

**Precision:** The results of the 5-FU control solution at three levels (240, 450 and 900 ng/mL) and the 5-FU spiked human plasma pools at two levels (511 (M) and 1046 (H) ng/mL) analyzed according to the standard CLSI protocol are shown below. The total CV was <6% for all concentrations of 5-FU. The within run precision calculated from the total precision protocol was <3.3% for all samples.



Correlation of the immunoassay with an LC-MS/MS assay validated according to FDA guidelines (Kosovec JE 2008) was performed with plasma samples from patients undergoing 5-FU treatment. Analysis was performed with Deming regression (EP Evaluator, David G. Rhoades Associates, Kennett Square, PA); slope = 1.03 (95%CI 1.00-1.06), intercept = 16.2 ng/mL (95%CI 1.4-30.9), R = 0.986.

## Correlation 5-FU immunoassay vs. LC-MS/MS



## Conclusions

- The first 5-FU monoclonal antibodies ever were generated.
- Clone 7.2A2 was selective and sensitive and could be used in the development of a competitive immunoassay to quantitate 5-FU in plasma without interference from endogenous compounds.
- The assay amply covers the relevant plasma concentration range of 500-3000 ng/mL, observed when patients are treated with continuous infusion 5-FU.
- The assay is sufficiently precise and analytical results correlate well with those obtained with a validated chromatographic method.
- This immunoassay method is suitable for quantitating of 5-FU in human plasma. It offers the advantages of being rapid, using a small sample, minimal pre-treatment and runs on widely available automated clinical analyzers.
- The ability of this assay to generate 400 or more determinations per hour enables efficient routine monitoring of 5-FU levels in clinical practice.

## References

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