Manual *'System Duetz'*

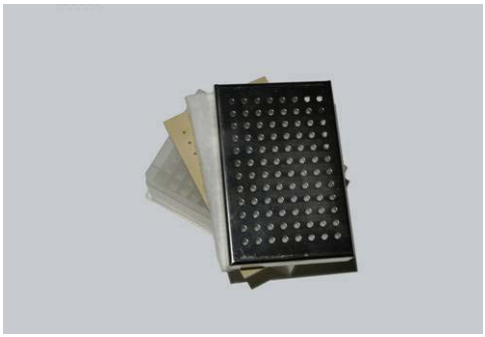
(maintenance, replication, and growth of microbial strains in microtiter plates)

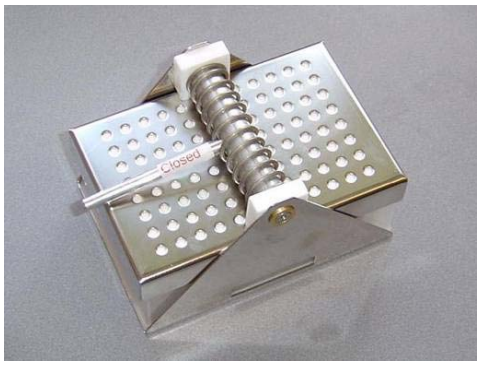
Contents:

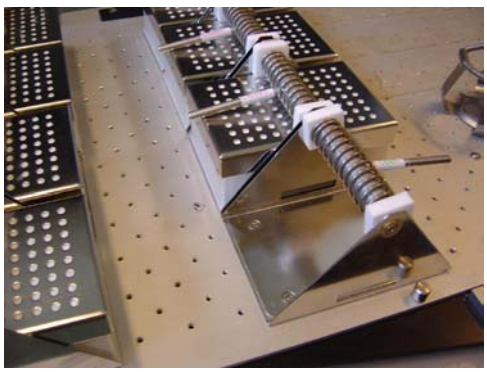
For the purchase of additional equipment and accessories contact your distributor

For technical questions and remarks contact duetz@enzyscreen.com

Further reading: Duetz et al. (2000) Applied and Environmental Microbiology 66:2641-2646



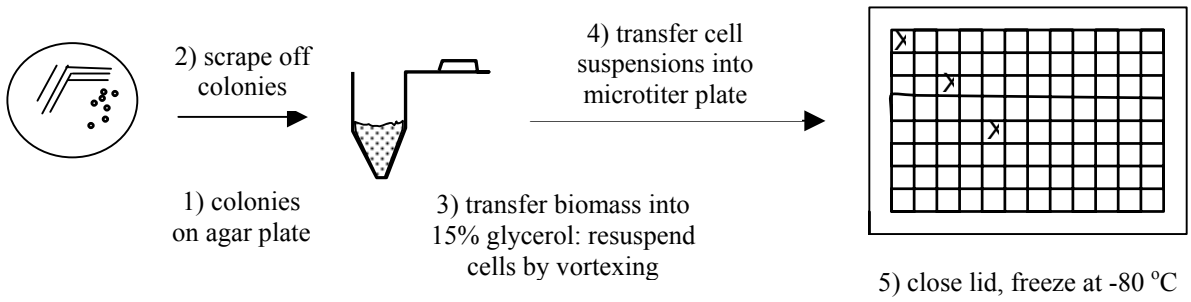




Stage 0: Preparation of frozen glycerol cultures stored in microtiter -format (master plate).

