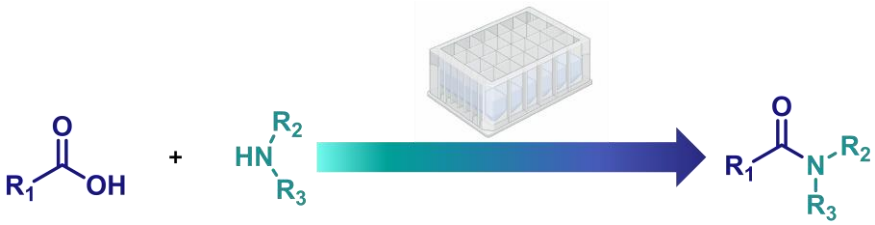
Application Note – Amide coupling 96-well plate kit

Introduction

Amide (carboxamide) group is one of the most important functionalities broadly occurring in small molecules, peptides, proteins and various natural/synthetic polymers. In the pharmaceutical industry, amide formation covers nearly 25% of the patent reactions, thus presents as the most widely used synthetic method in all phases of drug discovery. Amide formation between a carboxylic acid and an amine is initiated with the pre-activation of the carboxylic acid with a coupling reagent to form an active intermediate, such as acyl halides, acid anhydrides and active esters, which undergoes a nucleophilic replacement of the amine to form the amide product. Numerous coupling reagents, including carbodiimides, aminium/uronium salts, phosphonium salts, organophoporous reagents, acylazoles and other heterocycle-incorporated reagents, have been developed and become readily available from commercial vendors. Amide formation reactions are mostly run in a polar aprotic solvent, such as dichloromethane, acetonitrile and *N*,*N*-dimethylformamide. Before purification, a manual workup is usually required to remove the water-soluble byproduct of the coupling reagents, which can be lengthy and tedious. More recently, increased concerns over the coupling reagents as immune sensitizers have arisen, which prompt research in finding safer alternatives and/or improving existing protocols to meet the elevating safety standards in laboratories.

Using a solid-supported coupling reagent for amide formation has emerged as a suitable and welcoming solution in both batch and flow setup for minimizing the exposure to sensitizing chemicals, while reaction workup and purification of the amide product are also largely simplified, as the byproduct of the coupling reagent remains covalently bound to the solid support.



Using the approach described in this application note, the Synple Chem 96-well plate reaction kit offers a high-throughtput method to carry out amide coupling reactions between amines and carboxylic acid.

References and Publications:

(1) Montalbetti, C. A. G. N.; Falque, V. Amide Bond Formation and Peptide Coupling. *Tetrahedron* **2005**, *61* (46), 10827–10852. Link.

(2) Valeur, E.; Bradley, M. Amide Bond Formation: Beyond the Myth of Coupling Reagents. *Chem. Soc. Rev.* **2009**, *38* (2), 606–631. Link.

(3) Joullié, M. M.; Lassen, K. M. Evolution of Amide Bond Formation. *Arkivoc* **2010**, 2010 (viii), 189–250. Link.

(4) McKnelly, K. J.; Sokol, W.; Nowick, J. S. Anaphylaxis Induced by Peptide Coupling Agents: Lessons Learned from Repeated Exposure to HATU, HBTU, and HCTU. *J. Org. Chem.* **2020**, *85* (3), 1764–1768. Link.

Kit Composition

- **1x Reaction plate (A)**

96 deep-well plate (2 mL). Contains Si-Carbodiimide for the reaction.

(Greiner Masterblock 96-well plate, 2 mL, Polypropylene).

- **1x Purification plate (B)**

96 deep-well filter plate (1.9 mL). Contains Si-Carbonate for purification.

(Millipore Multiscreen Deep, 96-well Solvinert, Hydrofobic PTFE, 1.9 mL).

- **1x Collection plate (C)**

Empty 96 deep-well collection plate (2 mL) for product collection.

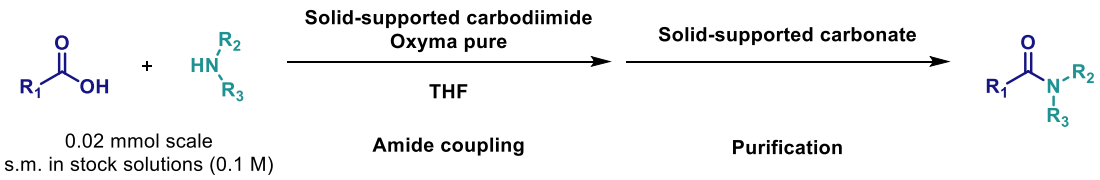
(Greiner Masterblock 96-well plate, 2 mL, Polypropylene).

