Miniaturisation of assay development and screening

In recent years we have seen a strong increase in the number of chemical compounds and molecular targets used for lead discovery by high-throughput screening among most Pharma and Biotech companies. This has caused a strong push towards volume reduction, throughput increase and cost reduction among these organisations. Since microtiter plates (MTPs) serve as the main carrier for biological test reactions in biomedical research, there has been a strong demand towards high- and ultra-high-density MTP formats. This paper describes the evolution of the various plate formats used for miniaturised assay development and screening in modern drug discovery. Whereas the 96w-MTP has been the standard plate format for compound testing in the last decade, current trends clearly point towards 384w- and 1536w-MTP formats for compound testing and towards 384w-MTP format for compound storage. Similar trends can be seen for standard research laboratories with lower throughput requirements, eg laboratories in industry and academia with pharmacological background. Also here, volume and cost reduction have been the main drivers for the move towards higher density plate formats. In summary, the 384w-MTP has become the major plate format used in biological assays for drug discovery in industry and academia.

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Current drug discovery relies on massive screening of chemical libraries against various extra- and intracellular molecular targets to find compounds with the desired mode of action. In recent years, high-throughput technologies for combinatorial and multi-parallel chemical synthesis, automation technologies for isolation of natural products, and also availability of large compound collections from commercial sources have tremendously increased compound collections among Pharma and Biotech companies up to several hundreds of thousands, in some cases even up to more than one million distinct chemical entities. At the same time, sequencing of the human genome as well as sequencing the genomes of various pathogens, such as microbes, bacteria and viruses, has delivered hundreds to thousands of novel molecular targets for pharmaceutical

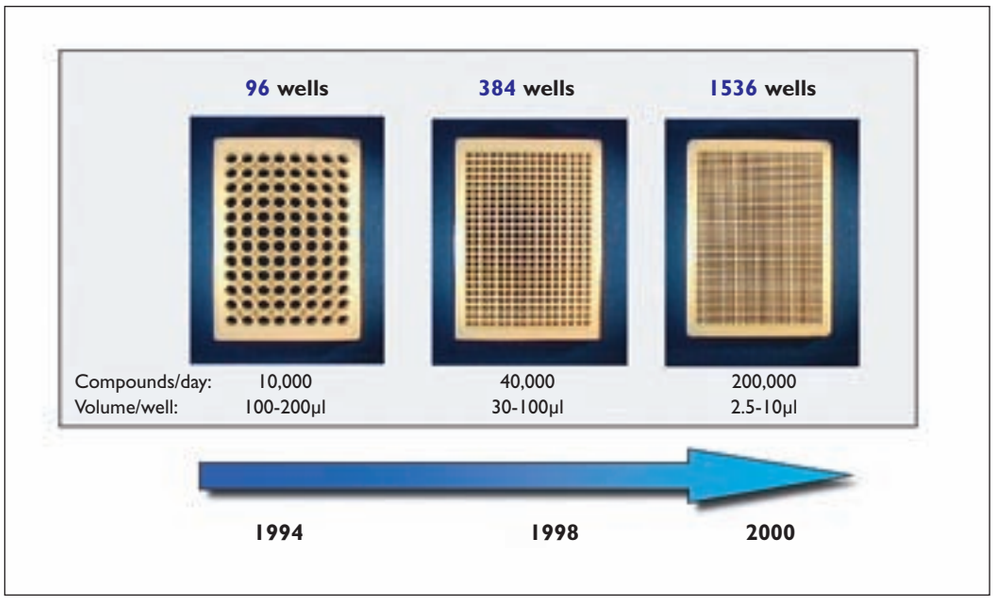

Figure 1
The history of Microtiter Plates (MTP) used for HTS. The figure shows the different types of microtiter plates used for compound storage and high-throughput screening. In the early and mid-1990s, the main screening format has been the microtiter plates with 96 wells per plate (96w-MTP). This plate type has been largely replaced in recent years with 384w- and in some cases even with 1536w-plates (384w-MTP, 1536w-MTP). Despite that all these plate types have the same footprint, they can carry different numbers of distinct reaction wells and thereby enable miniaturisation and higher throughput in the screening process

intervention with sufficient understanding of the molecular function to pursue drug discovery efforts on these targets.