Option A (highest quality, e.g. for heterogeneous culture collections):

1. Grow the individual strains on an appropriate agar-based medium (agar medium dispensed in regular 4 or 9 cm Petri dishes or 12 or 6 well microtiter plates). If working with non-swarming bacterial or yeast strains, one may consider to grow multiple strains on a single agar plate divided into sections using a marker[a)].
2. After incubation during an appropriate number of days: scrape off a number of colonies or lanes with e.g. a sterile spatula or inoculation loop, and transfer the biomass into sterile Eppendorf tubes filled with e.g. 0.5 - 1.0 ml P-buffer (50 mM, pH 7) containing 15% (v/v) glycerol.
3. Close Eppendorf tubes, vortex them, preferably until the cell suspensions are homogeneous. Do not worry if the cell suspensions are not completely homogeneous: this may be solved in the next step
4. Transfer the suspensions into the wells of a sterile square-deepwell 96-well microtiter plate [b)], using sterile 1 ml filter tips. If required, resuspend remaining clunts/aggregates by repeated filling/emptying of pipette tip [c,d, e)]
5. Put the plate(s) (with a lid on top) into a –80°C freezer, preferably directly on a metal surface (more rapid freezing). The next day, put the frozen plate in a 14 x 14 x 5 cm cryobox for long-time storage[f)]



Footnotes:

a) Variant step 1-3: grow strains in individual Erlenmeyer flasks or tubes in an appropriate growth medium. Centrifuge cultures in sterile 10 or 50-ml tubes. Resuspend cells in e.g. 0.5 - 1.0 ml P-buffer (50 mM, pH 7) containing 15% (v/v) glycerol.

b) Advise: use only 93 wells for your cultures; fill the remaining 3 wells (e.g. A1, C3, and E5) with a sterile glycerol/buffer solution (sterile controls). These sterile wells serve to check if sterile conditions are sufficiently maintained throughout the various steps of your future screening/assay procedures. If possible, ensure that the strains stored in one microtiter plate are more or less similar with respect to their growth rate and medium requirements.

c) The best square-deepwell plates for this purpose are those from Bel-Art, which have thick walls and withstand freezing at -80 °C well (no breakage, no deformation). Additional advantage: they fit well into regular 14x14 cm cryo-boxes. The sole negative feature of these Bel-Art plates is their lid system: the lids have a sleeve that should be repaired (filled with molten PP, e.g. obtained by melting a pipette tip in a flame), and the corresponding ridge on the plates should be removed (using a knife or gripper).

d) Alternatively (e.g. if space in your -80 °C freezer is limited) use regular (low) PS or PP sterile microtiter plates

e) Consider to prepare 2 duplicate master plates: one for regular use, one as a backup (preferably store in a separate -80 °C freezer).

f) Use a cryobox without holes (or close the holes using tape) in order to prevent ice-crystals from depositing on the microtiter plate and its contents in the course of months/years.

Option B (fast, e.g. for large clonal libraries):

1. Fill all wells of a regular low PP or PS microtiter plate (round wells with a volume of approx. 0.35 ml) with 0.18 ml of an appropriate agar-based growth medium
2. After solidification of the agar-based medium, inoculate each well with strains using sterile (blunt) toothpicks or inoculation loops
3. Allow the strains to grow on the agar surfaces for a number of days (put the plate in a dessiccator or plastic box together with a beaker of water in order to minimize evaporation [loss of water from the agar would jeopardize the next steps]).
4. Resuspend the cells by adding 50 µl of a 15 % (v/v) glycerol solution (in buffer or water) to all wells using a multipipette fitted with wide tips, followed by repeated (slow) filling and emptying of the tips; this requires quite some skill: the tips should be kept a small distance from the agar surface but close or inside the colony.
5. Transfer the plate (with a lid on top) into a –80°C freezer, preferably directly on a metal surface (more rapid freezing). The next day, put the frozen plate in a 14 x 14 x 5 cm cryobox for long-time storage.

PS. Other options include growth in liquid medium dispensed in microtiter plates, followed (after growth) by addition of an appropriate amount of a sterile 50% (v/v) glycerol solution. Do not hesitate to ask for technical advise (duetz@enzyscreen.com)

Master plate maintenance:

Preferably, a new master plate is prepared every few years according to option A. However, for the majority of strains, loss of viability over the years is minimal (since the remaining cell mass remain frozen). Therefore, one can also decide for a more opportunistic approach: if a particular strain in a well appears to have lost viability (no colony formation after replication on an appropriate medium), this particular strain should be taken from its original source, and grown individually (as decribed in steps 1-3 of option A) and a small volume (e.g. 100 or 200 ul) should be transferred into the appropriate well of the master plate (no need to remove the dead cells). As a consequence, the frozen surface in this particular well will be at a slightly higher level, but this will not pose any problems during the sampling (*stage 2*) as all pins of the cryo-replicator can move up individually.

