# Method Development Strategies for Automation of Ligand Binding Assays

M. Montjovent, H. Williams, A. Markus, M. Groeschl, H. Faust, S. Wood and P. Struwe
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

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Applied Translational Medicine

## Introduction

Regulated laboratories proposing Ligand Binding Assays (LBAs) in support of biotherapeutics development face increasing demand to support projects with improved quality and efficiency.

In comparison to small molecule analysis, large molecule bioanalysis encounters many unique challenges:

- Large numbers of study samples to be analyzed: screening/confirmatory/ titer assays for quasi-quantitative ADA assays or long half-life large molecules such as mAb PK assays
- Limited dynamic range of calibration curves
- Standard format used: 96-well plates, duplicate analysis

Automated assays cover a wide range of applications such as:

- Enzymatic Immunoassays (EIA)
- Electrochemiluminescence Assays (ECLA)
- Radioimmuno Assays (RIA)

Benefits of automation include improvement in:

- Throughput
- Traceability
- Reliability
- Reproducibility
- Robustness

However, one of the greatest challenges of moving towards an automated method is stability. Even with dedicated on-board incubators, washers and readers the high throughput of samples can lead to delays in analytical steps, which all need to be stress-tested and refined.

The work presented here describes:

- Strategies used for automation of LBAs in Celerion's facility
- Examples of stability assessments made with respect to automation
- A stability comparison of manual and automated methods in terms of precision (case study)

## Method

### Strategies for Automation of LBAs

Strategies adopted at Celerion Switzerland AG for the development of automated LBAs comprise the following key steps (Figure 1):

- Definition (aims, resources, time schedule)
- Establishment of the manual method
- Program development
- Qualification
- Validation
- Archiving

After a planning phase, methods are first developed manually with automation in mind. Various incubation conditions are tested to identify critical steps and assay bottlenecks are mitigated by developing robust methods with standardized processes which utilize reagents with proven stability. Lastly, the archiving of validated automated processes allows development of similar assays with optimized conditions. Transfer of existing manual methods to automation is also proposed and is illustrated hereafter as a case study.

