WHITE PAPER

Cell-based assays: Purposeful screens for better results

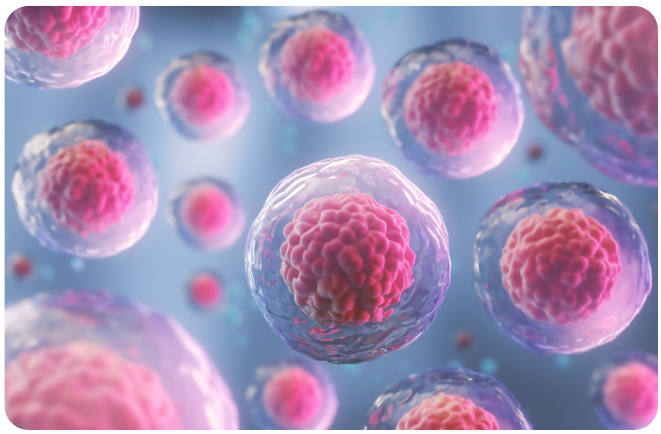
Key considerations for designing cell-based assays

Over the last few decades there has been a growing trend in drug discovery to use cellular systems and functional assays, in addition to biochemical assays, for the finding and characterization of new potential therapeutics. It's no secret that disqualified candidates are greater in number than those that make it through the drug discovery process. One thing that this has taught the drug discovery community is that biology is incredibly more complex than we often give it credit for, and that it is in our benefit to use systems that are as physiologically relevant as possible earlier in the drug discovery process.

Here, we gather insights gained over the years through close interactions with researchers to provide you with elements to consider when setting up cell-based assays. Properly designing and interpreting your assay helps ensure accurate and reliable results, ultimately helping to increase your success rate.

Target context

An important aspect to consider when selecting a biological model system is the repertoire of accessory proteins associated with a potential protein target. Targets do not exist alone, and most often their interaction with partner proteins is an important component of their behavior and impacts their response to modulation by test compounds. This is particularly true for targets that have an absolute need for a cofactor to be active. When selecting a biological model system, it is important to keep in mind that your target is part of an ensemble, and therefore to select a cellular model



where your target protein will find the same working partners as it is supposed to collaborate with *in vivo*, so that the target modulators that will be developed *in vitro* will make sense when later used in the patient.

In addition to considering accessory proteins, it is also important to choose the right allele of your target for screening. As humans, we share many similarities, yet we are each very different and genetically unique from each other. Originally, we all shared the same genes however over time different versions of these genes have appeared in different populations, these changes are what have made us genetically unique. Even the smallest of changes on a protein can have dramatic effects on both the function of that protein as well as the way it responds to modulators. For this reason, when deciding on which target to screen, it is important to select the allele that is most representative of the human population. Furthermore, a counter-screen should be performed early enough in the screening process with the major alleles to make sure that the molecule developed is also active at the other target allelic versions.

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Selecting your cell model

There are many things to consider when selecting a cell model. Do you use an established cell line or go with primary cells that perhaps are closer to mimicking an *in vivo* model? Is your target adequately expressed?

Target expression - endogenous vs. recombinant

The very first consideration to make when developing a cell model is whether to use a model in which the target is already present (endogenous expression) or a model in which the target is absent and needs to be added (recombinant expression). Table 1 outlines the differences between endogenous and recombinant expression in terms of a cell model and offers some pros and cons to each.

Table 1: Endogenous vs. Recombinant Expression.

