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Report on continuous surveillance for drug development (D - 3)

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1. Introduction

One of the key points in standardization of the HTS procedures is the definition of a work plan, including the period from reception of the chemical library to be evaluated to communication of the results (Inglese et al., Nat Chem Biol. 2007; 3:438-41). A general example of such a work plan is shown in Figure 1.

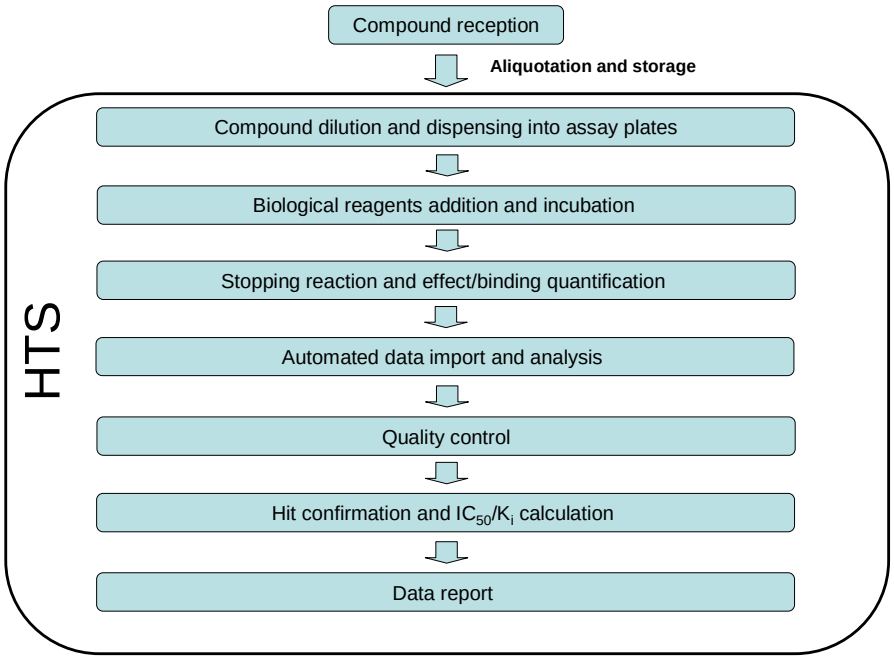


Figure 1: Flow diagram of the steps involved in HTS surveys.

In public-private collaborations in the field of drug discovery it is essential to have available standards that enable use of a common language for exchange of protocol data, molecules, hits, leads and candidates.

High Throughput Screening (HTS) of chemical libraries is a standard method of identifying new hits in drug discovery procedures. However, because of the wide diversity of the multiple components of HTS (biological assay, format of the assay, nature of the library, dispensing methods, detection instrumentation, data analysis algorithms...), standardized operating procedures and strict quality control methods are required.

Prior to carrying out HTS, the miniturized and automatized assays must be validated. Such validation comprises several steps in which the different experimental conditions are established. By way of example, validation of a radioligand binding study involves the following steps:

- Protein linearity study: carried out to establish the amount of protein to use at each experimental point, compromising between the optimal specific signal and amount of protein.
- Saturation study: the radioligand used in the assays is validated by checking that the affinity values obtained are consistent with those reported in the bibliography for this radioligand acting at the target under study.
- Calibration studies: concentration-response curves are elaborated for standard compounds with known affinity for the target under study, and the values obtained are compared with those reported in the relevant literature.
- Vehicle tolerance studies: concentration-response curves are constructed for the solvent in which the compounds in the chemical library are dissolved, with the aim of determining the tolerability of the assay to the solvent and thus establishing the dilution protocol to follow during execution of the screening procedure.
- Studies of inter and intra-experimental variability: assays are carried out in different plates with known standards, in order to calculate the coefficient of variation between the different plates and within the same plate.