The strong increase in both the number of available compounds as well as molecular targets has caused a fundamental change in the drug discovery process applied at Pharma and Biotech companies. Various technologies for miniaturisation, lab automation and robotics enable testing of chemical compounds in biological systems by the means of high-throughput screening (HTS) and ultra-high-throughput screening (uHTS). Whereas HTS is defined by the number of compounds tested to be in the range of 10,000-100,000 per day, uHTS is defined by screening numbers in excess of 100,000 compounds tested per day. Taken together, the technologies of HTS and uHTS are seen as key elements for filling the drug discovery pipeline in industry with new chemical compounds and new modes of action.

The need for miniaturisation

A strong increase in the number of chemical compounds for testing and the concomitant increase in the number of molecular targets for lead finding can be accommodated only via substantial miniaturisation of HTS assays. In the past, microtiter plates (MTP) with 96 wells per plate (96w-MTP) have been the main compound handling and screening format among most pharmaceutical and biotech companies (**Figure 1**). This commonly used plate format and derivatives thereof are clearly defined by the MTP standards of the Society of BioMolecular Screening (SBS), (www.sbsonline.org). For this as well as the plate formats with even higher density, various types of plate materials exist, depending on the desired application, readout technology and interference with the biological test system (Greiner Bio-One (www.greiner-bioone.com), Nalge-NUNC (http://www.nalge-nunc.com), Evotec (www.evotec.com), among many others). The typical working volume for 96w-MTP is in the range of about 100-200µl total volume with a standard volume of about 150µl per well (**Figure 1**). This volume range can be handled routinely with manual and automated liquid handling systems (Beckman-Coulter, www.beckman-coulter.com), Cybio, www.cybio.de), Caliper Lifesciences, www.caliperls.com), PerkinElmer, www.perkinelmer.com), Thermo Electron, www.thermo.com), Evotec, www.evotec.com, among many others) by pipetting or dispensing of the appropriate volumes with sufficient precision and accuracy. Depending on the applied readout technology and the measurement time per well, this volume range equals about 10,000 compounds or compound concentrations tested per eight-hour working day at a tick rate of about 4-5min per plate (combined plate readout and plate handling time). This tick rate equals about 2-3sec average readout time (including plate loading and plate unloading from the plate reader) per well in the 96w-MTP format.

The last decade has seen a strong trend towards plate types with higher densities at the same footprint. The first development was the 384w-MTP

which accommodates four times more samples than a 96w-MTP (**Figure 1**). The typical working volume for 384w-MTP is in the range of about 30-100µl total volume with a standard volume of about 50µl per well. This volume range can still be handled by most of the routinely used liquid handling systems and can also be filled still by manual pipetting. Depending on the applied readout technology and the measurement time per well, this volume range equals about 40-50,000 compounds (single compound per well) or compound concentrations (single concentration per well) tested per eight-hour working day at a tick rate of about 4-5min per plate (combined plate readout and plate handling time). This tick rate equals about 0.6-0.7sec average readout time (including plate loading and plate unloading from the plate reader) per well in the 384w-MTP via a sequential readout technology (well by well is read out by the MTP reader). Alternatively, various imaging technologies can read out the whole plate simultaneously via CCD (charged coupled device) technology and can achieve readout times independent of plate format of just a few minutes with systems such as the ViewLux (Perkin-Elmer, www.perkinelmer.com) and LeadSeeker (GE Healthcare, www.gehealthcare.com) systems, among many others. Despite the benefits of volume reduction on the amount of used assay reagents and chemical compounds, assay quality as defined by signal over background (S/B) or signal over noise (S/N) can be negatively affected by reduction of volume and the statistical quality of the assay can be hampered. The majority of all assays, biochemical or cell-based, can be adapted towards 384w-MTP without any problems and this plate format has been established as the format of choice for compound storage and screening assays among most Pharma and Biotech companies.

