1 Considerations while setting up cell-based assays

Marian Kelley
Clinical Pharmacology & Experimental Medicine, 145 King of Prussia Road, Radnor, PA 19087, USA

1.1 Introduction

Cell based assays appear to be increasing in number and importance within the Pharmaceutical Development arena. There are innovative new platforms available, along with historical and established methods using cells as an integral part of the assay design. The data generated by these assays are being used to support such diverse endeavors as the characterization of an immune response in support of pharmacokinetics or phase IV safety, measures of cell activation, proliferation, and death, cell surface marker expression, and confirmation of useful biomarkers thereby contributing to decision-making in the Drug Developmental process.

Because both the assays themselves and the intended use of the data are so diverse it is difficult to standardize a single validation strategy. The stage of the development process, sample and data types, and how the data will be used, all influence what elements will be included in assay development and the validation plan. Generally speaking it is prudent to

Validation of Cell-Based Assays in the GLP Setting: A Practical Guide Editors, Uma Prabhakar and Marian Kelley

require the data used as the basis for scientific or clinical decision making to be both accurate and reliable. To most effectively accomplish this it is recommended to incorporate a general Good Laboratory Practice (GLP) format for performing a defined assay development program, which is used to generate a validation plan incorportating, *a priori* acceptance criteria.

This chapter will discuss the development and validation of cell-based assays including the lead in to development, basics that should be accomplished during development, execution of the validation plan and the final report.

1.2 Lead in to assay development

(A) Cells: The major requirement to develop a cell-based assay is the cells themselves. Cell function assays such as those investigating cytokine secretion, apoptosis and cell-surface marker modifications depend on a reliable source of cells, whether fresh or cryo-preserved, to make accurate and defensible conclusions. Cellular histology platforms also require a systematic and well-defined procedure for collection and preparation to ensure the consistent and reliable source of cells. When the source of cells is patient samples it is imperative that well thought out processes for collection, shipment and storage are implemented to ensure accurate and reliable data.

In some cases, such as proliferation and neutralizing antibody (NAB) assays, a cell line is used as the source of the assay read-out. Without a secure source of this cell line that can be expected to provide a consistent assay reagent the entire development and validation process is compromised.

A description of the cell line is essential and should include how it was developed, media and growth conditions, storage and recovery. References are useful if available. A full description of the cell banking process is helpful. When using cell-lines for a cell-based assay special care must be taken to preserve the cells by creating a Master Bank. Cell Banking is performed to preserve the characteristics of the cell line to be used. It is recommended that the cell-banking program be implemented as early as possible in the life cycle of assay development. Often it is not known how many passages a cell line can withstand before drift occurs. Cell banking also offers insurance that the cell line can be re-established in the event of a catastrophe like microbial contamination, cross contamination with other cell lines, or loss of desired characteristics.

As soon as a cell line is introduced into the lab an initial Master Bank should be frozen. The number of ampoules will be dependent on how quickly the cells multiply but at least 3–5 ampoules should be frozen within the first week. Initial evaluation of the cells should be as complete as possible, but at least examine sterility (mycoplasma, fungi, etc) growing

conditions, viability, and the ability to be frozen and recovered from liquid nitrogen. Once convinced of the cell line's integrity, the main Master Bank should be prepared by thawing an ampoule from the initial Bank. Since it is preferable to expand these cells to a high concentration with as few passages as possible, while maintaining high viability, it may be advisable to thaw several of the ampoules, if the inventory of the initial Master Bank allows it. The number of vials contained in the Master Bank is dependent of the life expectancy of the assay. It is always prudent to bank more than the expected requirement, even if the Master Bank must consist of several different and increasing passage numbers due to the slow growth of cells. Once established, the Master Bank is used as the supply for the Working Stock. Early in the process while the working stock is in culture the number of passages should be monitored closely and tested at intervals to determine that its integrity is being maintained. Well before the Master Bank is depleted a sequential Master Bank should be prepared, if necessary.

Tests critical to the determination of the continued integrity of the cell lines' required characteristics should be conducted thoughtout the cells' expansion to assess the optimum permissible passage number.

If the cells once thawed lose viability some rescue methods may be employed. Dead cells can be removed by centrifugation or other method. Cells can be nursed to higher viability by expanding in smaller culture volumes/ culture plates. Higher concentrations of sera, if used, or other growth supplements, may encourage growth. Be aware that such rescue methods could encourage the growth of a variant cell line and further re-characterization would have to be performed.

(B) Assay format: The type of assay to be used will define the development process and validation needed. The data generated by the assay may be quantitative and consist of a continuous numerical value, such as data reported from a regression of a standard curve. Proliferation assays frequently are reported based on this format. Other qualitative formats allow for a discrete or descriptive, numeric-reporting format, where the data is spaced across the axis or used a descriptive, non-numeric term (e.g., high or low; yes or no). Of course, intrinsic to the assay format is the sensitivity requirement. This must be determined at the initiation of development, based on the intended use to confirm that the platform selected and data reporting will afford the sensitivity to meet the needs of the study.