As a single determination of each of the compounds in the library is obtained in HTS assays (i.e. a single concentration with no replicates), there is a higher incidence of false positives and false negatives than in low throughput assays. After carrying out the biological assay, the data are therefore subjected to strict quality control to minimize the occurrence of false positives and false negatives. The quality control procedures include:

- Evaluation of the stability of the signal/noise ratio during the assay.
- Evaluation of concentrations of compounds of known activity in all of the assay plates.
- Data dispersion controls: the values of Z and Z' are determined as indicators of the data dispersion throughout the assay (Zhang et al., J Biomol Screen 1999;4:67-73).
- Analysis of the distribution of hits: this type of analysis is useful for identifying systematic errors throughout the assay.

Once the plates have passed the quality control, the hits obtained are confirmed by a second assay in which a single concentration of the hits is evaluated. Those plates in which the variation between the assays is less than 10% are considered as validated hits. The affinity of the validated hits for a target to study are evaluated by construction of concentration-response curves in which the IC_{50} and the K_i (or one of these) are calculated.

Although standardization of the entire HTS procedure and the different quality controls contribute to the rapid and reliable identification of new hits, the

procedure would be worthless without correct logistical handling of the compounds in the library. This involves the following steps; defined for the project:

- Dissolution of the chemical library in the format required for generating plate assays that can be used in HTS, e.g. compounds dissolved in DMSO in 96-well plates, with columns 1 and 12 free for insertion of the different assay controls.
- Aliquoting of the chemical library: The number of assays to be carried out must be predicted so that there are sufficient numbers of samples available for assay during long periods. Aliquoting will avoid subjecting the compounds to different cycles of freezing/thawing thus preventing uptake of water, which would diminish their solubility and/solubility.
- Use of suitable recipients for storage: e.g. opaque screw top glass vials for powdered compounds and polypropylene plates for compounds in solution.
- Labelling and location of the compounds: the label must contain the information necessary for the identification of compounds and execution of the assays. The inclusion of bar codes in the labels will facilitate later handling of the plates during the HTS procedure.
- Storage under suitable conditions: e.g. in the case of powdered compounds, protected from light, and in the case of dissolved compounds, at -20ºC.
- Handling of stock solutions: of vital importance for determining the remaining amounts of each compound in the chemical library and for planning the acquisition/synthesis of new batches of compounds before they run out. Also useful for locating compounds in the storage sites.

2. Objectives and methodology

The **objective** is to establish criteria for development, to guarantee the transferability of technological innovations that arise from the project to businesses. It is hoped that this will promote links between the University and businesses and that the researchers' work will be guided by business-related criteria as well as scientific criteria.

The criteria will be established for the two priority areas of study in the project with greatest potential for transfer:

- Definition of criteria for development of minituarized assays for compound evaluation.

- Definition of criteria for management of compounds for the treatment of metabolic illnesses.

The working **methodology** will be based on the organization of round table meetings and work agreements with businesses and other parties (such as intermediaries, business or specialist sector associations, etc.).

3. Results achieved

A conference was organized jointly by the USC partner in the DIOMED project and Pharmaindistry, to present and promote the public-private initiatives that are being produced in the field of drug discovery at regional, national and international levels. The conference was part of the activities of the Galician Network for Drug R& D, within the ChemBioGal and DIOMED projects. The companies participating were Laboratorios ESTEVE, Laboratorios Almirall, Palau Pharma, Oryzon Genomics, BrainCo Biopharma, Progenika Biopharma, Vivia Biotech, GalChimia and Janssen Cilag.

The First Guide to Early Drug Discovery Centres (Preclinical Screening) in Spain, promoted by the Spanish Platform for Innovative Medicines, and in which public and private institutions have participated was also presented at the conference. It is hoped that the guide will be converted into a useful working tool for all those involved in drug research.

Also, regular meetings have also been held with different companies (which the partner USC maintain a continuous collaborations in HTS), with the aim of defining criteria for developing working methods and protocols for handling compounds.