Stage 1: Sterilization of the cryo-replicator (s): warning: do not autoclave !!!

Option A (first-time use, or if the replicator has just been used to sample a frozen master-plate [*stage 2*])[a)]

1. Submerge the pins of the cryo-replicator is an appropriate tray filled with ethanol (filling height 5 mm).
2. Flame all pins of the replicator shortly in a gas-flame (move all pins quickly through the flames).
3. Allow the replicator to cool down to room temperature for a minimum time period of 12 minutes before use. (important, use a timer)[b)]

Option B (first time use, or if the replicator has been used to sample a frozen master-plate [*stage 2*] or to transfer colonies [*stage 3*])

1. Pre-heat a flat metal heating plate to a temperature of 300 °C (clearly mark this plate "hot !!" in order to prevent colleagues from burning themselves).
2. Clean/wet the end of the pins of the replicator: hold the replicator under an angle of approx. 60° above the sink (see photo 2) and brush and rinse the ends of the pins of the replicator using demineralized water. Try to prevent that the blue plates and the springs become wet: this may cause deposits of traces of minerals and so gradually lead to more friction of the pins in the guiding plates.
3. Put the cryo-replicator (ends of pins are still wet) on the hot plate for 5 minutes (make sure that all pins touch the surface of the hot plate: if necessary press the replicator down using a stand, see photo 1). Important: the replicator may be damaged if it is left on the hot plate for a time period much longer than 5 minutes; use a timer with alarm to prevent this from happening).
4. Transfer the cryo-replicator from the hot plate into the replicator press (or onto one of the two positions on top of the replicator-press) and allow to cool down to room temperature for a minimum time period of 12 minutes before use. (important, use a timer)[b)].

***Photo 1**: sterilization of the replicator on a hot plate: the alu bar presses the grip down and so ensures that all pins are well in contact with the hot plate.*

***Photo 2**: washing of the replicator using demineralized water: it is important that all cell debris is well removed before sterilizing and using the replicator again. It is advisable to avoid wetting the aluminum plates or the springs during washing.*

Footnotes:

a) Some microbial strains may be resistant to this classical method of sterilization of replicators (immersion in ethanol and subsequent flaming). This is particularly relevant if the replicator has been used beforehand to transfer agar-medium grown colonies; not all cells within clumps of biomass that may have remained on the pins may be killed by the ethanol. Subsequent (short) flaming may not heat the pins sufficiently to ensure all pins are sterilized properly either (the flame itself causes some heating of the pins but the evaporation of the ethanol causes some cooling as well). For these reasons we recommend to use the "hot plate method" (option B).

b) During the cooling down of one replicator, consider to use a second and third replicator that have cooled down already. This prevents idle waiting when working with large collections of strains.

Stage 2: Sampling of the master plate / revival on an agar-based medium.

1. Place a cryo-replicator (sterilized and allowed to cool down to room temperature as described before) into the replicator-press (in a laminar flow cabinet).
2. Take the microtiter plate with the frozen cultures (master plate) from the –80°C freezer.
3. Transfer the master plate into the laminar flow cabinet, take the lid of the plate and position the plate under the cryo-replicator (photo 3).
4. Lower the cryo-replicator into the master plate and press the replicator onto the frozen surfaces of the cultures for three seconds; apply a force sufficient to move up each pin for at least 5 mm relative to the frame of the replicator in which the pins are allowed to travel (photo 4) [a)].
5. Move the replicator back up to its highest position in the press.
6. Remove the master plate, put the lid on, and immediately transfer the master-plate back into the –80°C freezer (make sure that the master plate is out of the –80°C freezer for no longer than 2 minutes: a longer time period may cause partial melting and so affect the future viability of the cells sampled from the frozen master culture).
7. Position a regular PS 96-well microtiter plate (volume wells 0.35 ml) of which each well is filled with 0.18 ml of an appropriate agar -based medium under the replicator (photo 5) [b,c,d)].
8. Press the replicator down onto the agar surface at a force sufficient to move up each pin for a few mm relative to the frame of the replicator in which the pins are allowed to travel (photo 6).
9. Incubate the agar-microtiter plates for a number of days (dependent on the set of strains used) at an appropriate temperature in order to allow the transferred cells to grow into colonies of a reasonable size (put the plate in a dessicator together with a beaker of water in order to minimize evaporation).