	Endogenous expression	Recombinant expression
Expression Level	**Determined by Cell Type, Usually Low Expression Level** PRO More physiological, can provide more predictive pharmacology CON Expression level may not be sufficient for some assay types	**Selected by** ▪ Expression vector design ▪ Clonal selection from low to high levels PRO Flexibility on system's response CON High expression levels can lead to Pharmacological distortions by over-titration of interaction partners
Signal Detection	**Need to Identify an Assay Suitable for Working with Endogenous Proteins and Their Relevant Expression Levels** PRO More physiological CON Can be difficult and/or costly to detect	**Permits Redirection of the Receptor Coupling** PRO Allows for screening platform standardization CON Can distort pharmacology **Fusion of Target Protein to a Reporter** PRO Non-expensive assay CON Can distort pharmacology **Stable Co-Expression of Reporter System Such as Aequorin (Calcium Flux Assays)** PRO Non-expensive assay, no fluorescent compound interference CON Need to establish the cell line or to perform transient transfection **Stable Co-Expression of Reporter Systems Such as Transcription-Based Reporter Gene Assays** PRO Non-expensive assay CON Can increase non-specific hit rate
Isoform Considerations	**Multiple Target Isoforms Present** PRO Presence of multiple splicing variants is more physiological; better reflects the *in vivo* environment CON Presence of other receptor isotypes may prevent the specific detection of the modulation of the desired receptor isotype	**Expression of Specific Selected Isoform** PRO Allows for screening of a precise isoform CON May not fully reflect the *in vivo* environment

For recombinant expression, when working with stably transfected cells it is advantageous to isolate clones, as doing so allows for better stability of the functional response of the cells over time. Clonal selection is typically done by limiting dilution where, for example, an average 0.1 cell/well is dispensed into 96-well plates. After a few weeks the cells are examined and the probability that wells containing cells truly originate from a single cell, and thus is a true clone, is calculated. This calculation is based on Poisson distribution, using the number of wells with and without cells. Once true clones are identified, the cells can then be grown and kept in culture for up to three months, with vials of frozen cells prepared every month. At the end of three months, frozen vials can be thawed in parallel and cells tested to control if the functional characteristics of the selected clone are stable over time in culture.

Cell type

The next consideration when selecting a cell model is the type of cells to use – primary cells, an immortalized cell line, or iPSCs. The cell type should be as close as possible to the *in vivo* environment, but also needs to be both easy to grow and to provide a robust assay to ensure adequate data quality and the avoidance of false positives. Because of this, the selection of a cell type is always a compromise.

Primary cells are, in theory, the cultured cell option that is most like the *in vivo* environment in which the test compound will need to be effective. While this may sound ideal, primary cells have their share of limitations. Such limitations include difficulty in securing enough cells for screening and subsequent development of assays, as well as variability between donors. While such variability may be advantageous in later stages, for hit selection it is best to use something more uniform as it leads to less variable data. Immortalized cell lines have the advantage of uniformity, but their characteristics may not be representative of the *in vivo* environment.

Induced pluripotent stem cells (iPSCs) offer the potential of combining the advantages of being more physiologically relevant than immortalized cell lines, while being accessible in larger quantities and with less variability than primary cells.

Target expression level

Ensuring the appropriate expression level of the target is essential to obtain good, reliable data. In some cases, having enough of the target expressed is critical to the establishment of a good, quality assay. In other cases, too much of the target can be detrimental to the assay quality. For example, in radioligand binding assays it is common for endogenous receptor expression levels to be too low to generate a signal within the assay window. In such cases, a recombinant cell model that has a high level of receptor expression is required. Another example that shows the role expression levels can play in assay readout is the over-expression of a receptor that an agonist is partial to. In such cases, the agonist may appear to have a more complete agonist activity and can even present the same response as genuine full agonist. This is because the over-abundance of the receptor can compensate for the weak efficacy of the agonist.

The level of expression of the target can have a definitive impact on the pharmacological behavior of the model system, and there is not a unique cellular system that will be suitable to all assays. Therefore, it is important that each target/assay combination is assessed on a case by case basis to ensure the optimal decision is made.

Repertoire of accessory proteins

As previously mentioned, targets do not exist alone. Most often, their interaction with partner proteins is an important component of their behavior and impacts their response to modulation by test compounds. When developing a cell model, it is once again important to look at the accessory proteins to your target to ensure that they are adequately expressed in your model.