Several companies have managed to adapt their processes in part or in total towards 1536w-MTP formats for compound handling and screening[1,2]. The typical working volume for 1536w-MTP is in the range of 2.5-10µl total volume with a standard volume of about 5µl per well (**Figure 1**). Depending on the complexity of biological test systems (number of volume addition steps), this volume range needs special equipment to handle the addition of small volumes in the range of 0.1-1µl with sufficient accuracy and precision (Sysmelec (www.sysmelec.com), GeSim (www.gesim.de), Caliper Lifesciences (www.caliperls.com), Deerac Fluidics (www.deerac.com), Genomic Solutions (www.genomicsolutions.com), TTP Labtech (www.ttplabtech.com), among many others). Depending on the applied readout technology and the measurement time per well, this volume range equals about 100-200,000 compounds or compound concentrations tested per eight-hour working day at a tick rate of about 4-8min per plate (combined plate readout and plate handling time). This tick rate equals about 0.1-0.3sec average readout time (including plate loading and plate unloading from the plate reader) per well in the 1536w-MTP via a sequential readout technology (well by well is read out by the MTP reader). These fast reading times per well are only feasible with a

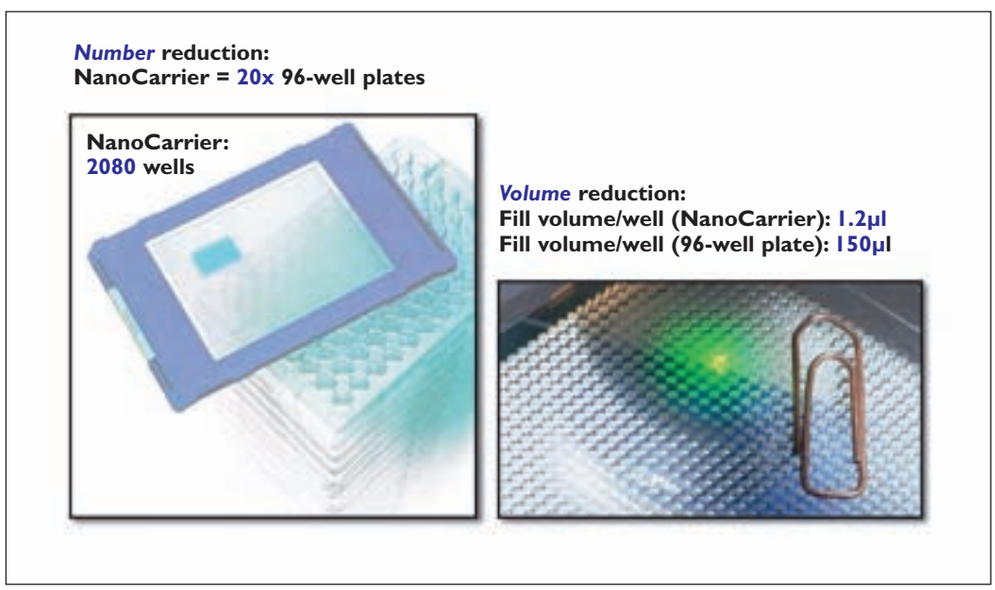

Figure 2
The Novartis/Evotec NanoCarrier (NTP) concept. The figure on the left panel shows a standard NanoCarrier plate with 2080 wells (2080w-NTP) surrounded by wells loaded with water to minimise evaporation of liquid. For demonstration purposes, 96 wells of the NTP are filled with blue dye solution and the plate was placed on top of a pile of twenty 96w-MTPs to demonstrate the high density format of the 2080w-NTP. The figure on the right panel shows for demonstration purposes a paper clip placed on top of the 2080w-NTP. Each well of the 2080w-NTP is filled with a total volume of 1.2µl per well

Figure 3: Comparison of various microtiter plate formats for screening.
The figure shows the effect of volume reduction for various types of plates used in routine high-throughput screening (96w-384w-1536w-2080w plates). Volume reduction has a pronounced effect on the number of plates and the amount of liquid to be handled per full screening campaign, the amount of chemical compound necessary for testing, the amount of biological assay reagent and, ultimately, the reagent costs associated with screening of large compound collections. The numbers are based on a commercially available protease assay with a fluorogenic peptide as a substrate and 1.0 million compounds on the screening deck. The exact plate formats are (including the controls): 88/96w-, 252/384w-, 1408/1536w- and 1760/2080w plate format

