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White Paper

# Meeting Evolving Clinical Demands for Assay Performance: Advances in Acridinium Ester Technology on the ADVIA Centaur Systems

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# Driving Innovation in Chemiluminescent Immunoassay Design with Evolving Acridinium Ester Technology

## Background

The continuous evolution of acridinium ester (AE) chemiluminescence technology by Siemens Healthcare Diagnostics, and its applications in immunoassay testing, is a success story. The story bears comparison to the evolution of the microprocessor, whose development over the years has increased the power and utility of desktop and laptop computers. Likewise, advances in AE technology have resulted in significant improvements in the performance and reliability of commonly used clinical assays. Microprocessor evolution has also helped drive the introduction of novel forms of personal computing, such as tablet devices and smartphones. Similarly, Siemens evolution of the AE molecule supports the introduction of new assays, including:

- A Thyroid-Stimulating Hormone assay (TSH3-Ultra) that delivers enhanced low-end precision and sensitivity
- An assay for cardiac troponin I (TnI-Ultra) that improves analytical sensitivity by a factor of 5 over its predecessor
- The Enhanced Liver Fibrosis (ELF™)\* test, the first fully automated liver fibrosis test that detects the direct biomarkers of liver fibrosis
- An assay for Vitamin D Total that demonstrates good clinical concordance with LC/MS/MS methods

Chemiluminescence has been a leading technology of choice for multiple diagnostics vendors, and is likely to remain so as new immunoassay systems emerge. Siemens ADVIA Centaur® Immunoassay Systems utilize AE chemiluminescence technology because of its flexibility and ability for optimization.

In this paper, we review clinical and other benefits delivered by some of the latest AE-dependent assays from Siemens. The history of acridinium ester molecule evolution, reviewing specific details of AE properties, ongoing research findings, and modifications to the AE molecule that continue to drive critical advances in assay performance and diagnostic testing are also detailed.

## Introduction

Immunoassay technology is in a state of constant development, driven by novel marker discovery, and clinical demands for greater assay sensitivity, specificity, and precision. The AE chemiluminescence technology featured on ADVIA Centaur Immunoassay Systems has successfully resolved many of these challenges and is pushing the innovation barrier through the innate characteristics of the AE molecule. The AE molecule is extremely flexible because it is based on a family of chemiluminescent structures that can be selectively optimized for each individual assay.

### Features of evolving acridinium ester chemistry

**High quantum yield (HQY).** High signal-to-noise ratio for improved sensitivity and low-end precision.

**Hydrophilicity.** Improved efficacy of wash step for low nonspecific binding.

**Hydrolytic stability.** Long reagent shelf life and extended onboard stability.

**Versatility.** Labeling versatility for an extensive assay menu.

**Small size.** Direct labeling with AE for use in a broad range of assays.

**Rapid kinetics.** Light emission complete in 1 to 5 seconds for high throughput.

Ongoing modification of the AE technology used on ADVIA Centaur Systems continues to meet the growing needs for improved detection. The latest developments in Siemens AE chemistry have established the groundwork for assays with even better low-end precision and sensitivity, and for other significant assay improvements, including enhanced reagent stability, higher throughput, and smaller sample volumes (see Sidebar A). AE molecule evolution is also enabling the introduction of novel assays, and is facilitating their design through reliance on well-proven principles and performance.

## ADVIA Centaur TSH3-Ultra Assay: A Step-Change in Low-End Precision

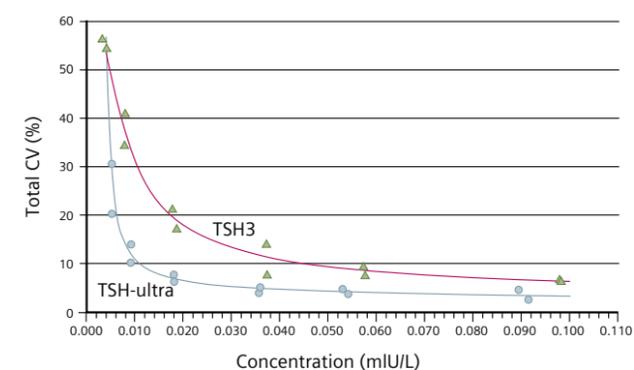
Unlike the latest ADVIA Centaur TSH3-Ultra assay, some of the older or alternative chemiluminescent technologies cannot provide the clinical performance currently required for accurate low level assessment of TSH levels. Low-end precision in a Thyroid-Stimulating Hormone (TSH) assay is also important to optimal management of patients with thyroid disease.

