

Equipment Required for High Throughput Enzyme Screening Methods

A) 96-well plates and covers:

- 500 μ l NUNC 96 well plate (VWR 62406-510)
- NUNC pierceable mat cover (VWR 62406-512)

B) Multichannel Pipettes and Tips (VWR)

C) Eppendorf Thermomixer R (which holds 96 well plates) (VWR)



Technical Package For The 96-Well Plate High Throughput Enzyme Screening Method

An economical HTS system has been developed to screen enzymes for activity toward various compounds in less than 10 minutes of human labor time and 5 hours of HPLC time (using a short 3 min HPLC method developed by ARD). The same system can be used to screen for enantioselectivity and for subsequent optimization studies.

This system relies on the one-time preparation of a series of enzyme screening kits (96-well plates containing buffered solutions of a library of enzymes. Screening Kit A, which contains 94 economical proteases, lipases, and acylases, will be screened through first. Screening Kits B and C, which consist of more expensive and specialty enzymes, can be used for subsequent screening in the event a hit is not acquired through Kit A.

ONE TIME PREPARATION OF SCREENING PLATES

A) Preparation of Master Stock Plate (For Screening Kit A)

Enzyme Stocks: [100 mg/ml]

- a) To make a 1 ml initial stock, dissolve 100mg of each enzyme powder in 1ml of 500mM potassium phosphate (KHP) buffer (pH 7.2).
- b) Vortex Enzyme stocks for 10 minutes until homogenous solution is obtained.
- c) Pipette stock solution in 2 ml 96 well plate (Master Stock Plate).
- d) For enzymes that already come in solution form, simply pipette 1 ml into your Master Stock Plate.
- e) Seal plate with pierceable mat cover (VWR 62406-512)

B) Preparation of HTS Reaction Plates

- a) Using a multichannel pipette, pipette 10 μ l of each enzyme solution (mix prior to pipetting each time) from the Master Stock Plate into a 350 μ l BD Falcon deep well conical bottom 96 well plate (VWR 62406-510).
- b) For immobilized enzymes that are harder to pipette, cut the end of the pipette tip to allow for easier pipetting from the stock to the screening plates.
- c) Seal plates with pierceable mat cover (VWR 62406-512) and store at -20°C .

Note: Every freeze-thaw cycle of enzyme solutions reduces the activity of these enzymes, and hence by having enzyme reaction plates ready to go, we not only save the time needed in thawing the entire Master stock plate and pipetting the enzyme solutions into the reaction plate, we also preserve the activity of these enzyme solutions for each reaction by going through only one freeze-thaw cycle.

C) Preparation of HTS Reaction Plates for Neat Reactions

Some screening plates should be lyophilized prior to freezing to allow for screening in neat reactions. Upon lyophilizations, these plates can be stored at $4-8^{\circ}\text{C}$.

RUNNING A HTS REACTION (10 minute process)

A) Prepare substrate stock solution: [10mg/ml]

Use any solvent that will dissolve your substrate.

B) Running Reaction

- 1) remove reaction plate from freezer and add 80 μ l of KHP buffer (0.1M)
- 2) allow plate to mix for 2 minutes while preparing substrate stock (to allow enzyme solutions to mix with buffer)
- 3) add substrate and incubate at 30°C, 750 rpm for 24-72 hours.

Reaction are conducted on a 100 μ l reaction scale:

| | |
|-------|--|
| | 10 μ l Enzyme stock (100mg/ml) |
| | + 10 μ l Substrate stock (10mg/ml) |
| | + 80 μ l Buffer (0.1 M) |
| Total | 100 μ l |

Final Reaction Conditions in each well:

**10% Organic Solvent
1 mg/ml Substrate
10mg/ml Enzyme
pH=7.2**

USING THE PLATE READER (HPLC Conditions)

Instrument: Agilent 220 Microplate Sampler with CC Mode (See attached instructions on how to use software)

Method: TFA3MIN.M or Gluco3min.M short (or any other short method available)

Solvents: 0.1% TFA in H₂O / 0.1% TFA in ACCN

Run time: 3 min / sample

Plate time: Each plate will take about 5 hours to run. (96 reactions)

Note that TLC or any other identification method may also be used.