Managing cell procurement

When in culture, cells can vary over time and the greater the number of passages the higher the potential drift. For this reason, it is important to carefully manage stock vials to ensure that at the time of an assay, cells are still at a passaging time in which they keep the same functional response characteristics. Preparing in advance stocks of frozen cells, ready to be thawed and used in the assay can be of great help in ensuring cell procurement consistency over time. This also allows working with transiently transfected cells; some frozen aliquots being used to validate the transfection process in advance of the screen[1]. Another thing to keep in mind is the possibility of cell line contamination, and therefore it is wise to confirm the cell line identity[2].

Cell culture conditions

Another important thing to be aware of is that cell culture conditions, like the type of medium and serum used, or even the lot number of serum used, can impact the cell phenotype. Different nutrient environment can lead to different levels of repertoire of protein expression, ultimately impacting the pharmacological response of the cells.

Cell culture media

Cells are grown in media specifically formulated to provide them with what they need to synthesize the proteins and polynucleotides needed for growth and replication. In addition to the nutrients within media, cells need two additional additives to thrive: serum and glutamine.

Typically, fetal bovine or horse serum are added to the media, providing cells with growth factors needed to inhibit apoptosis signals and stimulate growth and cell attachment. An increasing number of media is commercially available in which serum is replaced by recombinant growth factors, however serum is still the most widely used means of providing growing cells with these growth factors.

Serum, by acting on the protein translation machinery, will affect protein levels in cells. When considering this along with the concept that the level of expression can impact the pharmacology, it becomes obvious that culture conditions can have a great impact on the general response of a cellular system, and therefore needs to be carefully controlled to generate reproducible assays.

A common mistake often made when culturing cells is the omission of glutamine from culture media. Most culture media include glutamine in the standard formulation. However, as glutamine is not very stable, some culture media are provided without glutamine, and therefore glutamine needs to be added to the media before culturing the cells. Lack of glutamine leads to lack of cell replication and adherence to the plate, as glutamine is necessary for cells to start the KREBs cycle.

When to serum-starve

To analyze phosphorylation pathways via receptor/kinase stimulation or inhibition, it may be necessary to serum-starve cells. Doing so allows these pathways to be less active and reach a lower level of basal phosphorylation. By reducing the basal phosphorylation levels, it allows for stimulants to lead to a detectable increase of phosphorylation. Such serum starvation is critical for some pathways but not needed for others. It is also cell type dependent, and so should be optimized when establishing any new assay.

Quite often different culture media are used by different labs to culture the same cell line. Habit plays the biggest role in this as researchers most often use conditions that have worked in the hands of lab coworkers or in publications from trusted labs. As the environment impacts the phenotype of cells, it may be worth exploring the use of a different culture media than the one traditionally used in the lab. This practice may improve the cell's response in a particular assay.

Cell culture surfaces

Many cell types can be grown on "tissue-culture (TC) treated" plates. This surface is made by treating the plastic surface of the plate with a plasma to incorporate oxygen ions into the surface, resulting in a hydrophilic surface to which cells can adhere. While common, this approach results in a quite artificial growing environment, and it is not uncommon for some cell types to need to be able to create stronger contacts with the culture plate surface than a standard TC treatment would allow for. Therefore, plates can be coated with various synthetic or natural proteins such as Poly D-lysine (PDL), collagen or complex protein mixtures. Such coatings not only provide contact points, but also affect cellular phenotype by stimulating cell surface receptors for these proteins.

2d vs 3d cell culture

In their natural environment within the body, cells are surrounded by extracellular matrix and other cells. In the traditional 2D cell culture method cells are grown in a monolayer on a surface allowing for very few cell-to-cell contacts and creating direct cells' exposure to both the plate surface and the culture media.