The Siemens TSH3-Ultra assay is based on a re-engineered AE molecule that shows both greater quantum yield and greater hydrophilicity than the AE variant used by its predecessor. Even though the older TSH3 assay demonstrated third-generation performance, the newer TSH3-Ultra assay delivers significantly improved low-end precision and sensitivity, as well as reduced sample size.

This was confirmed in a performance comparison study between the TSH3-Ultra assay and the TSH3 assay. Figure 1 shows the precision profiles used to determine each assay's functional sensitivity.

- TSH3-Ultra assay low-end precision is nearly an order of magnitude better: The profile shows significantly improved low-end precision across the entire concentration interval tested (CV improvement by a factor of approximately 2).
- The profile for the TSH3-Ultra assay shows a shift to the left corresponding to a 3-fold improvement in functional sensitivity, from approximately 0.018 mIU/L down to 0.008 mIU/L (functional sensitivity for TSH assays is defined as the lowest concentration with a total CV of 20% or better).
- The TSH3-Ultra assay also decreases the sample size required from 200 µL to 100 µL.

Figure 1. Precision profiles for the TSH3-Ultra assay and its predecessor. The curves depict total CVs (within-laboratory) as a function of TSH concentration.



## ADVIA Centaur TnI-Ultra Assay: Five Times More Sensitive than its Predecessor

Cardiac troponin I measurements determined by high-sensitivity assays may be used as an aid in the diagnosis of acute myocardial infarction (AMI), and in the risk stratification of patients with acute coronary syndromes.

The AE-based ADVIA Centaur TnI-Ultra™ assay is a highly precise assay that can measure very low concentrations of cardiac troponin I. This excellent precision exceeds the industry benchmark established by the Joint European Society of Cardiology/American College of Cardiology Committee of ≤10% CV at the 99th percentile of normal. The assay uses the HQYAE molecule (Table 1) to help improve analytical sensitivity by a factor of 5 over the previous ADVIA Centaur cTnI assay.

Table 1. Analytical values of the TnI-Ultra assay versus its predecessor.

Values	ADVIA Centaur TnI-Ultra Assay	ADVIA Centaur cTnI Assay	Improvement
10% CV	0.03 ng/mL	0.33 ng/mL	Ten-fold improvement in precision
99th Percentile	0.04 ng/mL	0.07 ng/mL	Nearly 50% lower values
Analytical Sensitivity	0.006 ng/mL	0.03 ng/mL	Five times more sensitive

## ADVIA Centaur ELF Test: Setting a New Standard in Liver Fibrosis Assessment

Siemens AE-related research has helped in the development of the innovative Enhanced Liver Fibrosis (ELF™) test that measures direct biomarkers of liver fibrosis to assess the severity of liver fibrosis in patients with chronic liver disease.

The current standard of care to assess liver fibrosis is an invasive liver biopsy. However, numerous problems with obtaining and interpreting liver biopsies have fueled the search for additional and less invasive methods for assessing the severity of liver fibrosis.

The ELF\* test is the first standardized routine test to use a blood-serum sample to help assess the severity of liver fibrosis in patients with chronic liver disease. The test combines three direct serum biomarkers to obtain a single ELF score calculated by ADVIA Centaur Systems that correlates with severity of fibrosis as assessed by liver biopsy. The test has been clinically validated in an international multi-center study with a mix of patient groups, and was found to be accurate in differentiating mild, moderate, and severe fibrosis.

\* Not available for sale in the U.S.

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## ADVIA Centaur Vitamin D Total Assay: Fast, Accurate Vitamin D Results on a Primary Analyzer

A modified AE molecule (ZAE, Figure 6) is a central component of the Siemens Vitamin D Total assay, which offers the equimolar detection of both D2 and D3 necessary for assessment of vitamin D status. In recent years, vitamin D has become an assay of general health status, and multiple publications have linked vitamin D deficiency to several disease states, such as cancer, cardiovascular disease, diabetes, and autoimmune diseases.

The AE-reliant ADVIA Centaur Systems Vitamin D Total assay eliminates the need for a specialty analyzer for vitamin D testing. The assay measures total 25(OH) Vitamin D with equimolarity (~100 D2 and D3) to ensure accurate results with good precision, and offers clinical concordance with LC/MS/MS methods. Results are available in 18 minutes, representing a significant improvement in turnaround time over legacy methods.