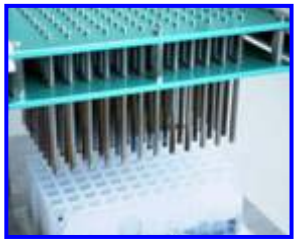


Photo 3

Photo 4

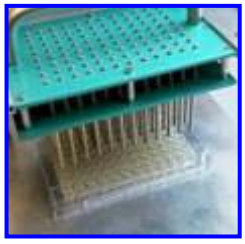


Photo 5

Photo 6

Footnotes:

a) The contact period of three seconds allows the melting and subsequent transfer of approx.0.3 µl per culture.

b) Use 2% agar rather than 1.5% in order to attain a surface of sufficient hardness (to prevent the pins of "sinking" into the agar). Usually a complex growth medium (e.g. Nutrient Broth or Luria Bertani medium) at 6-20 g/l gives good results.

c) Instead of microtiter plates, the use of large Petri dishes (e.g. round, PS, diameter 15 cm, or rectangular plates with the same dimensions as an microtiter plate) filled with an appropriate agar-based growth medium can be considered if:

   i. the goal of the study is solely to determine if the tested strains are capable of growth with a certain medium (e.g. in antibiotic susceptibility studies, or biodegradation studies)

   ii. the biomass will be used to inoculate liquid cultures (*stage 3*), but all strains are known to form distinct colonies (no swarming, no filament formation, no spores) and are all capable of growth on the same medium. In this case a complex medium at a concentration of no more than 3 g/l should be used; if a higher concentration is used, the colonies may become too large and are then more likely to cross-contaminate each other

d) Direct inoculation of frozen stock cultures into liquid medium often results in the failure of growth of part of the strains. However, for some species (test yourself), this time-saving short-cut is feasible.

Stage 3: Replication of colonies into liquid growth medium.

1. Place a cryo-replicator (sterilized and allowed to cool down to room temperature as described before) into the replicator-press (in a laminar flow cabinet).
2. Place the inoculated agar (microtiter) plate prepared as described in *Stage 2* under the replicator.
3. Lower the cryo-replicator into this plate and press the pins onto the colonies of the cultures at a force sufficient to move up each pin for at least 5 mm relative to the frame of the replicator in which the pins are allowed to travel: for most types of strains a significant part of the colony will stick to the pins[a)].
4. Remove the agar (microtiter) plate.
5. Place a sterile square deepwell microtiter plate [b,c)] under the replicator. The wells of this plate were previously filled (using a multipipette) with 0.5-1 ml of an appropriate liquid growth medium
6. Press the replicator down onto the bottom of the wells (move the microtiter plate laterally several times in order to remove as much cell material as possible from the pins).
7. Put the sandwich cover on, place the plate+lid in the clamp and close the clamp (photo 7 -9).
8. Incubate at orbital shaking (5 cm shaking amplitude, 300 rpm) for an appropriate time period (strain and medium dependent).
9. For harvesting, see *stage 4*.