**Figure 1. Strategy for LBA Method Automation.**
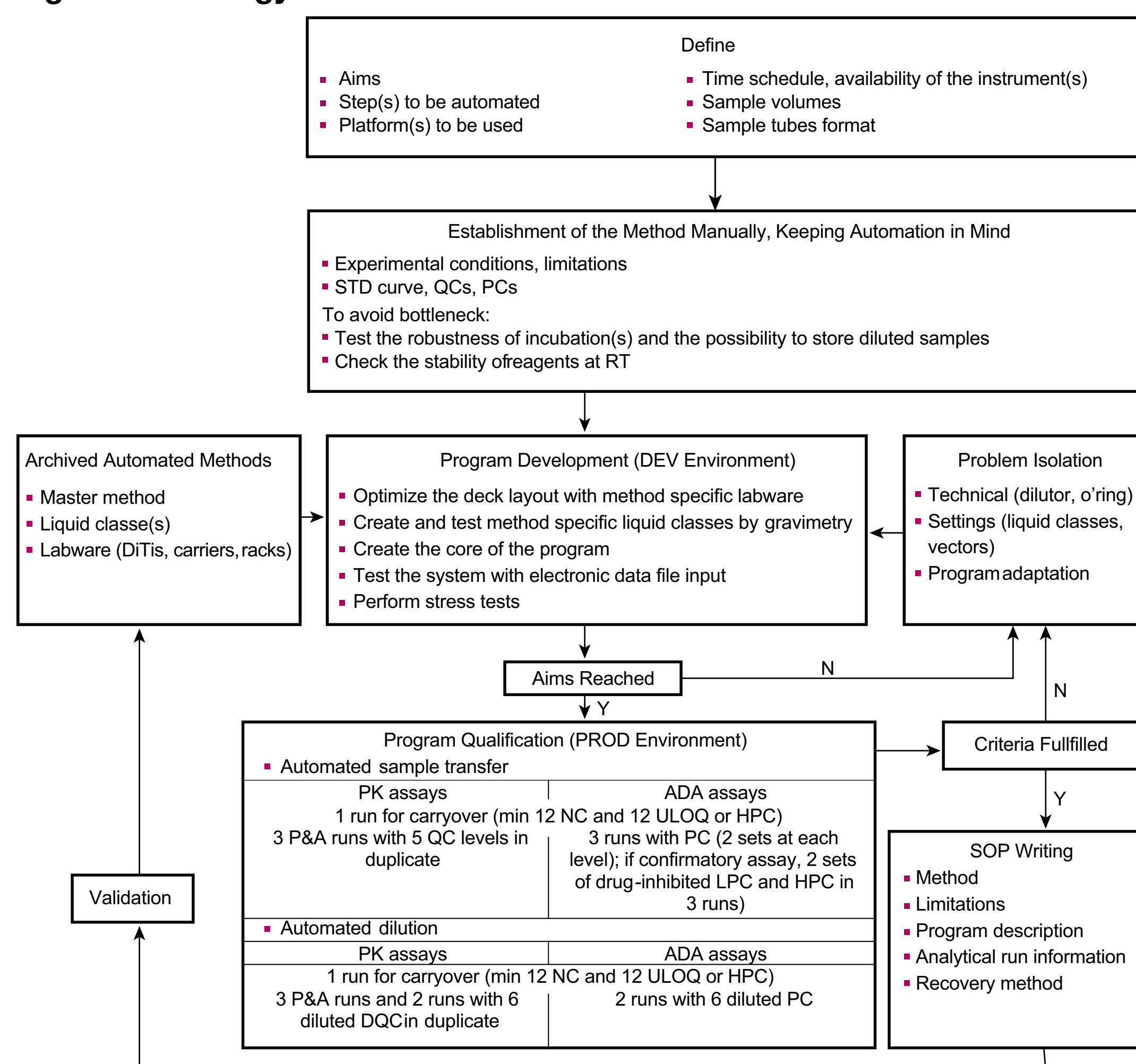


### Stability Assessments for Automation - 3,3',5,5'-tetramethylbenzidine (TMB) Substrates (EIA)

For the development of automated EIA, different commercially available TMB substrates were compared with our in-house preparation. Criteria of comparison were:

- Kinetic curves
- Stability

Kinetics were assessed at two concentrations of coated HRP-conjugated antibody to mimic high and low positive samples (0.02 and 0.008 µg/mL, Figure 2A and 2B, respectively). Stability was evaluated by exposing TMB substrates on the benchtop of an automated platform before incubation on the assay plates coated with HRP-conjugated antibody ($t_0$ and 5 hr benchtop exposition, Figure 3A and 3B, respectively).