## A History of AE Molecule Evolution

When automated non-isotopic technologies began to displace 125I-labeled radioimmunoassays (RIA) and other immunoradiometric assays in the clinical laboratory, chemiluminescence based on direct labeling with AE played a prominent role because of the advantages offered by the AE molecule (see Sidebar A). In particular, AE's small size enables its use in a broad range of assays and contributes to the generation of high quantum yields. This enables highly sensitive detection with performance similar or superior to RIA while eliminating many of the problems of working with radioactive isotopes.

Advances in AE-based chemiluminescence technology encompass an impressive legacy of contemporary and historical research. Discoveries made in the research environment have led to an understanding of how to modify the AE structure for enhanced quantum yield, hydrophilicity, hydrolytic stability, emission kinetics, and flexibility in assay design. These modifications to AE technology include better sensitivity and low-end precision, more diverse assay architectures, faster throughput, smaller sample volumes, enhanced onboard reagent stability, longer shelf life, and overall greater assay robustness.

The chemical versatility of the AE molecules have been vital in allowing improvement of existing assays as well as development of novel testing advances. Moreover, improved methods for synthesizing the AE molecules and optimizing the conditions under which they react and emit light have been developed. Many of these AE-related advances are detailed in some 40 issued and pending U.S. patents, as well as in numerous peer-reviewed journal articles (see Reference List).

## AE Properties and Assay Design

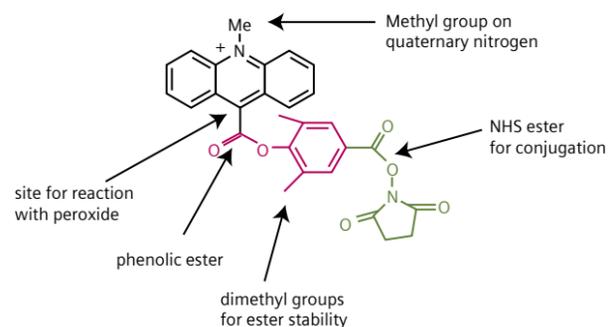
AE-based assays have a proven record of performance that can be directly related back to unique properties inherent to the analog of AE used. Five principal AE forms are shown in Figures 2 through 6. Internal differences among these five molecular forms contribute towards better assay performance and fall into two categories:

**Sensitivity:** Improvements in low-end precision and sensitivity are relevant to assays for analytes such as thyrotropin (TSH) and troponin I (TnI) that are central to thyroid and cardiovascular disease testing.

**Robustness:** Relevant for all analytes are improvements in reagent stability, aqueous solubility (hydrophilicity), lower non-specific binding, and lot-to-lot consistency. Direct labeling with a small chemiluminescent molecule is preferable to using a larger enzyme, as is done in many other non-isotopic immunoassay systems. In terms of molecular weight, the size of an AE is only slightly larger than 125I, whereas enzymes can be several hundred times larger; even a small enzyme like horseradish peroxidase (HRP) is larger by a factor of up to 120 compared to dimethyl AE (DMAE). This is especially critical for competitive immunoassay formats, where direct labeling with AE makes it possible for the labeled analyte to compete with the analyte from the patient sample for antibody sites on an equal basis. Moreover, conjugate preparation and analyte labeling are simpler and more straightforward when working with AEs.

The original AE molecule developed by Siemens Healthcare Diagnostics, dimethyl AE (DMAE), is shown in Figure 2. It is composed of three parts: a tricyclic acridinium ring system (in black), a dimethylphenolic ester (lavender), and an N-hydroxysuccinimidyl (NHS) ester (green). The acridinium ring, with its quaternary nitrogen, is responsible for the chemiluminescence reaction. Conjugation is effected through the NHS ester. The two methyl groups flanking the phenolic ester provide steric shielding and thus greatly enhanced chemiluminescent stability.

Figure 2. "DMAE," 1989. DMAE-NHS. Siemens original dimethyl acridinium ester. The tricyclic acridinium ring system is indicated in black, the dimethylphenolic ester in lavender, and the N-hydroxysuccinimidyl (NHS) ester in green.



