Immunoassays

DOI: 10.1002/ange.200501641

Sandwich Immunoassay as a High-Throughput Screening Method for Cross-Coupling Reactions**

*Paola Vicennati, Nicolas Bensel, Alain Wagner, Christophe Créminon, and Frédéric Taran**

Combinatorial and parallel methods have become an important focus of research in catalysis. In particular, techniques that allow the simultaneous screening of numerous catalyst candidates have gained particular attention as they may accelerate the identification and optimization of active catalysts. To fully realize the potential of combinatorial approaches for catalyst discovery, general and powerful high-throughput screening (HTS) is therefore essential.[1] In this context, several techniques, including IR thermography,[2] capillary electrophoresis,[3] mass spectrometry,[4] image analysis,[5] and chemosensing[6] have been developed. The use of chromogenic[7] and fluorogenic[8] substrates is also a very popular approach for monitoring chemical transformations.

We recently demonstrated that, aside from spectroscopic or chemosensor-based methods, techniques that exploit the specific-binding properties of antibodies might be valuable tools for the high-throughput screening of enantioselective catalysts.[9] Herein, we report a new versatile enzyme immunoassay (EIA) format that is suitable for the fast screening of coupling reaction.

One of the most convenient methods to screen reactions that involve bond formation is fluorescence resonance energy transfer (FRET).[10] Although this method has many advantages, false positives caused by intermolecular quenching from the catalytic system may occur.[11]

As our aim was to provide a more-general analytical tool, we chose to adapt sandwich immunoassays, a well-known diagnostic technique for antigen detection, for the high-throughput screening of cross-coupling reactions. Typically, sandwich immunoassays require two specific monoclonal antibodies (mAbs), one that ensures the capture of the antigen onto a solid phase and the second that acts as a detector. Both antibodies can simultaneously bind the antigen through the recognition of two distinct epitopes. The principle of this assay might easily be extended to catalyst discovery through the use of substrates that are conveniently tagged with haptens. If hapten moieties (tags) are linked to the chemical functional groups **A** and **B**, which react through covalent-bond formation in the presence of a catalyst, the double-tagged product **A**–**B** is formed. This product should then be detected through a direct sandwich immunoassay with the help of two specific antitag antibodies (Figure 1).

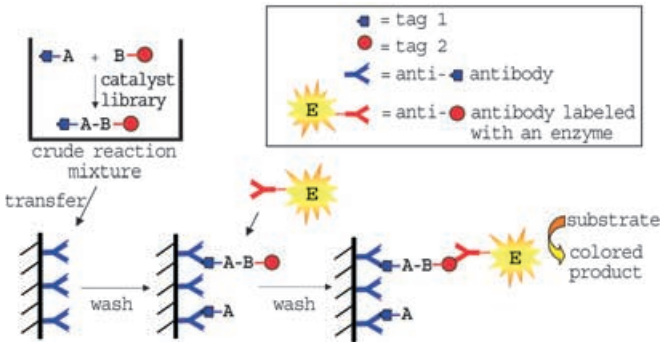

Figure 1. Schematic depiction of the HTS procedure by using a sandwich immunoassay.

In the first step, the crude chemical mixture is added to a microtitre plate that was previously coated with an antitag 1 antibody. The wells are washed with phosphate buffer (10mM, containing 0.05% tween 20) to remove reactant **B** and any unbound material. The second antibody (antitag 2), labeled with an enzyme is then added. The concentration of the product **A**–**B** can now be determined from the absorbance signal, which is related to the activity of the solid-phase-bound enzyme. Thus, the yields of a reaction that involves any kind of covalent bond formation can be easily determined by using an inexpensive and automated absorbance plate reader.

To illustrate the efficiency of this concept, we applied this method to the screening of palladium-based catalysts for the Sonogashira reaction, which was chosen as the model coupling reaction. The alkyne and aryl iodide partners were tagged with imidazole-based (tag 1)[12] and guaiacol-based (tag 2)) haptens, respectively, to form **1** and **2**. Specific monoclonal antibodies had been produced previously in our laboratories for these haptens.[13] The feasibility of this assay depends on the possibility of the product **3** being bound by the two antibodies. Although routinely used for protein or virus detection, sandwich immunoassays for small molecules are less developed[14] because of a common misconception that very small haptens, such as compound **3**, are not large enough to be simultaneously bound by two antibodies. To the best of our knowledge, the smallest molecule previously detected by sandwich immunoassay is angiotensin II, an octapeptide with a molecular weight of 1048 Da.[15]

As several antibodies raised against tags 1 and 2 were available, we investigated a variety of combinations of antibodies (see Supporting Information). The couple,