In recent years, 3D cell culture is being used in more and more assays, either as spheroids or scaffolding to create a 3D cellular structure. Some models have gone so far as to control the flow of nutrients, CO_2 and oxygen, the evacuation of waste, and mixing different cell types to more closely mimic the true nature of tissues. Such systems lead to phenotypes and cellular responses that are much more predictive of the real behavior of cells in a complete organism and are therefore a better tool for identifying new potential therapeutic molecules.

While 3D systems may sound like the fool-proof way to go, there are limitations in both throughput and cost of such elaborate cell culture systems. Researchers are faced with the challenge of having to decide between what is desirable and what is technically and financially possible.

Designing your assay

When using radioligand and competition binding assays to analyze the binding of molecules to targets, isotherm curves are generated, and from these curves thermodynamic values such as the K_d (saturation binding) or K_i (competition binding) are extracted. These values are often considered as the "true" affinity of the molecule for the target.

When using functional assays, the extracted values looked at are typically EC_{50} or IC_{50} rather than K_d or K_i. These values tell us the concentration of the test molecule that will result in 50% of the maximal stimulatory (EC_{50}) or inhibitory (IC_{50}) effect. As chemists need some stable guidance to be able to decide if a given modification to a molecule under development is improving or degrading the quality of the molecule, there is a natural tendency to look at EC_{50} and IC_{50} values as if they were "absolute" values (like K_d and K_i values in radioligand and competition binding assays). This is, however, far from reality: EC_{50} or IC_{50} values only represent the concentration needed to get 50% of the maximal effect, on a given cell type, in given culture conditions, for a specific assay, run under specific assay conditions, and with a specific stimulation/ inhibition time.

In addition to keeping in mind the very relative values of EC_{50} and IC_{50} parameters obtained in functional assays, there are several other things to consider when designing your assay such as the kinetic factors, the level of amplification, and the dynamic range.

Traumatized cells need rest

When cells are seeded in multi-well assay plates, they will need some time not only to adhere but also to recover from the trauma caused by trypsinization. The trypsinization process is quite far from physiology, trypsin itself directly activates the Proteinase-activated receptors, shaves some of the proteins from the cell surface, and cells need time to re-synthesize proteins and for signaling pathways to calm down. Cell-to-cell contact also plays a great role in the control of signaling pathways and needs some time to re-establish. Sometimes keeping cells in culture for a few days before running the functional assay can be critical to recover a response close to physiology.

Kinetic factors

In most functional assays, you will need to decide when the data will be gathered to create the dose-response curves, and to determine EC_{50} and efficacy values. Whether you are performing a calcium flux assay, a kinase pathway analysis assay, or a cAMP accumulation assay, the EC_{50} and efficiency value can vary according to the exact time at which data will be recorded for the analysis. For this reason, it is important to be aware of the kinetic factors at play and determine the optimal one to be used in the assay. It is also important to note that care should be taken when comparing EC_{50} values from different sources, as different stimulation times may have been used even if not always reported.

Need for multiple assays

Different molecules can regulate the same target in very different ways, a phenomenon referred to by many different names, most often biased agonism and permissive antagonism or biased inhibition. Figure 1 illustrates typical differences that can be observed between different assay types. Once these considerations have been well understood and integrated, it becomes evident that one cannot be restricted to using a single assay type to estimate a molecule's potential ability to provide a solution to a medical need.

Level of amplification

It is important to keep in mind that there is some signal amplification when moving downstream within a pathway, as at each stage one stimulated molecule will speak to multiple downstream molecules. Figure 1 summarizes a theoretical example of what can be found when comparing a molecule activity in different assay types.

Dynamic range and the hooking effect

Dynamic range should always be taken into consideration when setting up an assay. The smaller the dynamic range, the lower the likelihood that unknown sample concentrations will fall within the standard curve.