(C) Critical Reagents: It is important to identify which reagents are critical to the assay method so that their qualification, sourcing, and lot-to-lot acceptance criteria can be established up-front. Additional assessments conducted during this phase include read-out signal (color intensity, MTT etc) incubation times, reagent concentrations etc.

1.3 Assay development

The output of the development lead in described above is a high level plan forward. The development phase is typically the most intensive and results in a defined method that enters the validation stage.

Cell-based assays differ from ligand-binding methods and can be characterized based on the type of assay format or platform. They also differ significantly from each other since they may consist of a single "layer" or be multi-layered. The simplest example is a one-layered cell-based assay. This type describes the immunohistological slide platform, or the cells line with single stimulus, e.g., a cytokine's effect on a cell-line, which elicits an expected response. An example of a one-layered cell-based assay uses the agonist cytokine where a dependent cell line proliferates in a dose-dependent manner to the addition of increasing amounts of the cytokine.

Some assays developed to detect neutralizing antibody build upon the one-layered assay by adding an inhibitory facet to the proliferation assay mentioned above. An example of a two-layered cell-based assay is the assay to detect antibodies to a cytokine therapeutic. In this case the method would include the cell line, its optimized stimulatory element followed by a serial dilution of an expected inhibitory element such as patient sera or spiked quality control samples containing antibody to the therapeutic protein.

When antagonists are being developed as a biologic therapeutic, frequently the complementary neutralizing cell-based assay must be developed and is composed of three layers. This is the case for some monoclonal antibody (MAB) therapeutics since the action of the antagonist monoclonal is to inhibit the action of a stimulus (the MAB- related agonist) on the cells. Assays developed to detect neutralizing antibodies to the MAB would add an additional layer to the basic cell-based assay. The "normal" process of a responsive cell type responding to the target of the MAB would be inhibited by the addition of an optimized amount of therapeutic drug. The cellular response to the target is salvaged by the addition of samples containing varying concentrations of antibodies to the monoclonal therapeutic (see figure one: three-layered assay).

Assays using cells that constitutively produce a cytokine, for example, may be referred to as four-layer assays since the basal concentration of cytokine (1) is another parameter that would need to be monitored during validation and sample analysis. In this instance the basal concentration of cytokine may be enhanced (2) with the addition of a specific cytokine and the therapeutic drug would reverse (3) that increase. Detection of neutralizing antibodies (4) to the therapeutic drug comprises the fourth layer (see figure two: four-layered assay). Specific monitoring for each step is necessary to assure the consistent behavior of the method.

The "formula" for optimizing a cell-based assay method is guided by the number of layers attributed to the method. All the layers leading up to the

final "read-out" must themselves be optimized sequentially and in a way that permits the final "read-out" to be useful. The final "read-out" must be a dose-dependent response (quantitative or qualitative) that is attributable to and can characterize the test in question. The requirements for the optimization of the layers are likely similar to each other. The cell number, concentration of agonistic therapeutic (layer 1), then concentration of inhibitory factor (layers 2 or 4), or concentrations of antagonistic therapeutic (layer 3) must be tested in a dose-dependent manner to select the optimal dose (concentration) to be used in the final format. It is insightful to understand that the more layers an assay contains the more complicated the selection of the optimal concentration for each layer. The concentration that produces the highest response is frequently not the best choice. For instance, in cases where sensitive neutralizing antibody detection is needed, the aim is to detect low concentrations of antibody. Adding in very high concentrations of drug to be neutralized skews the assay to require a high antibody response rate. To be able to detect low antibody response rates, the method developer will need to balance the added drug to be neutralized by the lower apparent antibody present together with an acceptable response range.

Other parameters that are optimized during development but are independent of the layering aspect of the assay method include the cells themselves, i.e., cell passage, viability, sensitivity in the presence of subject sera, response variability etc.

All the elements up to now have been performed in development. The final assay method now becomes the focus of the validation stage. By compartmentalizing the development in this fashion, the validation experiments may become focused on documenting a reliable, robust and reproducible assay.

1.4 Sample handling

Special attention must be paid to how the samples targeted for analysis in cell-based assays are collected, processed, stored, shipped, or frozen. Since each of these conditions is dependent on the platform to be used, the specifics of sample handling are best presented in the context of the particular assays described in this manual. A description of the investigation into appropriate sample conditions are documented the validation report.

1.5 Validation plan and conduct

Validation is typically preceded by a validation plan, which summarizes *a priori*, the performance parameters to be tested. The extent of the validation and the acceptance criteria are dependent on several factors, among them,

the needs of the study, the nature of the methodology, and the observed variability (Lee et al, 2006). Generally, the stringency of the validation parameters should correlate to the drug development stage where the assay is to be used. Less rigor would be expected for assay supporting Drug Discovery or early development. In most of the cases in this manual the focus is on clinical samples support of late stage clinical studies. Therefore, in following the stage-appropriate validation a more inclusive validation would be expected.