[*] Dr. P. Vicennati, Dr. F. Taran
Service de Marquage Moléculaire et de Chimie Bio-Organique
DSV/DBJC CEA Saclay, 91191 Gif sur Yvette Cedex (France)
Fax: (+33) 1-6908-7991
E-mail: frederic.taran@cea.fr
Dr. C. Créminon
Service de Pharmacologie et d'Immunologie
DRM/DSV CEA Saclay, 91191 Gif-sur-Yvette Cedex (France)
Dr. N. Bensel, Dr. A. Wagner
Novalyst Discovery
23 rue du Loess–BP 20, 67037 Strasbourg (France)

[**] Financial support from the European community (*IBAAC* project) is gratefully acknowledged. The authors also thank D. Buisson and S. Gabillet for assistance with the HPLC and product synthesis.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

mAb 203 (antitag 1) and mAb 46 (antitag 2), provided the best specific signal and demonstrated an efficient simultaneous binding of product **3** (Figure 2). The wells of a microtitre plate were directly coated with mAb 203, and mAb 46 was conjugated to acetylcholinesterase (AChE). Through the use of a conventional sandwich immunoassay protocol and the Ellman reagent (colorimetric enzymatic substrate), product **3** was detected in a dose-dependent manner (Figure 2). This assay could detect **3** at concentrations as low as 10 nM (detection limits: ≈0.5 nM) and no signal was detected for reagents **1** and **2**. The high sensitivity and selectivity of this sandwich assay allowed us to cover a large array of reaction conditions without interference from the substrates, solvent, or catalysts. Thus the yields of the coupled products could be determined without the need for any workup of the reactions.

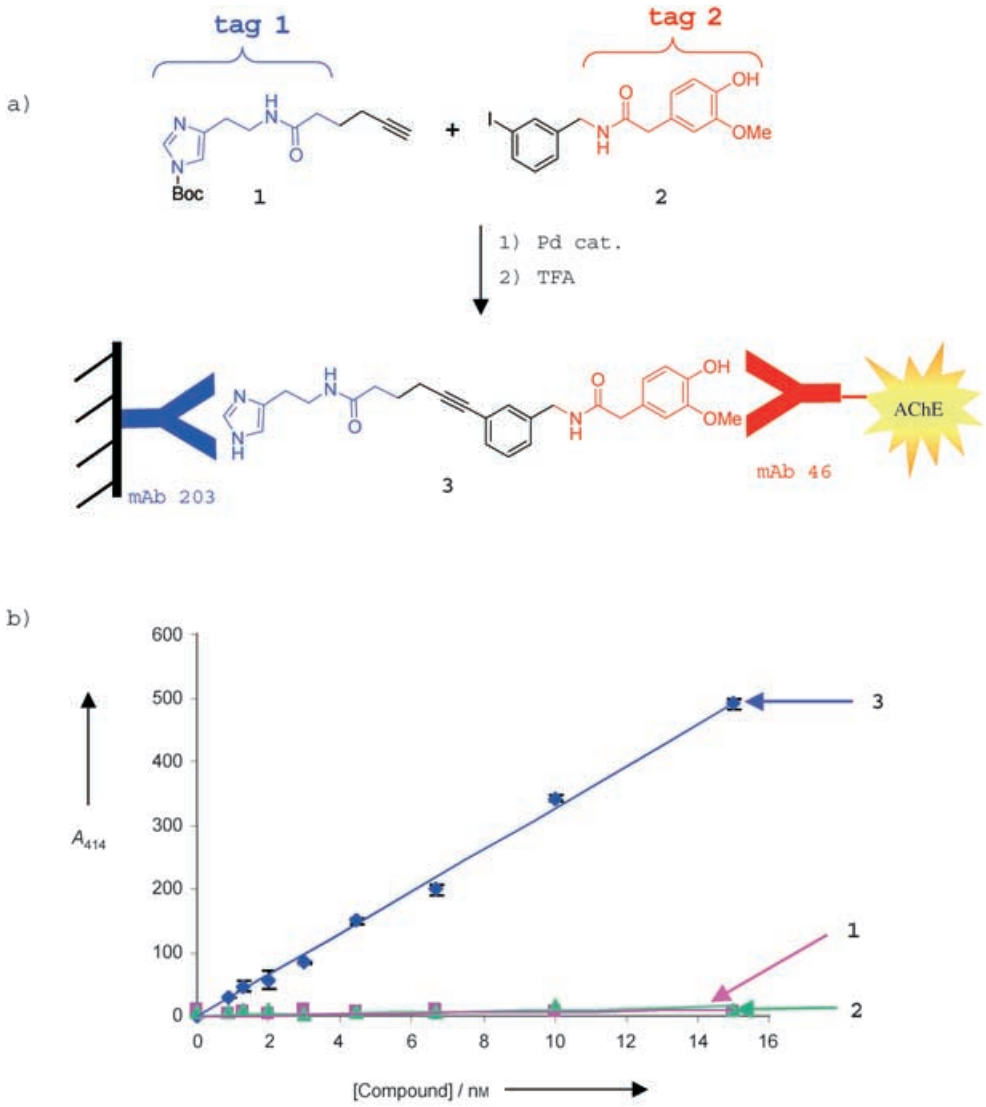

Figure 2. Schematic depiction of the HTS procedure by using sandwich immunoassay. a) the target reaction; b) the standard curves that were obtained with product **3** and reagents **1** and **2**. Boc = *tert*-butoxycarbonyl.