The light-emitting reaction for an AE molecule is both less complex and more efficient than that of other chemiluminescent structures, for example, luminol and its derivatives, which require catalysts and suffer from impaired light emission when coupled to a biomolecule. Exposing the AE to an alkaline H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide) solution triggers a flash. Before the flash occurs, the acridone, which is the light emitting species, separates from the remainder of the conjugate, so the subsequent light emission is not influenced by the size or structure of the particular antigen or antibody attached to the phenol group.<sup>6</sup> The event emits light at a wavelength maximum, depending on the specific AE variant, in the range of 430 to 480 nm, a range well suited for the high-efficiency photomultiplier tubes (PMTs) used in the ADVIA Centaur Systems. Furthermore, because the light emission is rapid and completes within 1 to 5 seconds, precise PMT-based measurement of the output can be achieved in a short time with a high signal-to-noise ratio, without losing information from the light emitted and without interference from the accumulation of background emission.

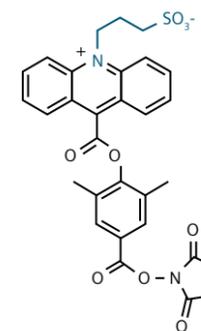
These properties are important characteristics for a chemiluminescent system. Because of their small size and chemical properties, direct labeling with AE molecules is a simple, inherently reliable approach, versatile enough to accommodate the wide range of analytes appropriate for measurement or detection by immunoassay, and supporting requirements for rapid turnaround and high throughput. AE molecules can be used to label small haptens, large analytes, and antibodies, allowing for a very broad menu of assays sharing a common signaling system.

## Early Improvements

The original Siemens AE molecule was made viable as an assay technology by the discovery that fortifying the phenol ring with a pair of methyl groups (highlighted in Figure 2) would protect the conjugates from hydrolysis at the bond between the acridinium ring and the phenol, resulting in excellent shelf life and onboard reagent stability.<sup>7</sup> To measure the improved hydrolytic stability yielded by this modification, experiments showed essentially no loss of chemiluminescent activity at 37°C over the course of a week for the dimethyl AE (DMAE, Figure 2), compared to a 90% loss under the same conditions for the unshielded AE.

The first improvement to the original molecule involved attaching an N-sulfopropyl (NSP) group to the nitrogen in the acridinium ring in place of the DMAE methyl group (Figure 3, in blue).

Figure 3. NSP-DMAE-NHS, 1997. The newly added N-sulfopropyl (NSP) group is highlighted in blue.

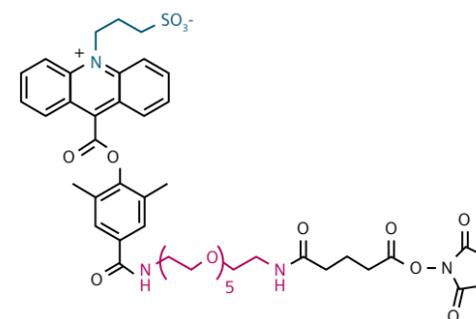


The second and third versions of the AE are shown in Figures 3 and 4. They represent successive improvements in the hydrophilicity (aqueous solubility) of the conjugates [Pat97, Pat03]. In particular, greater hydrophilicity and electrical neutrality can produce lower nonspecific binding and an improved signal-to-noise ratio. Such characteristics are conferred by functional groups such as hexa(ethylene) glycol (HEG) or N-sulfopropyl group.

Neither of these improvements sacrificed hydrolytic stability or other desirable characteristics (including kinetics and wavelength) of the original DMAE (Figure 2).

In addition to enhanced hydrophilicity, these 1997 and 2003 variants both exhibit about 50% better quantum yield (higher total light emission) than the original DMAE. Increased light output combined with reduced non-specific binding translates into improved low-end precision and sensitivity by further enhancing the signal-to-noise ratio. The HEGAE variant (Figure 4) was one of the innovations introduced into the ADVIA Centaur TSH3-Ultra assay, whose superior performance characteristics are partly the result of the increased quantum yield and hydrophilicity of its chemiluminescent technology.

Figure 4. "HEGAE," 2003. NSP-DMAE-HEG-NHS. The inserted HEG linker is shown in red.

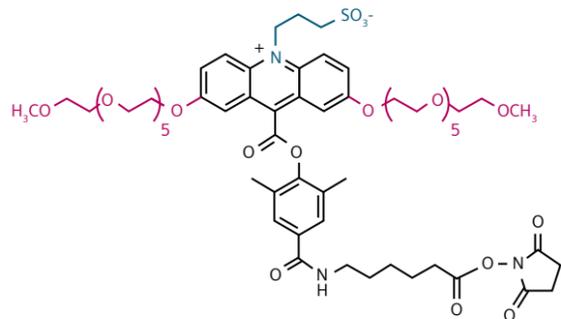


The search for better techniques of synthesizing the NSP variants shown in Figures 3 and 4 resulted in a versatile, highly robust manufacturing process, with considerably higher yields and greatly improved reproducibility. Details of the method are described in an article by Natrajan et al., published in *Green Chemistry* in 2011.<sup>12</sup> This enhanced manufacturing technique virtually eliminates a potential source of lot-to-lot variation associated with chemiluminescent technologies. It is also inherently “greener.” The new process reduces usage of propane sultone needed for synthesis of acridinium esters, minimizing potential exposure to personnel, and reducing toxic waste.