This information was used to elaborate the following annexes for exchange:

ANNEX 1:

DEFINITION OF STANDARDS FOR EXCHANGE OF INFORMATION FOR THE DEVELOPMENT OF MINIATURIZED ASSAYS FOR EVALUATING COMPOUNDS:

1. The miniaturized is set-up and validated by:

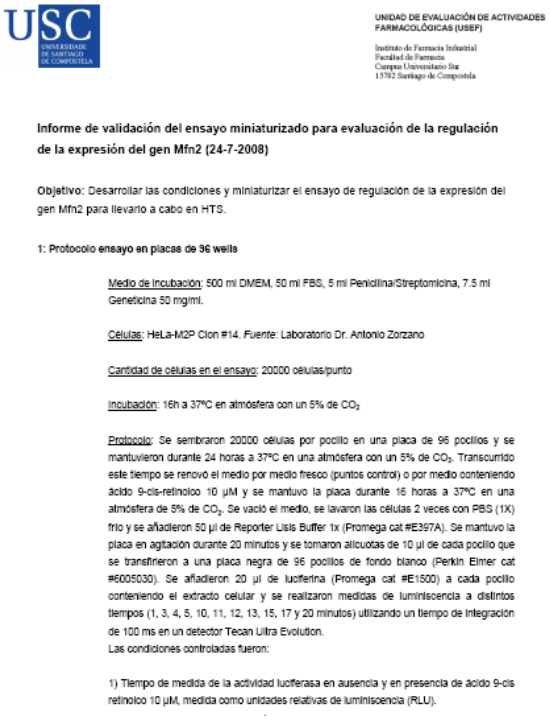
    - Fine tuning in the final volume of selected methodologies (inter-plate standard replication).
    - Library dilution protocol (80 cpds/plate; plate with columns 1 and 12 empty for quality control points)
    - Minimum volume robotic calibration, trasfer (minimum, maximum, air).
    - Inter and intra-experimental variability
    - Solvent interference
    - Signal stability

2. Prior to carrying out the screening campaign, a report is sent to the requesting group with details about the fine-tuning and validation of the assay. The validation of the assay is discussed with the group and new assays are carried out when needed.

3. Once the client's approval is given, the HTS campaign is undertaken.

ANNEX 2:

DEFINITION OF STANDARDS FOR ASSAYING COMPOUNDS THROUGH HTS, QUALITY CONTROL AND REPORT OF RESULTS, FOR DRUG DISCOVERY PROGRAMMES, INCLUDING THE TREATMENT OF METABOLIC DISEASES.



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Informe de validación del ensayo miniaturizado para evaluación de la regulación de la expresión del gen Mfn2 (24-7-2008)

Objetivo: Desarrollar las condiciones y miniaturizar el ensayo de regulación de la expresión del gen Mfn2 para llevarlo a cabo en HTS.

1: Protocolo ensayo en placas de 96 wells

Medio de incubación: 500 ml DMEM, 50 ml FBS, 5 ml Penicilina/Streptomicina, 7.5 ml Geneticina 50 mg/ml.

Células: HeLa-M2P Clon #14. *Fuente*: Laboratorio Dr. Antonio Zorzano

Cantidad de células en el ensayo: 20000 células/punto

Incubación: 16h a 37ºC en atmósfera con un 5% de CO_2

Protocolo: Se sembraron 20000 células por pocillo en una placa de 96 pocillos y se mantuvieron durante 24 horas a 37ºC en una atmósfera con un 5% de CO_2. Transcurrido este tiempo se renovó el medio por medio fresco (puntos control) o por medio conteniendo ácido 9-cis-retinoico 10 µM y se mantuvo la placa durante 16 horas a 37ºC en una atmósfera de 5% de CO_2. Se vació el medio, se lavaron las células 2 veces con PBS (1X) frío y se añadieron 50 µl de Reporter Lisis Buffer 1x (Promega cat #E397A). Se mantuvo la placa en agitación durante 20 minutos y se tomaron alícuotas de 10 µl de cada pocillo que se transfirieron a una placa negra de 96 pocillos de fondo blanco (Perkin Elmer cat #6005030). Se añadieron 20 µl de luciferina (Promega cat #E1500) a cada pocillo conteniendo el extracto celular y se realizaron medidas de luminiscencia a distintos tiempos (1, 3, 4, 5, 10, 11, 12, 13, 15, 17 y 20 minutos) utilizando un tiempo de integración de 100 ms en un detector Tecan Ultra Evolution.
Las condiciones controladas fueron:

1) Tiempo de medida de la actividad luciferasa en ausencia y en presencia de ácido 9-cis retinoico 10 µM, medida como unidades relativas de luminiscencia (RLU).