The Sonogashira reaction, one of the most widely used carbon–carbon bond-forming reactions, usually proceeds in the presence of a homogeneous palladium catalyst and Cu^{I} salts.[16] We were interested to evaluate the cross-coupling capabilities of heterogeneous palladium catalysts with the aid of the EIA described above. Although the low cost, ease of handling, and high recovery are previously established advantages of heterogeneous catalysts, their use in the Sonogashira reaction is not well documented.[17]

The catalyst library was prepared through the combination of a set of four homogeneous and eight solid-supported palladium sources as well as eight cocatalysts (copper, silver, or gold sources). These 96 catalytic reactions were run in a parallel manner, quenched by the addition of trifluoroacetic acid (TFA), diluted to the appropriate concentration with phosphate buffer, and assayed directly by a sandwich immunoassay (see Experimental Section). The results are shown in Figure 3.

The screening results indicate that under our reaction conditions, the activity of heterogeneous catalysts was similar to or greater than that of homogeneous catalysts. This was confirmed by HPLC analysis and subsequently reproduced on a larger scale. Among the tested catalytic systems, Pd/C combined with CuI or CuBr·Me_2S gave the best yields. We therefore carried out 192 more catalytic reactions in the presence of these two heterogeneous systems. The ligands, base, and solvents were varied to optimize the reaction conditions, the results of which are summarized in Figure 4. These experiments highlight the crucial role of all the tested reaction parameters. PPh_3/TMG in acetonitrile was found to be the most efficient system for both the palladium sources, and **3** was obtained in yields greater than 80%. The reactions were reproduced on the mmol scale without any significant decrease in yield.

To validate our technique, we compared the results of sandwich EIAs with those from HPLC analysis for 68 representative samples from the crude catalyzed reaction mixtures. A good correlation was obtained for EIA and HPLC analysis (Figure 5), with a linear regression that follows the equation EIA = 1.04 HPLC + 0.88 ($r^2 = 0.94$, $n = 68$), for the yield determination. The precision of the measurement was evaluated to be ±3%.

Figure 3. Sandwich immunoassay screening of the catalyst library for the formation of product **3** from reagents **1** and **2**. Catalyses were carried out in DMF/H_2O (95:5) in the presence of 3% of Pd and cocatalyst (10% (*w*/*w*)) at 80°C for 20 h. PEI = polyethylenimine.

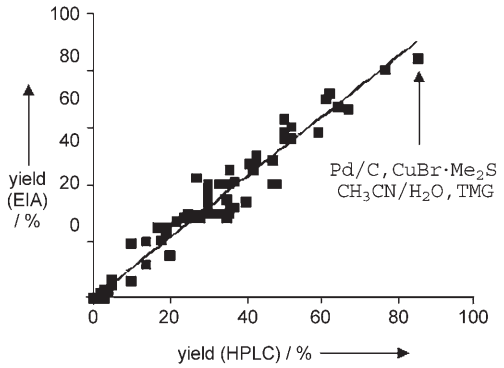

Figure 5. Graphical representation of the correlation between the yields determined by HPLC and EIA.

The most efficient catalytic system (Pd/C, CuBr·Me_2S, TMG, CH_3CN/H_2O) was finally and successfully applied to nontagged substrates. Good to high yields were attained for a variety of alkynes and aryl halides by using the optimized catalytic system (see Supporting Information).

In conclusion, we have demonstrated the usefulness of sandwich immunoassays as a highly efficient method of screening catalysts for cross-coupling reactions. We believe that antibody-based immunoassays remain one of the most desirable noninvasive methods for accurate, sensitive, and routine analysis. Although the assay presented herein is restricted to conditions and catalysts that are at least partially compatible with tag structures, we expect that its versatility and efficiency will be of great help in the discovery of catalysts and reactions. Improvement of the presented method would require the production of antibodies directed against chemically inert tags. Furthermore, the high sensitivity of this type of immunoassay allows the exploration of reactions that occur under very dilute conditions (up to 100 nM for the reaction described herein), an advantage that is of great importance in the search for new reactions compatible with bioconjugation.[18]