Reference Directions - Agilent 220 Micro Plate Sampler with CC-Mode Software

Load CC-Mode Software

Open up Instrument 1 online chemstation software as is normal with all 1100 Agilent Series HPLC systems. A new drop down menu, [CC-MODE], has been added to chemstation method and RunControl screen. Click on [CC-MODE] and choose the only option, [Start Software].

Prime 220 Micro Plate Sample Pump

Ensure that there are no air bubbles in the inlet & transfer tubing of the syringe valve by loading up the [maintance mode] under the drop down menu called [Sample], within the cc-mode software. This will create a pop-up menu called [Sampler Maintenance]. Press the [Continuous] button and allow several minutes and determine visually that there are no air bubbles within the syringe tubing that will cause insufficient sample drawn from your 96 well plates. After visual inspection, press [stop pump] and [Exit] to close the sampler maintenance screen.

Create a New Sample Run, Study.

Under [File] drop down menu, highlight [New Study] and follow the 7 steps required. Each step has its own popup menu that you must fill out. They include the Study name, entering the method name, prefix for data storage and adding the samples to the study. Various options are available to be entered in these fields. Some of the options are listed below.

Option - Import a CSV-File for adding samples to the study

Within the New Sample run that you create, you have the option of importing a CSV file to make sample addition easier. CSV files can be created in Excel where it is best to designate columns as fields. Example would be the following columns

| Plate # | Well Position | Sample Name |
|---------|---------------|--------------|
| 1 | A3 | Acid 5 hour |
| 1 | H6 | Ester ½ hour |
| 2 | B11 | Chiral mix |
| 2 | F10 | Blank ACN |

In the add samples to study screen when creating a new sample run, click the [Import samples via a CSV file] option. Next, you need to click [Configure] your import. For instance, above you created an Excel file and saved it as a CSV file onto a floppy disk, to configure the import choose Plate number, Well position, and Sample Name onto the right side of the popup menu by highlighting and adding using the arrows. Once system is configured click [OK]. Next to add your samples in this configuration, click [.....] and chose your CSV file on the floppy drive for instance.

Note that the sample names must be less than 16 characters.

Option - Create a New 96 Well Plate to be used on the Rack

- Step 1. Click on [Configuration] under the drop down menu. Select, [Rack Configuration], this will open the default rack window. Under the [Design] drop down menu, highlight [Edit Microliterplate]. A new popup window will appear, select a micro-liter plate for editing and click [OK]. The edit micro-liter plate screen will now appear. Things that you may need to change are the height of the plate and the depth of the well. Under [Height] choose the outside plate height and enter this value in units of millimeters. Under [Depth] this value will determine the depth the syringe will go into the well. For instance a plate that is 44mm in height with a very shallow well will require a depth of say 10mm. Similarly a plate 44mm tall with a deep well may require a depth of 35mm to enable the syringe to draw the sample. When finished, click [Save As] to rename the plate.
- Step 2. To add this micro-liter plate to for your rack to be used in a new study, you need to right click on a selected 96 well plate on the screen picture and highlight [Change Microliterplate]. Once you have entered the selected plate into the rack position desired 1-12, Save your new rack configuration by selecting [Save As] under the [File] drop down menu. To get back to the main screen under [File] drop down menu chose [Return to main screen].
- Step3. You need to load your rack configuration prior to starting a new study. To do this, under the [Configuration] drop down menu, highlight [Rack Configuration]. This will popup a window which you can now select the rack configuration that you created or saved prior. To do this, click on the [.....] button next to the Rack configuration field. Once selected, chose [OK] and then [OK] again to close the popup window. Now you may create a new study with the desired rack configuration and proper 96 well plate

Start your Sample Run, Study.

Ensure that you have your 1st method loaded and the instrument is running via the Chemstation Software, similar to whenever you would start a sequence. Within the CC-Mode software at the top of the window, be sure the right Study name and Rack Configuration is listed.

Under the CC-Mode software, click the traffic light icon, which is green indicating your ready to start your new study.