	96w plate (120-250µl)	384w plate (30-100µl)	1536w plate (2.5-10µl)	2080w plate (0.5-1.4µl)
Volume/well	150µl	50µl	8.5µl	1.2µl
Number of plates	11,364	2,841	711	569
Reaction mix	150.1 litres	50.1 litres	8.5 litres	1.2 litres
Compound/well (MW 500Da; 10µm)	750ng	250ng	42.5ng	6ng
Enzyme (MW 80kDa; 20nM)	240.2mg	80.2mg	13.6mg	1.94mg
Substrate amount (MW 1,000Da; 100µm)	15.01g	5.01g	851mg	121mg
Substrate costs (1mg = $100)	$1,501,000	$501,000	$85,100	$12,100

Example: Protease HTS assay with fluorescence readout and 1.0 Mio compounds tested:
Library size 1,000,000 compounds, control wells on each plate (88/96w-, 352/384w-, 1408/1536w- and 1760/2080w-plate format); in addition, excellent sensitivity for the confocal detection set-up with single-molecule detection in NanoScreening will allow even lower substrate concentrations

few readout technologies and some distinct MTP readers. Therefore, in most cases 1536w-MTPs are read out via CCD-based imaging of the whole plate at once and typical readout times for this technology are in the range of several minutes. Special care has to be taken, however, that volume/surface ratios in 1536w-MTP do not affect the biological test system. In many cases, lids have to be used on 1536w-MTPs to minimise evaporation during extended incubation times or incubation times at higher temperatures, eg 37°C. The main limitation of the 1536w-MTP format, however, is a potentially negative effect of volume reduction on the quality of the readout signal.

Further trends towards miniaturisation are still ongoing. Several examples with biological assays in 3456w-MTPs have been reported (total assay volume 1-2µl)[3]. An interesting alternative of ever-increasing importance is becoming the 384w low volume MTP (384lv-MTP). These plates use a 384w format of the plate, albeit conical shapes of the wells and can be used to run biological assays at fairly low volume (10-20µl total volume). The benefit of the 384w lv-MTP is, however, the applicability of 96/384 liquid handling and dispensing technology. Current industry trends point towards the 384w standard, 384w low volume and the 1536w MTP as the main plate formats for compound testing in the future.

Almost 10 years ago, Novartis (formerly Sandoz) entered into a technology development partnership with Evotec OAI (www.evotecoai.com) towards novel HTS readout technologies and strong miniaturisation of high-throughput screening systems. This concept implies the development of a readout technology almost independent of the assay volume as well as the development of liquid handling technologies in the nanolitre range. As a result of this development, the Novartis/Evotec NanoCarrier plate (NTP) was developed for HTS measurements via confocal fluorescence spectroscopy. Each NTP holds 2,080 wells (2080w-NTP) for compound testing and is surrounded by wells filled with water to minimise evaporation of liquid from the array of screened wells (**Figure 2**). In addition to

the compounds for testing, various wells of the 2080w-NTP are filled with controls (high/low control of the assay, dye control, etc) necessary for productive screening and in-process control. The typical working volume for 2080w-NTP is in the range of about 0.7-1.5µl total volume with a standard volume of about 1.2µl per well. Depending on the complexity of biological test systems (number of volume addition steps), this volume range implies that all volume additions are done in the range of only tens to hundreds of nanolitres per single step of reagent addition. These small volumes require unique liquid handling technologies not necessarily available for routine compound testing in 96w- or 384w-MTP formats. These small volumes require also unique detection methods with sufficient sensitivity for assay readouts at ultra low volumes, like confocal fluorescence spectroscopy with single molecule spectroscopy capabilities and detection volumes below 1 femtolitre[4-6] (**Figure 4**).