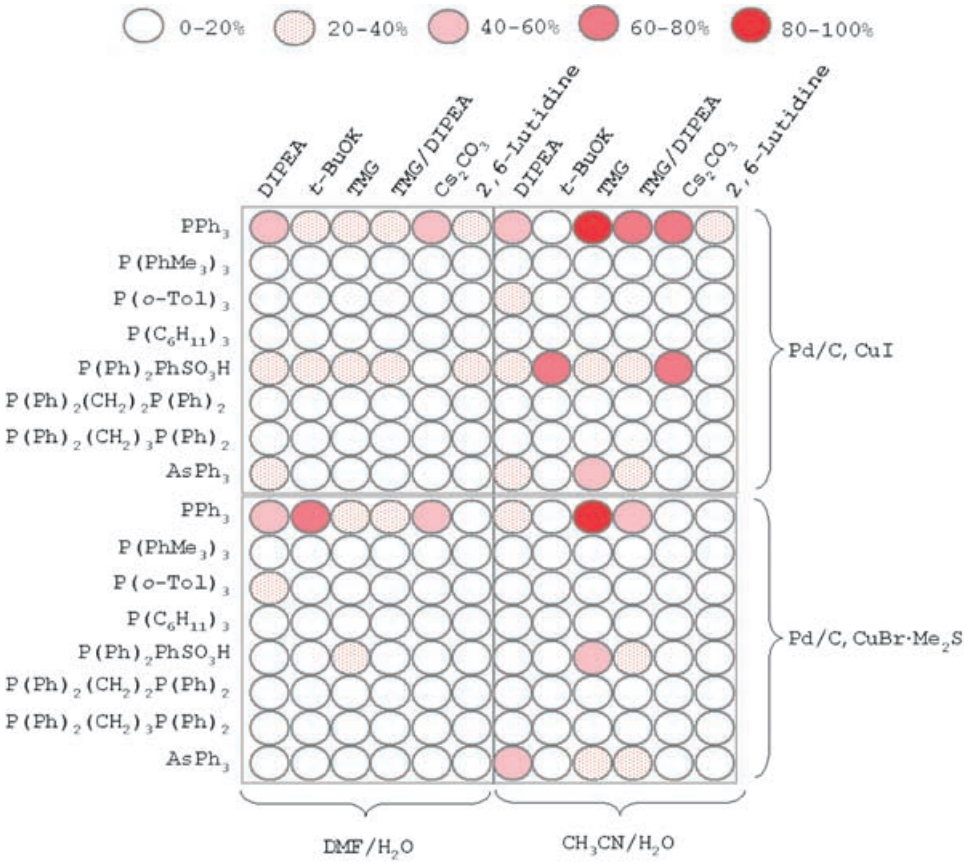

Figure 4. Sandwich immunoassay screening of the catalyst library for the formation of product **3** from reagents **1** and **2**. Catalyses were carried out in DMF/H_2O or CH_3CN/H_2O (95:5) in the presence of Pd source (3%) and cocatalyst (10% (*w*/*w*)) at 80°C for 20 h. TMG = methyl-1-thioβ-D-galactopyranoside, DIPEA = diisopropylethylamine.

Experimental Section

Typical procedure: Pure base (1.5 equiv; 6.6 μL in the case of DIPEA), ligand (0.15 equiv; 19 μL

from a 0.2 M stock solution in DMF), and alkyne **1** (1.2 equiv; 36 μL from a 0.84 M stock solution in DMF), supported Pd source (3 mg of 10%), cocatalyst source (1 mg), and a mixture of DMF/H_2O (95:5; 1 mL) were added to a 24-vial Miniblock reactor (solution phase synthesizer) that contained aryl iodide **2** (10 mg, 25.2 mM). After two cycles of purging with argon, the reaction mixture was stirred for 20 h at 80 °C.

The crude mixtures were then quenched by TFA (100 μL), diluted in EIA buffer (phosphate buffer (0.1 M) and BSA (1 mg mL^{-1}), pH 7.4) in a ratio $1:2\times10^6$. The diluted solutions (100 μL) were transferred to the wells of a microtitre plate that were previously coated with mAb 203 (antitag 1; direct adsorption to the polystyrene support). After 3 h of incubation at room temperature, the plates were washed with phosphate buffer (10 mM, containing 0.05% tween 20, pH 7.4) and the antitag 2 (mAb 46)–AChE conjugate (100 μL) was added. The AChE–mAb conjugate was prepared and stored as previously described.[19] After 12 h of immunological incubation at 4 °C, the plates were washed, and Ellman reagent was added. The resultant absorbance (related to the solid-phase-bound AChE activity) was measured at 414 nm. The immunological reagents (antibody for the capture step and enzyme conjugate antibody) were used in excess. All measurements for the standards and samples were made in duplicate.

Received: May 12, 2005
Revised: August 10, 2005
Published online: October 7, 2005

Keywords: antibodies · C–C coupling · heterogeneous catalysis · high-throughput screening · immunoassays

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