Once the validation is initiated experiments are expected to proceed uninterrupted and the experimentation documentation should reflect that. Analysts must be alert to cases when the assay method fails. One failure is likely not a cause for concern; however, there should be a plan for when failures do become a cause for concern and an investigation into the cause is required. At this point it should be clear in the documentation that the analyst has moved out of validation and back into development or failure investigation. Once the issue is resolved, a determination is made whether the resolution had a minor or major impact on the validation. If minor, the documentation should reflect a return to the on-going validation. If major, note that the original validation failed and a new validation must be implemented. After completion of the described experiments a validation report is required that captures the performance of the assay and any deviations from the described assay method or validation plan.

The validation plan may include:

- Introduction including purpose of the assay
- Background information
- Description of the assay and critical reagents
- Description of validation experiments
- Target criteria for the validation parameters to be included
- Positive and negative controls for each layer of the assay are needed to monitor the assay robustness
- Analysts conducting the validation
- Data handling technique
- Notebook and raw data references for assay development
- Archival location
- Management approval

Validation experiments

Controls

- Positive and negative controls for the cell-based assay method are used to monitor the robustness of the underlying assay and accept a run.

- Validation controls are used to assess the parameters of the assay to support the claim of validated method. The validation controls should reflect the intended samples, typically in human or animal serum.
  - Intra- and inter-assay precision including between runs, days and operators
    - Validation controls prepared using unique donors are assayed multiple times in a run, and over several days, conducted by several analysts to assess the precision of the replicate controls. The positive and negative controls used to monitor each layer of a cell-based assay may also be assessed to document the overall precision of the assay.
  - Assay cut-off to determine sensitivity or the difference between an positive and a negative sample
    - It is recommended employing as many unique donors (e.g., animal, normal human or target disease populations) as possible and in several assays to determine the appropriate cut-off. Adding two standard deviations to the mean read-out provides a false positive rate of about 5%, which ensures an acceptably sensitive assay.
    - Sensitivity may also be determined empirically by spiking quality control at a high concentration and titrating in several assays. The sensitivity is the lowest titer (or concentration) of the quality control (QC) that produces a value with acceptable precision. This experiment may also establish dilutional linearity of the sample.
  - Assay range and limits of quantification, if relevant, including the lower and upper limits (LLOQ and ULOQ)
    - When an assay is quantifiable, the standard curve range and upper and lower limits using spiked controls are assessed. Every run employed for the validation that includes the standard curve and independently prepared quality control samples should be compiled in two tables to document the overall performance of the curve and the controls during the validation.
  - Specificity and Selectivity
    - These parameters are closely related and are assessed to verify that the assay is specific for the intended use (will not tag a closely related but unintended target) and can preferentially select the intended target from a complicated milieu. While assay cut-off experiments are conducted in unspiked target matrix, these experiments employ multiple spiked matrices.

- The impact of drug interferences can also be assessed during the investigation into specificity.
- Specificity of the cell line, if applicable, is a parameter unique to the cell-based assay platform. Especially when the method makes claims of responding specifically to a cytokine or other stimulus this claim must be supported by testing the cells in the presence of factors found in a relevant matrix.

- Robustness
  - To understand the inherent reproducibility of the method, the impact of typical changes and varied conditions that can occur during sample analysis is assessed. The conditions tested depend on the assay format, and can include such parameters as incubation times and temperatures, cryopreservation and histology techniques, matrices etc.

- Stability
  - Cell-based assays, as described in this manual, have very specific requirements depending on the platform used. In all cases some investigation into the stability of the target in the milieu selected, (e.g., whole blood, peripheral blood mononuclear cells, tissue samples for histology, serum etc) must be conducted to assure the validity of the data reported.
  - Some references are also made to stability of response. To understand and anticipate the variability expected, some investigation should be conducted on the stability of the target response, e.g., cell surface expression on tissues on fresh, shipped, frozen/thawed and preserved samples, etc.

1.6 Validation report

Once completed and the experiments conducted to establish the validity of the assay are found acceptable it is necessary to write a report to document the assay validation. As a suggestion, a validation report typically contains an introduction and history of the assay to date. Also important to include are dates of the conduct of the validation, references to the analysts involved and the raw data notebooks to support assay reconstruction and where they are archived, a description of the experimental investigation and tables supporting the validation, any deviations made to the original validation plan. The report should be signed by the author and management and centrally archived for easy retrieval.

1.7 Conclusion

When data generated using cell-based assays are used as the basis for scientific and clinical decision making, investigators must apply and rigorously monitor appropriate controls for all "layers" of the assay to ensure its continued validity. Additionally, assay validation is a dynamic process. It is expected that questions will arise over the time the assay is being employed, for instance, as new disease states are being investigated. By applying the concepts of GLPs cited here the resulting assay should be a well-developed and well-documented method validation.

Reference

Lee JW, Devanarayan V, Barrett YC, Weiner R et al (2006). Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm Res*, **23**(2): 312–328.