Most pharmaceutical and biotech companies run around 50-200 high throughput screening campaigns per year with compound collections in excess of 0.5-1.0 million compounds. The importance of volume reduction via various microtiter plate formats is exemplified in **Figure 3**. This table compares assays at the various plate formats from 96w-MTP standard plates as a reference all the way miniaturised towards 2080w NanoCarrier plates. The example is based on a protease assay with a commercial fluorescent substrate and 1.0 million compounds for screening. With regards to the needs of automation and robotics, a full screening campaign equals either 11,364 plates in a 96w-MTP format or just 569 plates in a 2080w-NTP format (the plate format, including control wells per plate, is either a 88/96w-, 352/384w-1408/1536w- or 1760/2080w-plate format). The difference in assay volume equals either 150 litres of assay reagents per full screening campaign or just 1.2 litres, which has a strong impact on lead discovery campaigns where waste disposal can become a major cost issue. With regards to precious chemical compounds or isolates from rare natural products, the difference per well is either 750ng per data point (MW = 500; screening concentration = 10µM) in a 96w-MTP or just 6ng per data point in a 2080w-NTP. One element of utmost importance is the difference in amount of biological assay components, like recombinant proteins and cell lines. For biochemical targets in high throughput screening, the majority of the proteins have to be produced in eukaryotic expression systems like insect cells or mammalian cells. Under the same enzymological conditions, a conventional 96w-MTP based assay would need more than 200mg of recombinant protein for screening the full compound deck. The highly miniaturised 2080w-NTP would require less than 2mg of recombinant protein (higher sensitivity due to confocal single molecule detection methods not even included). For some target proteins, the request for very large amounts of recombinant protein can even become prohibitive for a lead finding campaign due to the high costs of eukaryotic protein

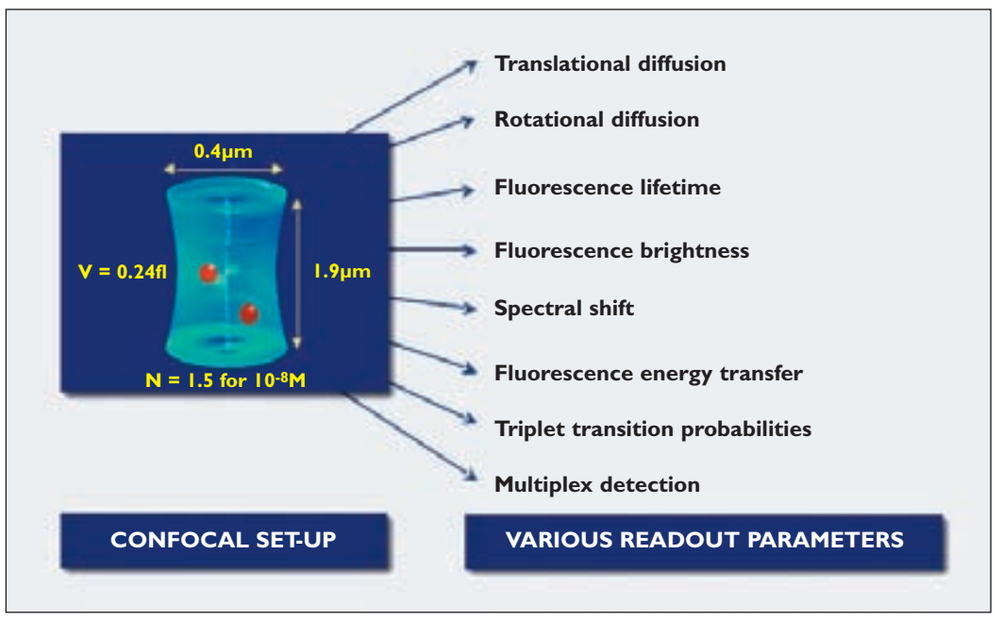

Figure 4
The principles of confocal fluorescence spectroscopy. The figure shows the confocal illumination field of about 1.9µm in height and about 0.4µm in diameter. This equals a detection volume of about 0.24 femto litres (10-15 litres). Only flurophors in this illumination field are excited by the confocal set-up in fluorescence spectroscopy via excitation by high-energy laser light of various sources. For 10nM solutions of fluorescent detection molecules, on average only 1.5 fluorescent molecules are present over time in the confocal detection field. This enables multiparametric readout technologies at very high quality