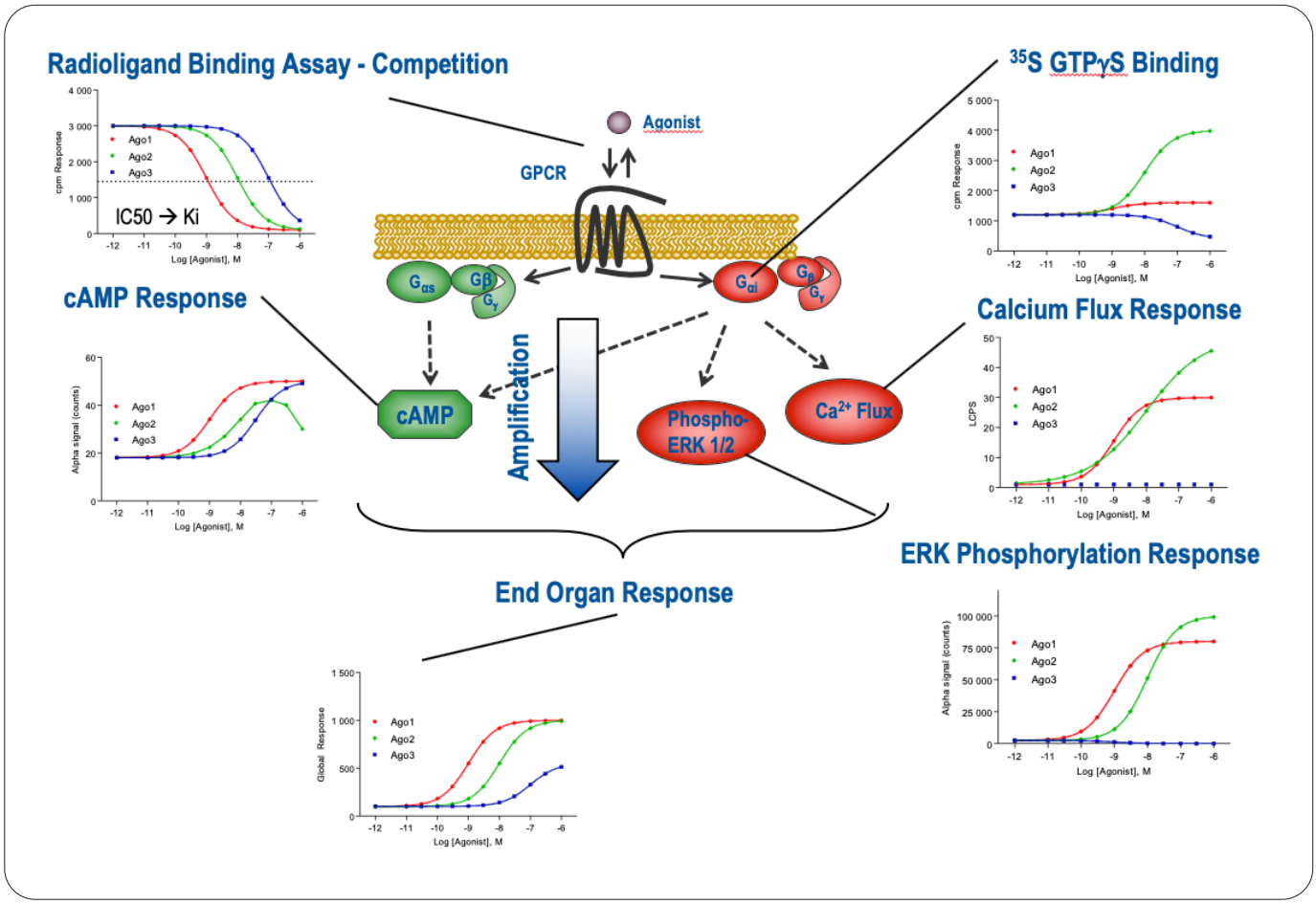


Figure 1: When analyzing the effect of the same molecule in different types of assays, its affinity/potency values (K_i for biochemical assays, EC_{50} for functional assays) and its efficacy values (partial or full agonist, inverse agonism) can be quite different in different assays, due to the different effects it may have on different pathways (biased agonism), and due to different levels of signaling amplification when going downstream the pathways. This is a theoretical example, forged from multiple cases met when interacting with researchers.