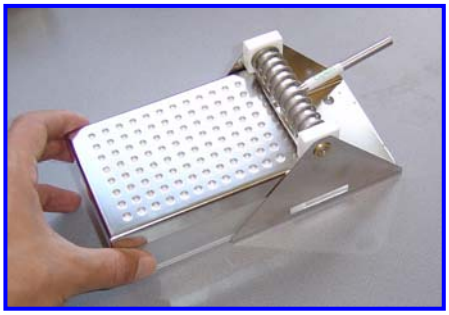


Photo 7

Photo 8

Photo 9

Footnotes:

a) Alternatively (e.g. if the cell mass of the colonies does not stick well to the pins, e.g. if the colonies are dry/crusty as is e.g. the case with some *Rhodococcus* species), one may consider to add 50 µl of a buffer to all wells using a multipipette (preferably tips with large orifices): resuspend (part of) the cells by filling and emptying the multipipette repeatedly and (finally) transferring the cell suspension to the deepwell microtiter plate.

b) See pages 8 of this manual for the type of deepwell plates to use and how to clean, detoxify and flatten them.

c) Autoclave the plates + lid wrapped in alu foil at 120 °C for 20 min. Subsequently, dry the plates (lids still on top, without the alu foil) in an oven set at ~ 100 °C for maximally 1 hour (not longer or hotter, because that would cause the plates to become brittle). The main purpose is to dry the cotton wool layer (important). Do not autoclave the clamp system. Do not store plates + lid in a tightened clamp (the spongy silicone layer will get irreversibly compressed when clamped for a long time).

Stage 4: Use of suspended cultures derived from *stage 3*.

Application A): Screening for the occurrence/level of a certain enzyme activity

1) Remove the microtiter plate + clamp from the orbital shaker.
2) In the laminar flow cabinet: open the clamp and take lid off.
3) Optionally: add 50-100 ul of fresh sterile growth medium, put a clean (sterile) lid on, and incubate for 1-3 hour (cells in the stationary phase will start growing again; re-induction), then restart procedure at step 1.
4) Centrifuge microtiter plate (without lid) at 4000-6000 rpm for 10-20 min. in a pre-cooled (4 ^{o}C) centrifuge (brake off) [a)].
5) Dispose of supernatant (plate upside-down above large waste tray, 'swing out' by hand to remove remaining supernatant)
6) Add 0.25-1 ml of an appropriate buffer containing the bioconversion substrate.
7) Resuspend cells e.g. by repeated filling and emptying of a multipipette fitted with wide tips.
8) Put on a (clean) sandwich lid, clamp together.
9) Incubate on the orbital shaker for an appropriate time period (e.g. 300 rpm, 5 cm amplitude)
10) Centrifuge plates (without lids) at 4000-6000 rpm for 10-20 min. in a pre-cooled centrifuge (brake off).
11) Remove 0.1-0.8 ml of supernatant into a microtiter plate of appropriate dimension, using a multipipette: in order to position the tips just (e.g. 1-2 mm) above the pellets (and not in the pellets), position a plastic or metal strip of an appropriate breadth in between the multipipette (between the tips, somewhere in the middle) and the upper side of the deepwell microtiter plate.
12) Analyze for product formation, e.g. by high-throughput LC-DAD-MS.

Application B): Screening for the occurrence/concentration of a metabolite

1) Remove the microtiter plate + clamp from the orbital shaker.
2) In the laminar flow cabinet: open the clamp and take lid off.
3) Centrifuge plates (without lids) at 4000-6000 rpm for 10-20 min. in a pre-cooled centrifuge (brake off)
4) Remove 0.1-0.8 ml of supernatant into a microtiter plate of appropriate dimension, using a multipipette: in order to position the tips just (e.g. 1-2 mm) above the pellets (and not in the pellets), position a plastic or metal strip of an appropriate breadth in between the multipipette (between the tips, somewhere in the middle) and the upper side of the deepwell microtiter plate
5) Analyze for product formation, e.g. by high-throughput LC-DAD-MS

Footnote:

a. For most modern centrifuges, rotors + swing-out buckets for microtiter plates are available. When selecting which of your centrifuges to equip for this purpose take into account the maximal speed at which the rotors + buckets may be used; around 3000 rpm is the minimum to spin down cells of most bacterial strains properly (4000-6000 rpm is preferable).