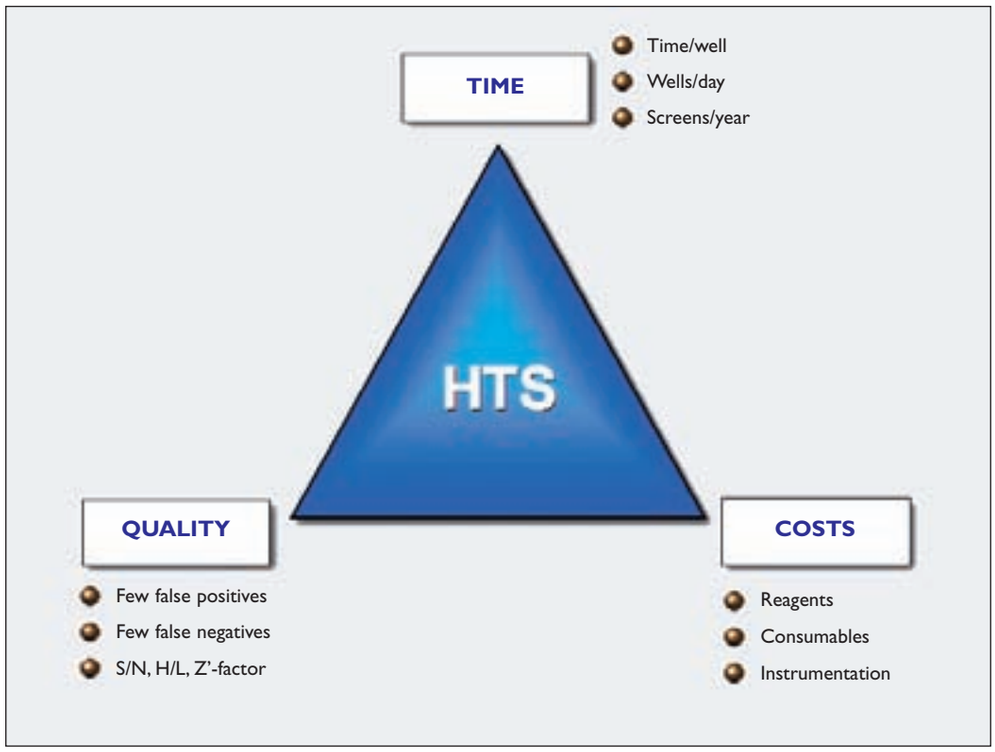

Figure 5
The 'magic triangle of HTS' The figure shows the key success factors for modern lead discovery via HTS, namely Time, Costs and Quality. As can be seen on the slide, all three factors are closely interdigitated and every change on either one of these factors changes the set-up all the other factors as well. Optimal lead discovery by HTS finds the right balance between these different elements

expression, mammalian cell lines in particular. The next factor to consider are the costs for the detection reaction in biological assay systems, for example fluorescently labelled substrates for proteases and kinases, antibodies for the detection of phosphorylation/dephosphorylation reactions, etc. The current example shows the difference in costs for the substrate based on a commercially available peptide substrate for a protease. Just the costs for the substrate would be either more than $1.5 million in a standard 96w-MTP format or just slightly more than $12,000 in a 2080w-NanoCarrier format. Taken together, despite higher initial investment costs for setting up miniaturised high-throughput screening systems, a detailed fully loaded cost analysis among the various assay technologies at Novartis Pharma AG has shown that the overall costs for screening clearly has been diminished mostly driven by assay miniaturisation (data not shown).