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2) Actividad específica calculada como la relación señal/ruido, medida según la fórmula:

RLU presencia ácido 9-cis retinoico 10 µM / RLU ausencia ácido 9-cis retinoico.

3) Dispersión de los datos. Se calculó la Z' (Zhang y cols. J Biomol Screen 1999; 4:67-73): La Z' es el parametro estadístico standard en el análisis de campañas de HTS. Se calcula a partir de la fórmula:

$$Z' = 1 - \frac{(3SD_{c+} + 3SD_{c-})}{(mean_{c+} - mean_{c-})}$$

donde SD_{c+} y SD_{c-} se corresponden con la desviación estándar de las medidas realizadas en presencia y en ausencia de ácido retinoico 10 µM, respectivamente y $mean_{c+}$ y $mean_{c-}$ se corresponden con las medias de las medidas realizadas en presencia y en ausencia de ácido retinoico 10 µM, respectivamente. El margen de aceptación del ensayo se encuentra entre valores de 0.5-1.

2: Validación ensayo en placas de 96 wells

1.- Actividad luciferasa

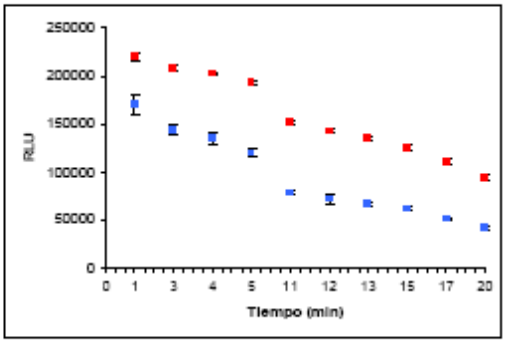

Fig 1: Tiempo sobre la lectura de la actividad luciferasa. Se muestran las unidades relativas de luminiscencia (RLU) en ausencia (■) y presencia (■) de ácido retinoico 10 µM determinadas a distintos tiempos tras la administración de luciferina. Los puntos representan la media±desviación estándar (barras verticales) de 4 pocillos.

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2.- Relación señal/ruido

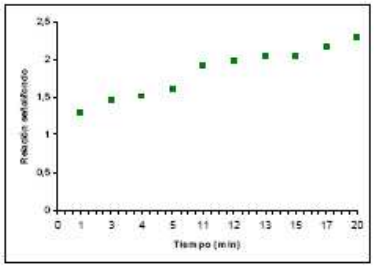

Fig 2: Relación señal/ruido (■) medida a distintos tiempos tras la adición de luciferina. Los puntos representan la relación entre la media de RLU de 4 pocillos en presencia y de 4 pocillos en ausencia de ácido 9-cis retinoico.

3.- Dipersión de datos

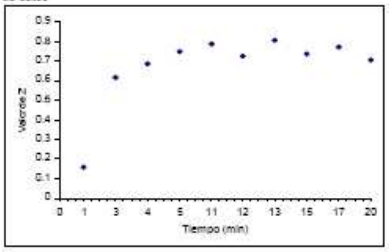

Fig 3: Valores de Z' (♦) calculados a partir de 4 puntos en ausencia y 4 puntos en presencia de ácido 9-cis-retinoico a distintos tiempos de medida tras la adición de luciferina.

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3: Conclusiones

1.- La actividad luciferasa decayó con el tiempo de medida, a tiempos superiores a 5 minutos se observa un descenso muy acusado de la actividad luciferasa. Los puntos en ausencia de ácido 9-cis retinoico presentaron un descenso más acusado que los puntos en presencia de este compuesto.