Furthermore, when using a homogenous detection system (i.e. and "no-wash" assay), there will be a point at which saturation will occur resulting in the "hooking" of curves at the highest analyte concentrations. When this point is reached, any additional analyte molecule added in the assay will bind separately to different antibody molecules, and prevent them from forming a sandwich, rather than enhancing the signal. This saturation leads to a signal decrease, and care must be taken to verify that the quantity of sample, and the concentration of samples worked with, is well located in the sensitive zone of the assay.

Understand the methodology

There is an increasing number of detection and quantification methods commercially available for analytes such as cAMP. While such options are certainly helpful, it is important that the user has a solid understanding of the method being used and its limitations. Figure 2 shows an example in which varying cAMP data is obtained as cell number fluctuates.

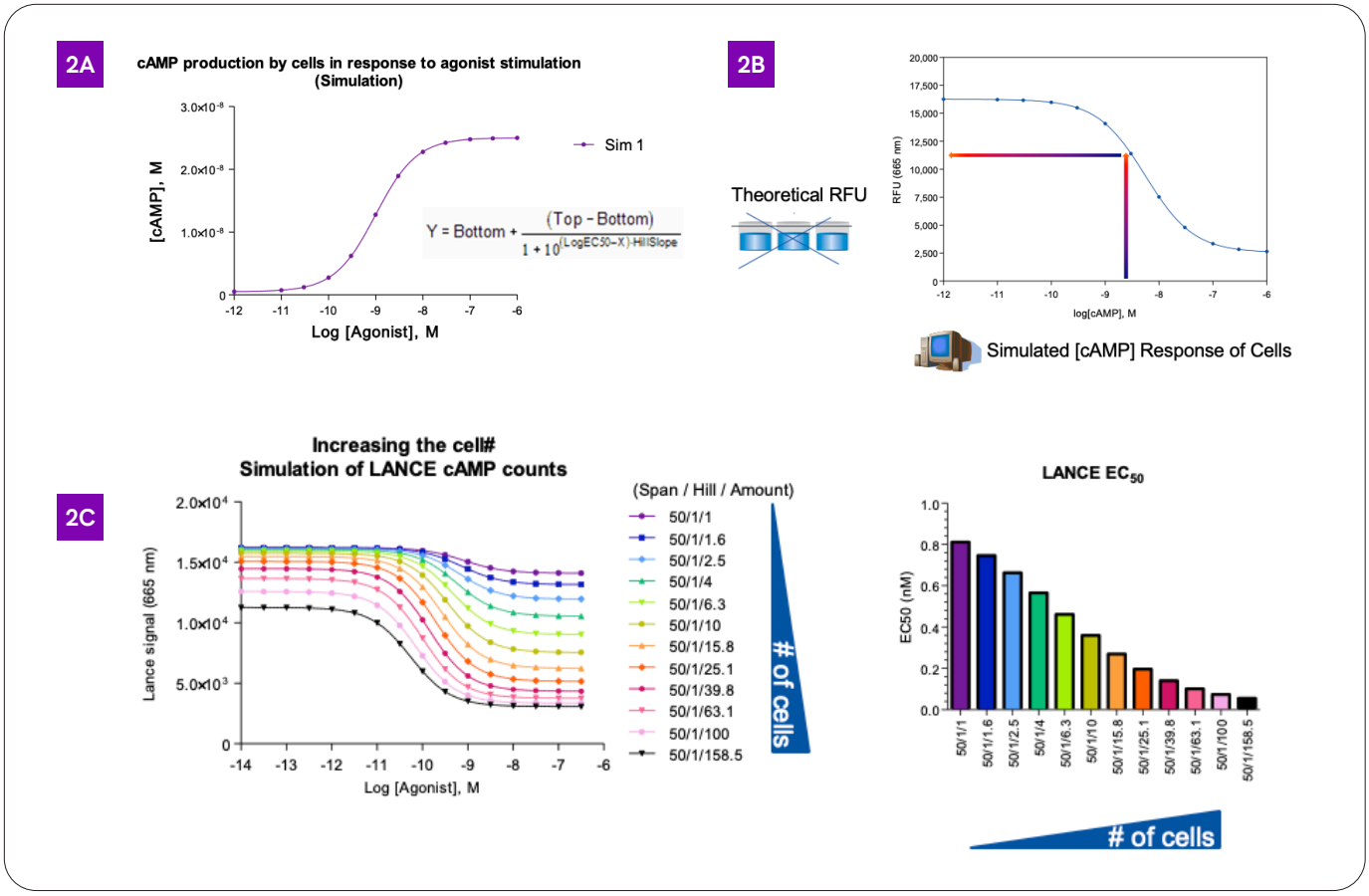
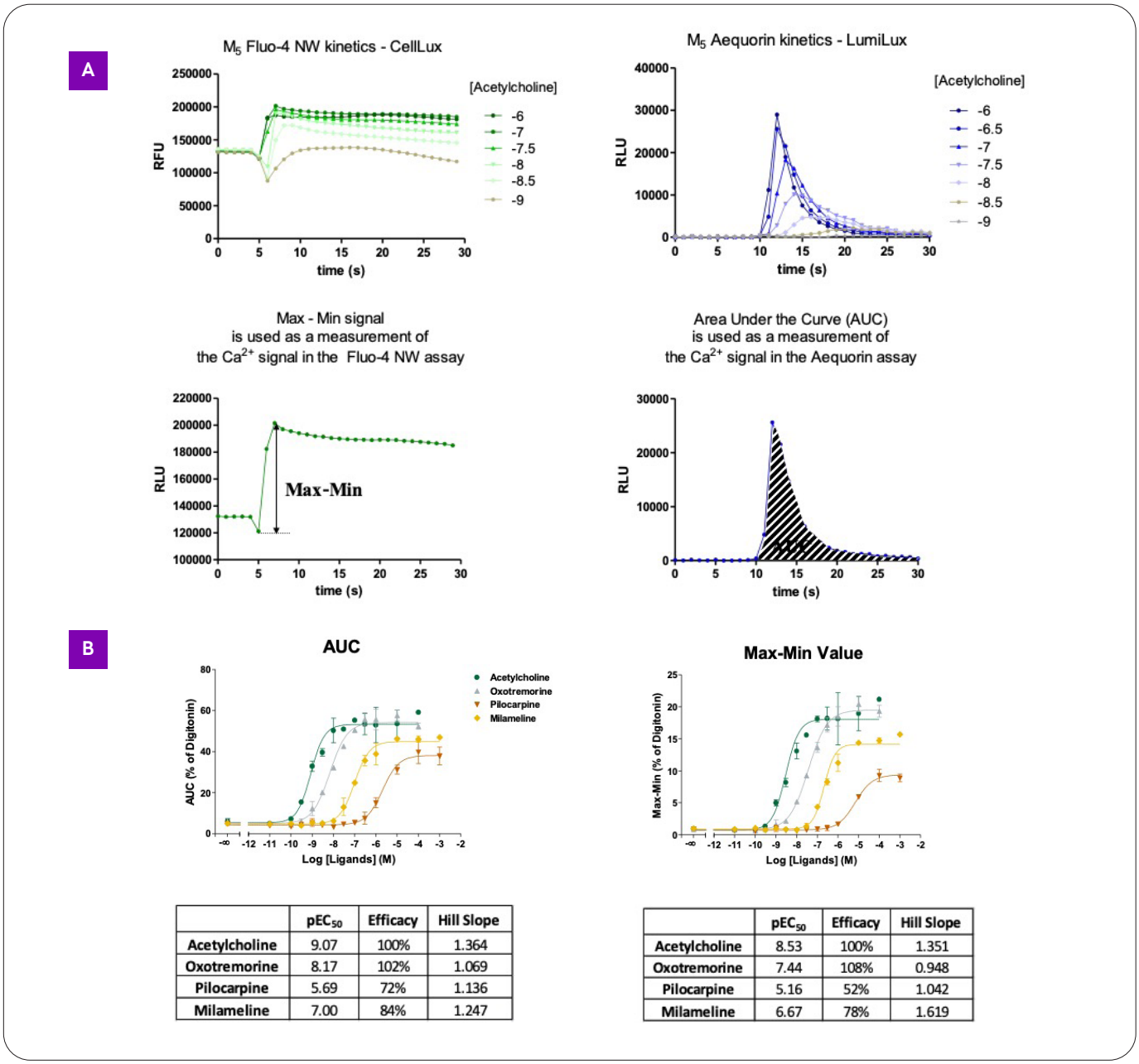