Square deepwell microtiter plates for suspended cultures: pre-treatment, cleaning

The square deepwell microtiter plates supplied by Kuhner AG are from Riplate (www.riplate.com).

- Kuhner AG has chosen these plates as i) they are of consistent quality, and ii) they are square up to the very bottom (important for an optimal oxygen-transfer rate).
- Kuhner AG has flattened the upper side up of the plates in order to prevent cross-contamination via small irregularities at the top of the plates that exist after manufacturing of the plates. If users of 'System Duetz' purchase replacement plates from another supplier, they are strongly advised to flatten these before use as well:
    - tape a piece of rough waterproof sand paper (quality 120) on a flat bench.
    - wet the sandpaper and the microtiter plate with plenty of water
    - move the microtiter plate over the sandpaper in an orbital way: use both hands and continue until the plastic is flat everywhere (takes about 3 minutes per plate; inspect carefully)
    - repeat this procedure with a piece of fine waterproof sand paper (quality 320)
- We strongly advice to detoxify microtiter plates (also the ones supplied by Kuhner AG) before first-time use. Although most manufacturers will contradict it, the polypropylene used often contains potentially toxic UV-absorbers and antioxidants (e.g. phenols and phosphites), whiteners (coumarins, benzoxazoles), and heavy metals.
    - soak the plates in boiling NaOH (0.1 M) in a large glass beaker or stainless steel pan (e.g. 5 or 10 liter), for 4 hrs
    - rinse the plates with plenty of demineralized water
    - soak the plates in HCl (0.1 M, 70 ^{o}C) in a large glass beaker or stainless steel pan (e.g. 5 or 10 liter), for 4 hrs
    - rinse the plates with plenty of demineralized water
- The plates treated in this way can re-used many times (20 times or more) if they are cleaned carefully without damaging the thin walls between the wells. Use an 8- or 12-channel device connected to the demi-water tap for cleaning; do not use metal brushes that may damage the walls of the wells (and cause cross-contamination during subsequent use); for persistent dirt, or remaining biomass use ear-sticks with cotton wool. For easy cleaning, soak the plates in water beforehand (e.g. overnight).

Other important reminders:

- recommended orbital shaking conditions: 300 rpm, shaking diameter of 50 mm
- open the lid only just before harvesting the cells; intermediate opening (e.g. to inspect the extent of growth) may lead to cross-contamination.

Further reading:

- Duetz, W.A., Rüedi, L., Hermann, R. O'Connor, K, Büchs, J., and Witholt, B. (2000). Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Applied and Environmental Microbiology 66:2641-2646

Proper handling and maintenance of the replicator

The pins of the replicator are made of a high-quality stainless steel. Nevertheless some maintenance may be required:

The surface of the end of the pins may change in the course of time. E.g. improper washing after the transfer of colonies and subsequent heating may cause a thin char layer. Also, excessive heating negatively affects the surface of the pins. A changed surface of the pins - in its turn- may have an influence on i) the heat-transfer during the sampling of the master plates and ii) de extent to which cells stick to end of the pins.
Such pin-to-pin variations are undesirable and can be counteracted as follows:

End of pins are black or damaged → polishing of the end of the pins using sandpaper:

1) tape a sheet of fine waterproof sand paper (e.g. quality 300) on a flat bench
2) wet the sandpaper with plenty of demineralized water
3) move the replicator over the sandpaper in an orbital way for e.g. one minute: use both hands, press the replicator down at such a force that the pins move up a few mm, and continue until the end of all pins are shiny and flat again

Pins are bent or broken → replace these pins with new ones

If the replicator is accidentally dropped or damaged in another way, it may be necessary to replace one or more of the pins and/or springs. If bent pins can not be bent straight again, replacement pins can be obtained via Kuhner AG. Also replacement springs can be obtained via Kuhner AG. Kuhner AG will also supply advice on the best procedure to replace pins and/or springs.

Warning: do not autoclave the replicator: this will cause severe damage!!

Read page 4 of this manual for a proper method to make the pins of the replicator sterile.

If you accidently have autoclaved the replicator, contact your distributor.