2.- La actividad específica, medida como la relación señal/ruido, aumentó con el tiempo de medida. Este aumento se debió al descenso más acusado de la actividad luciferasa que presentaron los puntos en ausencia de ácido 9-cis retinoico. A partir de los 5 minutos esta relación fue superior a 1.5, siendo válida para la ejecución de ensayos de HTS.

3.- Los valores de Z' aumentaron con el tiempo, estabilizándose en valores superiores a 0.7 a partir de 5 minutos tras la adición de la luciferina.

Por todo ello, se propone ejecutar la campaña de HTS según el protocolo descrito y realizando las medidas de la actividad luciferasa 5 minutos después de la adición de la luciferina.

ANNEX 3:

EXAMPLE OF DATA REPORT FOR HTS CAMPAIGNS.

INDIVIDUAL TESTS DATA REPORT

PCB

Study Completed: September 17th, 2008
Report Printed: September 18th, 2008

Work Order #: USEF0201/08U

USEF Internal #: CBG00001-CBG01120

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Pharmacology Data Report For PCB

Work Order Number: USEF0201/08U
Services Being Reported: Individual tests

Compound information:

USEF internal #	Compound code	DCI
CBG000001	Prestw-1	Azaguanine-8
CBG000002	Prestw-11	Meticrane
CBG000003	Prestw-21	Sulfaphenazole
CBG000004	Prestw-31	Chloramphenicol
CBG000005	Prestw-41	Procaine hydrochloride
CBG000006	Prestw-51	Trichlorfon
CBG000007	Prestw-61	Morantel tartrate
CBG000008	Prestw-71	Todralazine hydrochloride
CBG000009	Prestw-2	Allantoin
CBG000010	Prestw-12	Benzonatate
CBG000011	Prestw-22	Panthenol (D)
CBG000012	Prestw-32	Eprinzole
CBG000013	Prestw-42	Moxisylyte hydrochoride
CBG000014	Prestw-52	Carbamazepine
CBG000015	Prestw-62	Homatropine hydrobromide (R,S)
CBG000016	Prestw-72	Imipramine hydrochloride
CBG000017	Prestw-3	Acetazolamide
CBG000018	Prestw-13	Hydroflumethiazide
CBG000019	Prestw-23	Sulfadiazine
CBG000020	Prestw-33	Diprophylline
CBG000021	Prestw-43	Betazole hydrochloride
CBG000022	Prestw-53	Triflupromazine hydrochloride
CBG000023	Prestw-63	Nifedipine
CBG000024	Prestw-73	Sulindac
CBG000025	Prestw-4	Metformin hydrochloride
CBG000026	Prestw-14	Sulfacetamide sodic hydrate
CBG000027	Prestw-24	Norethynodrel
CBG000028	Prestw-34	Triamterene
CBG000029	Prestw-44	Isoxicam
CBG000030	Prestw-54	Mefenamic acid
CBG000031	Prestw-64	Chlorpromazine hydrochloride
CBG000032	Prestw-74	Amitryptiline hydrochloride
CBG000033	Prestw-5	Atracurium besylate
CBG000034	Prestw-15	Heptaminol hydrochloride
CBG000035	Prestw-25	Thiamphenicol
CBG000036	Prestw-35	Dapsone
CBG000037	Prestw-45	Naproxen

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METHODS

Methods employed in this study have been adapted from the methods provided from PCB to maximize reliability and reproducibility. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained.

Source: HeLa M2P overexpressing Mfn2 promoter clon#14 (provided by the sponsor of the study)
Control Ligand: 10 µM 9-cis-retinoic acid (Sigma R4643)
Vehicle: 1% DMSO
Incubation time/temp: 16h at 37°C, 5% CO_2 atmosphere
Incubation buffer: 500 ml DMEM, 50 ml FBS, 5 ml Penicillin/Streptomicin, 7.5 ml Geneticin 50 mg/ml.
Quantitation method: Luminiscence

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EXPERIMENTAL RESULTS

Assay results are presented as the percent of activity of 10 µM 9-cis-retinoic acid throughout the report. Compounds were tested at a concentration of 10 µM. Negative percentage values correspond to the inhibition of mitofusin-2 expression compounds elicited by compounds. Compounds showing activity higher than 60% of 9-cis-retinoic acid were considered as hits and were confirmed in an independent assay.