- **Oxyma pure** (Novabiochem® ≥99.0% HPLC): 85 mg

- **Additional required material (not present in the kit):**
  - THF (preferably stabilized with BHT) for stock solutions and purification step;
  - Pipettes or liquid handling robot for transferring liquid;
  - Centrifuge or vacuum manifold for purification step.

Reaction Scheme

This section describes the general course of the Amide coupling with the 96-well plate kit:



Reaction Planning

Precautions
The Amide coupling 96-well plate kit is not particularly air and moisture sensitive. The reaction and purification plates contain functionalized silica materials. Be sure the plates are kept horizontally when the plate seal is removed to avoid spilling the solid supported reagents. Be sure the seal is applied tightly during the reaction step and during centrifugation to avoid material leaking.

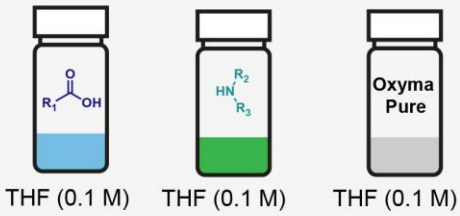
Solubility
Ideally, the chosen substrates must be soluble in THF in order to prepare the initial stock solutions. In case some substrates are not soluble in THF, they can be dissolved in DMF. Please consider that if DMF is used there can be a considerable drop in the reaction and purification performance.

Scale
The Amide coupling 96-well plate kit is optimized for a scale of 20 μmol (0.02 mmol). On such a scale, in the case of a positive outcome, approximately 2-8 mg of product will generally be obtained. Performing the reaction at higher scale is possible (up to 0.04 mmol), but the performance will drop consistently (worse conversion and/or purity). To perform the reaction at higher scale, is suggested to increase the concentration of the stock solution, rather than increasing the volume of liquids.

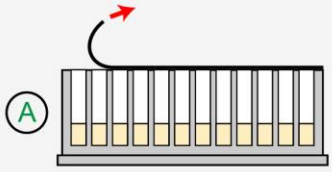
Reaction Procedure

1) Preparation

1. Prepare the chosen carboxylic acid and amine compounds as 0.1 M stock solutions in THF. Prepare a 0.1 M stock solution of Oxyma Pure by dissolving Oxyma Pure provided with the kit in 6.0 mL of THF.

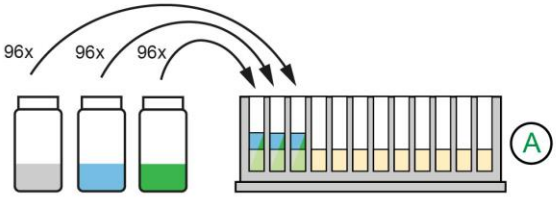


2. Unpack the **reaction plate A**, being careful to keep it in a horizontal position to avoid spilling the solids contained in the plate, and remove the foil seal.



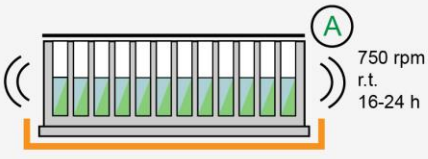
2) Reaction setup

1. With the help of a pipette or a liquid handling robot, add the required amount of carboxylic acid (300 μL) and amine (200 μL) starting materials into the appropriate wells of **reaction plate A**. Add Oxyma Pure (50 μL) to each well of **reaction plate A**.



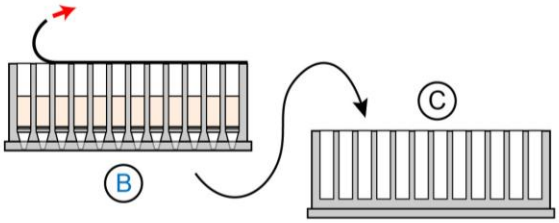
3) Reaction

1. Close the **reaction plate A** with the rubber sealing mat. Place the **reaction plate A** onto a shaker (or onto a shaker placed inside an oven or incubator) and shake the plate at 750 rpm at room temperature for 16-24 hours. At the end of this step let the solid settle down for two minutes.