Effect on productivity

Independent of the precise nature of the applied screening technology, lead discovery efforts can always be analysed and optimised along the same fundamental principles of performance management ('the magic triangle of HTS'): time, costs and quality of the process (**Figure 5**). Since high-throughput screening always deals with large amounts of samples to be analysed, the measurement time for a single well in the screening campaign is a key performance parameter. The same holds true for the number of wells which can be run on a single system per working day and the number of screens which can be run per year. Changing the time component of the HTS efforts among the types described above will have a direct and indirect effect on the costs of a screening campaign since instruments and other resources will be needed for either shorter or longer periods of time. A major cost driver for screening is not only the technological hardware for screening, like robotic plate handling and readout systems, but also the costs for reagents and consumables. Typical consumable costs are the costs for plates, tips, vials, etc. Typical reagent costs are the costs for the biological test samples, mostly protein, cells, substrate, etc can become detrimental in cases with some sensitive or hard to produce biological assay reagents. Ultimately, however, the most important factor in lead discovery efforts is the quality of the process. This is particularly important for screening which deals with very large data sets since only assays of high statistical quality can be used for proper data analysis. It is important to consider that the

number of false positives, the number of false negatives, and other statistical terms such as S/N (signal/noise), H/L (high/low) or Z'-factor[7] can be used to optimise the statistical quality of a high-throughput screening campaign. It should be noted that the quality of an assay is not only expressed by the statistical quality of an assay (exemplified by Z'-value), but also by the biochemical or biological sensitivity of a particular assay set-up for detection of compounds with intermediate of even weak affinity. In other words, assays should be developed not only towards maximum statistical quality, but also towards sensitive detection of weak inhibitors in a lead finding campaign to offer a potential new avenue for drug discovery with some new chemical series or scaffold. It is important that all three main elements of successful HTS – Time, Costs, Quality – are closely linked and interdigitated in reality. Every lead finding effort, but also every lead finding technology, can be evaluated according to these generic criteria for success.

With the help of the various automated screening systems, in particular NanoScreen and SpeedScreen[6,8,9], our organisation was able to strongly increase the number of screened entities per year in the 10 years. This clearly indicates that the organisation was capable of taking up the challenge with ever increased compound collections and the increased number of screenable targets. This was of course only possible due to the constant decrease in costs per data point in high-throughput screening. It has to be shown in the future whether the trend towards strong increase in number of data points and the strong increase in fully loaded costs per screening data point can continued. Despite that, it has become clear that automation and miniaturisation must have been capable of more than overseeding the initial set-up costs for large automation and miniaturisation systems. It should be pointed out that the cost analysis contains already a 2-5 year depreciation period for instruments and robotic equipment. We therefore can conclude from our internal cost analysis done at Novartis that our organisation has been very successful to validate and implement novel screening and liquid handling technologies and that this strategy has given already a remarkably good return on investment as expressed by the strong and continuous decrease in costs per data point. Another way of looking at this is given by the comparison of the Novartis costs per screen with external benchmarks for screening exactly the same amount of chemical compounds. Our analysis shows that the costs for the internal screening efforts at Novartis Pharma are significantly lower than the costs at external service providers (data not shown).

Summary

Most companies have successfully managed the challenge with ever increasing chemical compound libraries and larger number of screening targets via implementation of highly miniaturised systems for assay development and screening. Despite the demand for sophisticated technologies in both reagent handling as well as plate readout technologies, experience among various companies shows the successful implementation of these technologies into the drug discovery environment at these organisations. Current benchmark for compound storage and biological assays are 384w-MTP at most Pharma and Biotech companies. Some companies are using plate formats at even higher density, such as 1536w-, 2080w- or 3456w-MTP, but for most applications 384w-MTP and 1536w-MTP will serve the need of the majority of the customers.

In recent time, we also see a strong trend to further miniaturise assay development and screening not only at the centralised lead discovery departments at the major Pharma and Biotech companies, but also the same trend towards assay miniaturisation in the standard research laboratories at research laboratories in the disease areas of these organisations. This is, of course, only possible due to the ease of use and reliability of the current liquid and plate handling technologies currently available on the market.

Taken together, we can conclude that the trend for miniaturisation of assay development and screening over the last decade has been a great success. Technical obstacles in liquid handling and readout technologies have been overcome and the high quality of both assay plates as well as plate handling technology has become a key factor for the widespread use of the miniaturised technologies. In summary, productivity and cost analyses clearly show the beneficial contribution of assay miniaturisation for modern drug discovery.

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