Figure 2: cAMP variations in stimulated cells. (A) The response of a cellular system to produce cAMP can be simulated using a 4-parameter logistic equation, leading to a sigmoidal dose-response curve. (B) Using the standard curve of the cAMP assay, it is possible to calculate what is the level of signal that would be expected for each of the cAMP concentrations obtained in A. (C) Such expected levels of signal can be calculated according to different scenario, for example if the cAMP concentration would be increased by 50-fold following agonist stimulation, with a hill slope of 1, and each of the curves shown here corresponds to a different amount of cells engaged per well. When not enough cells or when too many cells are engaged in the assay, some of the cAMP concentrations obtained from the cellular system do not fit well in the sensitive zone of the assay, resulting in a "flattening" of the end or of the start of the curve. If the EC_{50} values are calculated using the fluorescent signal, as many users do (even though we advise converting data to cAMP concentrations before doing this analysis), it can be seen (histogram graph on the right side) that the number of cells engaged in the assay will have an impact on the EC_{50} value obtained from the system. Any kind of assay suffers from the same limitations to some extent, and the user should better keep this in mind to perform a correct analysis and interpretation of the data.

Impact of data analysis method

You have your target, you have chosen your cell line, and determined which assay is best for you. The assay is run, data is collected, and you are finally done. Right? Wrong. Data analysis is just as important to consider as what we have already discussed. Even if all experimental conditions are the same, the way in which data are analyzed can impact the reported results. Figure 3 shows an example of calcium flux assays in which the analysis of data using the area under the curve or the peak value of the calcium wave signal leads to different levels of efficiency for partial agonists.



	pEC_{50}	Efficacy	Hill Slope
Acetylcholine	9.07	100%	1.364
Oxotremorine	8.17	102%	1.069
Pilocarpine	5.69	72%	1.136
Milameline	7.00	84%	1.247

	pEC_{50}	Efficacy	Hill Slope
Acetylcholine	8.53	100%	1.351
Oxotremorine	7.44	108%	0.948
Pilocarpine	5.16	52%	1.042
Milameline	6.67	78%	1.619

Figure 3: Different analysis methods can lead to different results. (A) Typical kinetic curves of the calcium flux response recorded with a fluorescent calcium dye (left) or with the luminescent reporter protein aequorin (right). Typically, the fluorescent signal is analyzed using the peak value (max minus min value), as the dye sequestrates calcium and leads to an artificially prolongated signal. And the luminescent signal is analyzed using the area under the curve value, as the reported protein will be consumed over time, which accelerates the relative signal decrease rate. (B) when analyzing the luminescent aequorin response of the muscarinic M4 receptor to different agonists, using either the area under the curve (left) or the peak value (right), the relative efficiency of agonist, as well as the EC_{50} values will be different depending on the analysis method used.

Conclusion

We have discussed the importance of many parameters for setting up, using and interpreting cell-based assays. By keeping in mind these important parameters for making decisions about how biological and detection systems should be chosen, used and how the data should be gathered and interpreted, you will be able to make better use of your resources and increase the probability of success, resulting in less project failures and increased profitability.

References

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