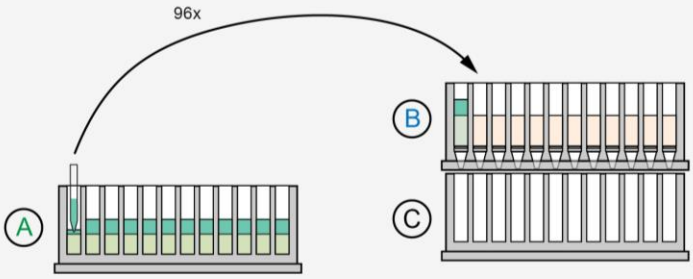


4) Purification

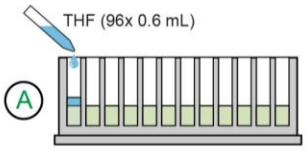
1. Unpack **purification plate B**, being careful to keep it in horizontal position to avoid spilling the solids contained in the plate, remove the adhesive seal and stack the plate over the **waste plate C**.



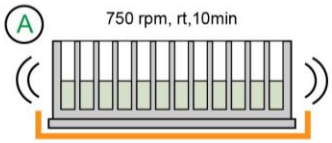
2. With the help of a pipette or a liquid handling robot, transfer the crude solutions from **reaction plate A** into the corresponding wells of **purification plate B**, being careful to transfer only the liquid, leaving the solid at the bottom of **reaction plate A**.



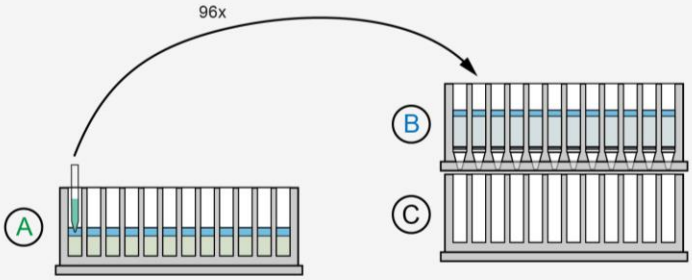
3. After the crude is transferred to **purification plate B**, with the help of a pipette or a liquid handling robot, add 0.6 mL of THF into each well of **reaction plate A**.



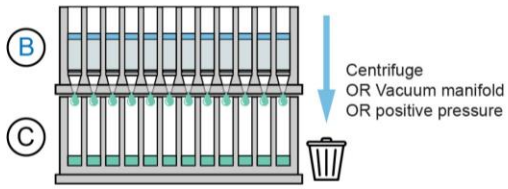
4. Cover **reaction plate A** again with the rubber sealing mat and shake the plate at room temperature for 15 minutes to wash the solid. Afterwards, let the solid settle down for two minutes.

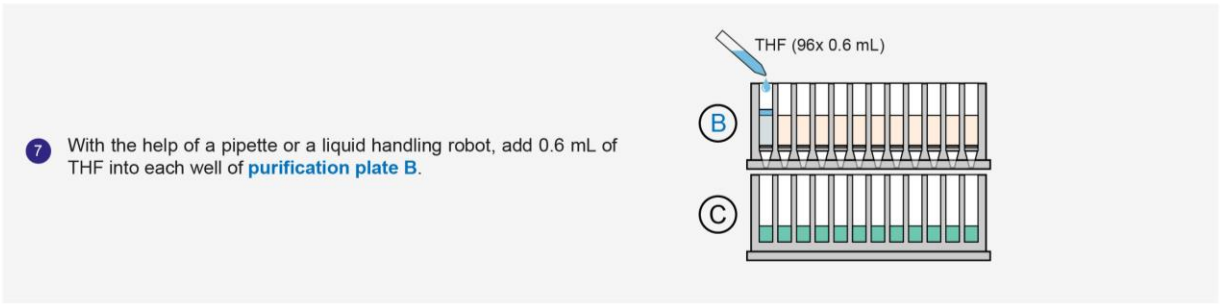


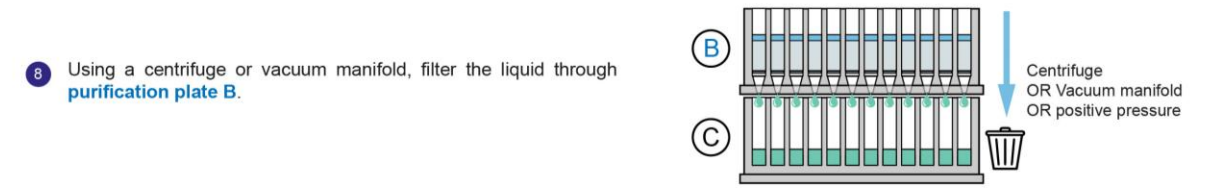
5. With the help of a pipette or a liquid handling robot, transfer the liquid from **reaction plate A** into the corresponding well of **purification plate B**, being careful to transfer only the liquid.