### Continuing Innovation

Figure 5 shows a still more recent structure featuring a pair of methoxyhexa(ethylene)glycol ethers (in red) attached to the outer rings of the acridinium ring.<sup>10,11</sup>

Figure 5. “HQYAE” (High Quantum Yield Acridinium Ester), 2007 and 2010. The methoxyhexa(ethylene)glycol ethers at C2 and C7 are shown in red.



This analog is called “high quantum yield” AE (HQYAE) because its light output is approximately 3 times that of the earlier NSP variants (Figures 3 and 4) and more than 4 times that of the original DMAE (Figure 2). The patented HQYAE variant is also more hydrophilic than its predecessors.

The alkoxy groups introduced at the C2 and C7 positions on the acridinium ring structure are responsible for the increased quantum yield, enhanced aqueous solubility, and lower nonspecific binding.

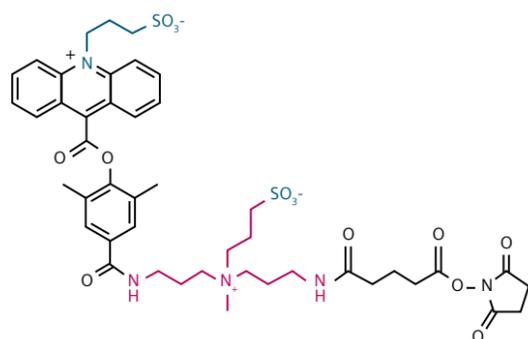
With both increased light emission and decreased non-specific binding, it is expected that the HQYAE will have a considerably better signal-to-noise ratio, and assays based on the newer structure should show improved low-end precision. As an example, the ADVIA Centaur Tnl-Ultra assay which uses the HQYAE variant shows analytical sensitivity improved by a factor of 5, from 0.03 ng/mL to 0.006 ng/mL using HQYAE.

HQYAE is also available for use in other assays where low-end sensitivity is essential, or where the inherent insolubility of the analyte might be an issue, such as the immunosuppressant cyclosporine.

Development of HQYAE, detailed investigation of its properties, and its subsequent incorporation into assays, represents major progress in the ongoing effort to continuously improve the direct-labeling AE technology used on the ADVIA Centaur systems.

Other AE-related developments include complementary approaches to improving solubility and limiting non-specific binding. A recently published study explored the impact of various surfactants on the kinetics and total light emission of AE variants.<sup>13</sup> The study resulted in a deeper understanding of the mechanisms involved, and led to discovery of promising alternative methods for improving aqueous solubility and signal enhancement. Figure 6 shows one of the key structures considered in this study, called ZAE (zwitterionic AE), featuring the insertion, after the phenyl ester, of a highly polar functional group—a sulfobetaine zwitterion (in red)—associated with the same quantum yield but increased hydrophilicity relative to HEGAE (Figure 4). ZAE is used in the Siemens Vitamin D Total assay, and is being applied in other newer assays, such as the Siemens HBsAg II\* assay for the qualitative detection of hepatitis B surface antigen.

Figure 6. NSP-DMAE-Z-NHS (ZAE), 2011. The linker a sulfobetaine zwitterion is shown in red.



### Conclusion

Robust acridinium ester remains the chemiluminescence immunoassay technology of choice, through its flexibility for use in the realization of improved performance, and in the development of novel immunoassays.

In every regard, the acridinium ester technology used on ADVIA Centaur Systems is a proven, solution with significant potential for further development. Benefits include direct labeling with a small molecule that uses a rapid and uncomplicated mechanism for high quantum yield light emission, hydrophilicity, and stability. Importantly, the modifiable AE structure has made possible new forms of the molecule whose characteristics have resulted in steady—and in some instances, dramatic—improvements in assay performance and reliability. This evolution of the AE molecule into its current multiplicity of forms is a key feature warranting continued use of this state-of-the-art immunoassay technology.



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