1. COMPLETE DATA ANNOTATION

USEF #	Compound code	DCI	% activity (10 µM)
CBG000001	Prestw-1	Azaguanine-8	-24,6168
CBG000002	Prestw-11	Metlorane	-4,0500
CBG000003	Prestw-21	Sulfaphenazole	-7,1096
CBG000004	Prestw-31	Chloramphenicol	-6,5544
CBG000005	Prestw-41	Procaine hydrochloride	-8,4524
CBG000006	Prestw-51	Trichlorfon	-1,7071
CBG000007	Prestw-61	Morantel tartrate	-18,5148
CBG000008	Prestw-71	Todralazine hydrochloride	12,2697
CBG000009	Prestw-2	Allantoin	4,9411
CBG000010	Prestw-12	Benzonatate	-0,8382
CBG000011	Prestw-22	Panthenol (D)	2,6472
CBG000012	Prestw-32	Epitizole	-8,1498
CBG000013	Prestw-42	Moxisylyte hydrochloride	1,4757
CBG000014	Prestw-52	Carbamazepine	-8,1796
CBG000015	Prestw-62	Homatropine hydrobromide (R,S)	-1,6459
CBG000016	Prestw-72	Imipramine hydrochloride	30,3091
CBG000017	Prestw-3	Acetazolamide	-2,6461
CBG000018	Prestw-13	Hydroflumethiazide	-7,4322
CBG000019	Prestw-23	Sulfadiazine	0,3243
CBG000020	Prestw-33	Diprophylline	-7,1396
CBG000021	Prestw-43	Betazole hydrochloride	-7,5535
CBG000022	Prestw-53	Triflupromazine hydrochloride	-13,2977
CBG000023	Prestw-63	Nifedipine	-30,5955
CBG000024	Prestw-73	Sulindac	28,0723
CBG000025	Prestw-4	Metformin hydrochloride	-5,5242
CBG000026	Prestw-14	Sulfacetamide sodic hydrate	-13,3989
CBG000027	Prestw-24	Norethynodrel	-26,3405
CBG000028	Prestw-34	Triamterene	-2,6661

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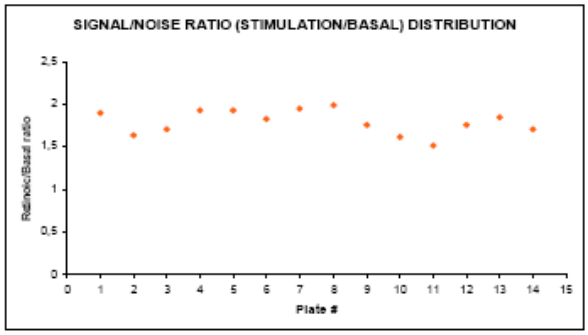

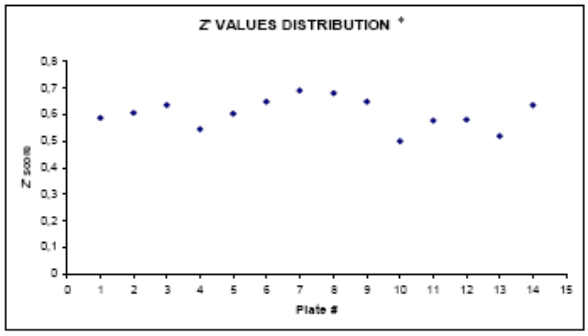

*: Zhang J, Chung TDY, Oldenburg KR. A simple statistical parameter for in evaluation and validation of High Throughput Screening assays. Journal of Biomolecular Screening 1999; 4:67-73.