6. Once all the crude material is transferred into **purification plate B**, using a centrifuge or vacuum manifold, filter the crude liquid through **purification plate B** to immobilize the Oxyma Pure and any excess of carboxylic acid on the solid support. Empty **waste plate C**, which contains waste liquid.







Substrate Scope

Tolerated functional groups

The functional groups tolerated are the same as those tolerated when using the Synple Amide coupling reaction cartridges with the Synple synthesizer (see Application Note – Amide formation https://www.synplechem.com/appnotesdownload).

Amines

This method is optimized for alkyl primary and secondary amines. Arylamines are generally expected to give worse results under this methodology, specially those bearing electron withdrawing groups. Still, positive outcome can be obtained in case of electron rich arylamines.

This method requires the use of free-amines. The efficacy of amine salts under the reaction conditons is currently under study.

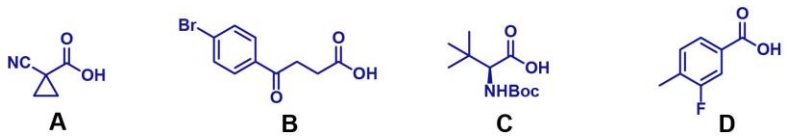
Solubility

The starting materials shall be soluble in THF in order to prepare the stock solutions. In case of insoluble substrates, DMF can be used to prepare the stock solution, but this will lead to a considerable decrease in the reaction performance.

Example results

To demonstrate the process, a test was conducted on a portion (6x4) of the 96-well plate kit. All the liquid transfer operations were performed in a fully automated way using an Opentrons OT-2 Liquid Handler. Filtrations were performed using a Thermo Scientific Multifuge X4 centrifuge (1000 rpm, 5 min). Yield of the final products was determined by ^{1}H NMR analysis of the crude.

Starting materials used in the experiment:



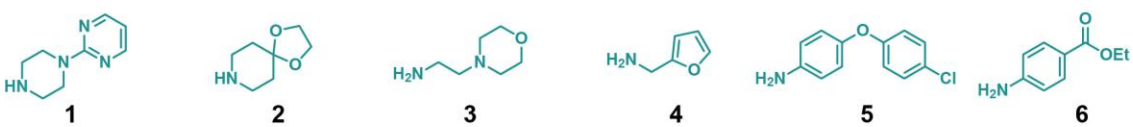
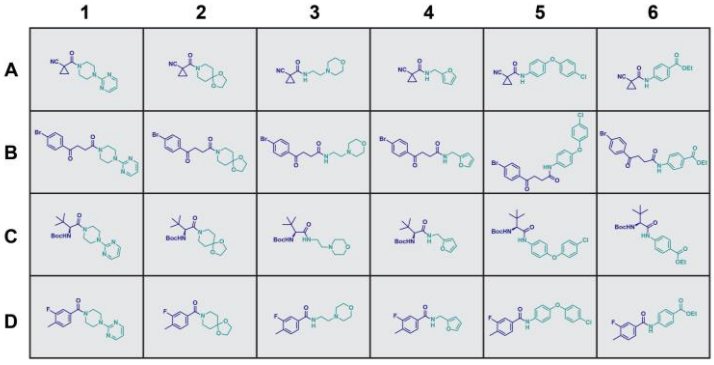


Plate scheme and results:



	1	2	3	4	5	6
A	66	34	55	62	64	23
B	77	91	69	84	78	17
C	72	77	77	83	44	12
D	80	93	84	87	74	5

Yields (%)[a]

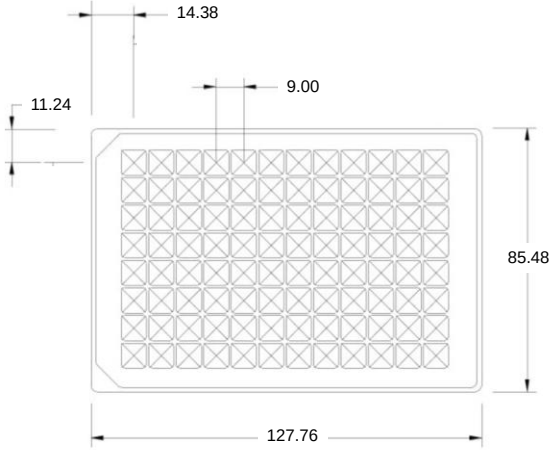
[a]Yields determined by ^{1}H NMR of the crude mixture.

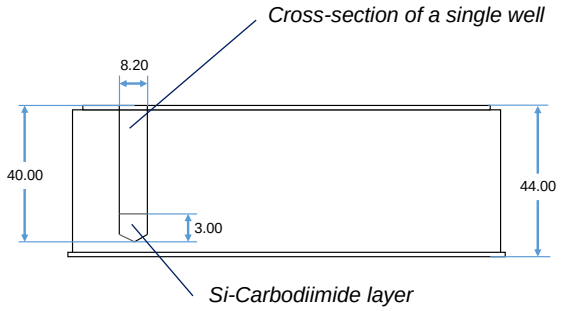
This experiment was conducted using both primary and secondary amines, together with alkyl and aryl carboxylic acids. Two aryl amines were also tested, even if the method is optimized for alkyl amines.
The desired product was obtained with ≥20% yield in 21 out of 24 cases (87.5%), with ≥50% yield in 18 out of 24 cases (75%) and with ≥75% yield in 11 out of 24 cases (46%). As expected, the aryl amine containing an electron-withdrawing group (column 6) gave the worst results (5-23% yield), but still some product is formed. The reaction was complete in 13 out of 24 cases, delivering the desired product in high purity (>90%). Although in excess (1.5 equiv.) the starting carboxylic acid was never observed in the crude product, proving the efficiency of the purification step, which has the scope of removing the unreacted carboxylic acid and Oxyma pure. An impurity due to the partial incompatibility of the polypropylene plate with the reaction solvent (THF) is always present, in an estimated amount of 0.5-1.0 mg. So far, there is no way to avoid the presence of such impurity in the reaction crude, which however can be easily removed through chromatography.

General parameters

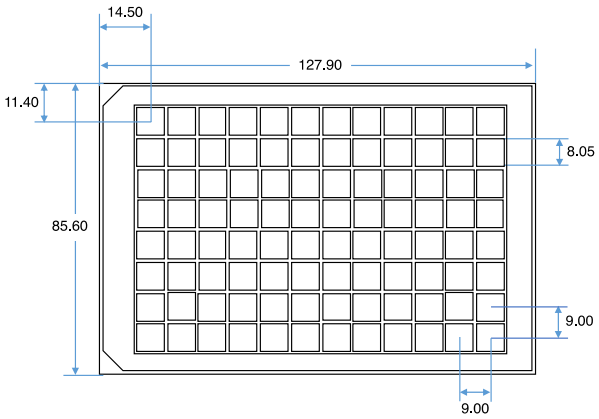
Parameters for Liquid Handling Robot (dimensions in mm)

Reaction plate A:

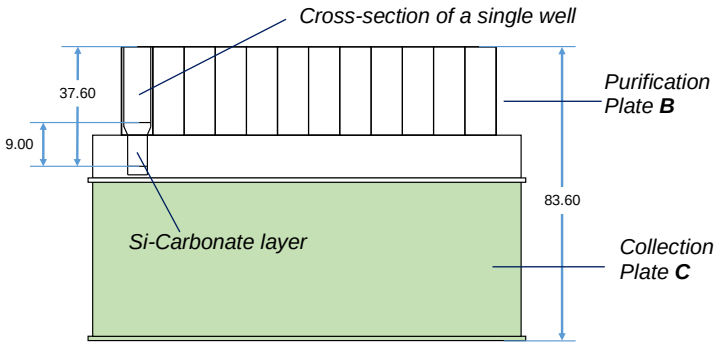




Purification plate B:



Purification plate B* + *collection plate C



Equipment for reaction step

The reaction step was performed using a Heidolph Titramax 101 shaker. Be sure that the rubber seal is correctly applied on top of the plate to avoid any loss of material and to minimize evaporation of the solvent during the reaction.

Equipment for filtration step

Centrifuge: the filtration by centrifugation was tested on a Thermo Scientific Multifuge X4 centrifuge, setting the rotation speed at 1000 rpm and centrifugation time at 5 minutes. Be sure that the rubber seal is correctly applied on top of the plate to avoid any loss of material from the top, and then apply the rubber bands around the two stacked plates to make them adhere as much as possible.

Vacuum manifold: the filtration with vacuum manifold was tested on a Merck Multiscreen® HTS, equipped with deep-well collar. The deep-well collar is necessary to make it compatible with collection plate included in the kit and with the waste reservoir.

Positive pressure manifold: this kit was not tested with positive pressure manifold. Positive pressure was applied manually and no issues in the filtration were observed.