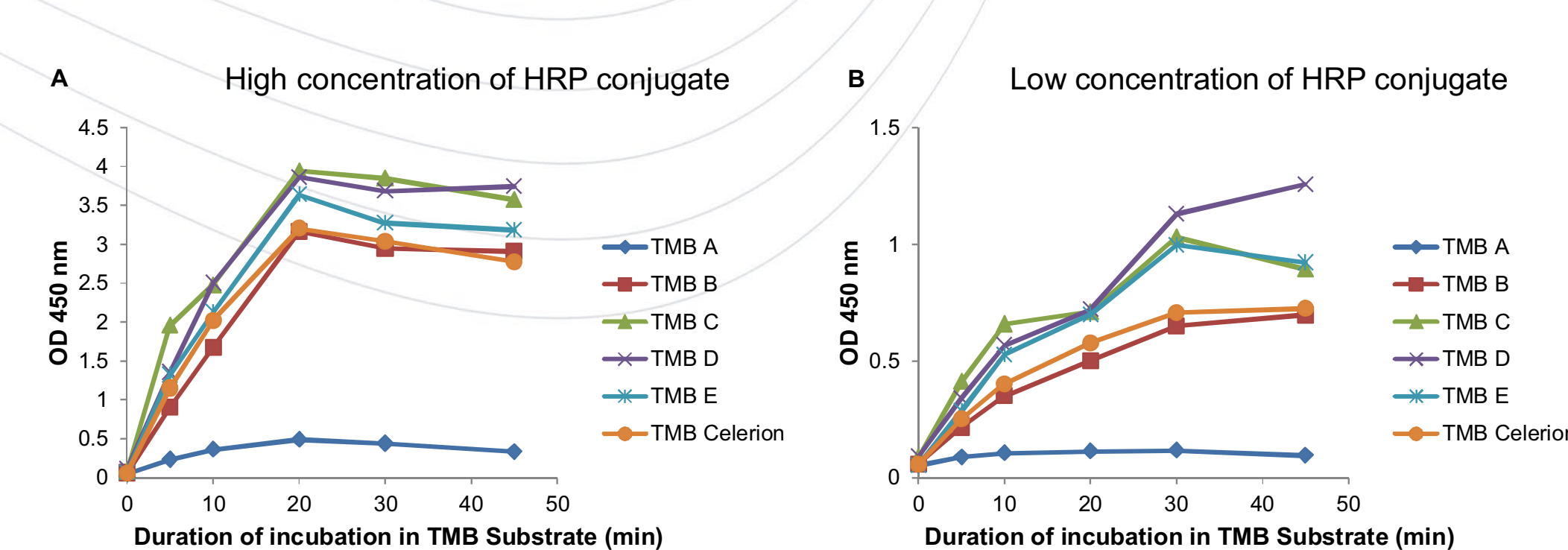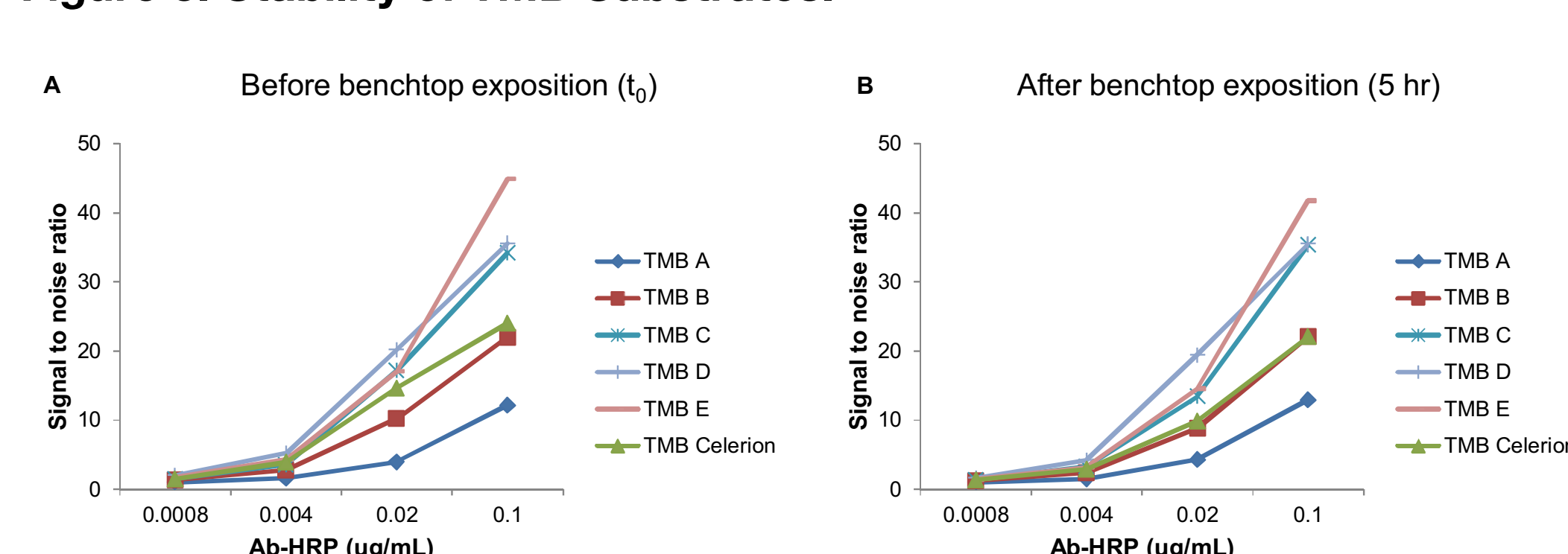**Figure 2. Comparison of Kinetic Curves in TMB Substrates.**



**Figure 3. Stability of TMB Substrates.**



Results are expressed as signal-to-noise values obtained after 12 min incubation with different concentrations of HRP-conjugated antibody. Incubation was extended to 30 min with TMB A due to the slow development observed.

The observations demonstrated:

- Commercially available preparations with slow or fast development have limitations of use when targeting wide analysis ranges
- TMB enzymatic reaction is almost completed after 30 min
- To avoid signal saturation, fine titration of capture/detection reagents should be preferred to TMB substrate dilution

Further investigations showed that the TMB substrate developed at Celerion could be stored up to 12 hr at RT and light protected (data not shown). Moreover, the kinetic of signal development observed with our preparation is suitable for wide analysis ranges when associated to optimized capture/detection reagent concentrations as well as conditions of incubations.

### Stability Assessments for Automation - Immune Complexes in Read Buffer (ECLA)

A key challenge encountered when developing high throughput ECLA methods is the stability of immune complexes in Read Buffer. Captured complexes lead to light emission when voltage is applied.

With a SECTOR® Imager 2400 (Meso Scale Discovery), the stepwise reading of the 24 sectors takes around 2 min per plate (Figure 4).

**Figure 4. Reading Sequence with SECTOR Imager 2400.**
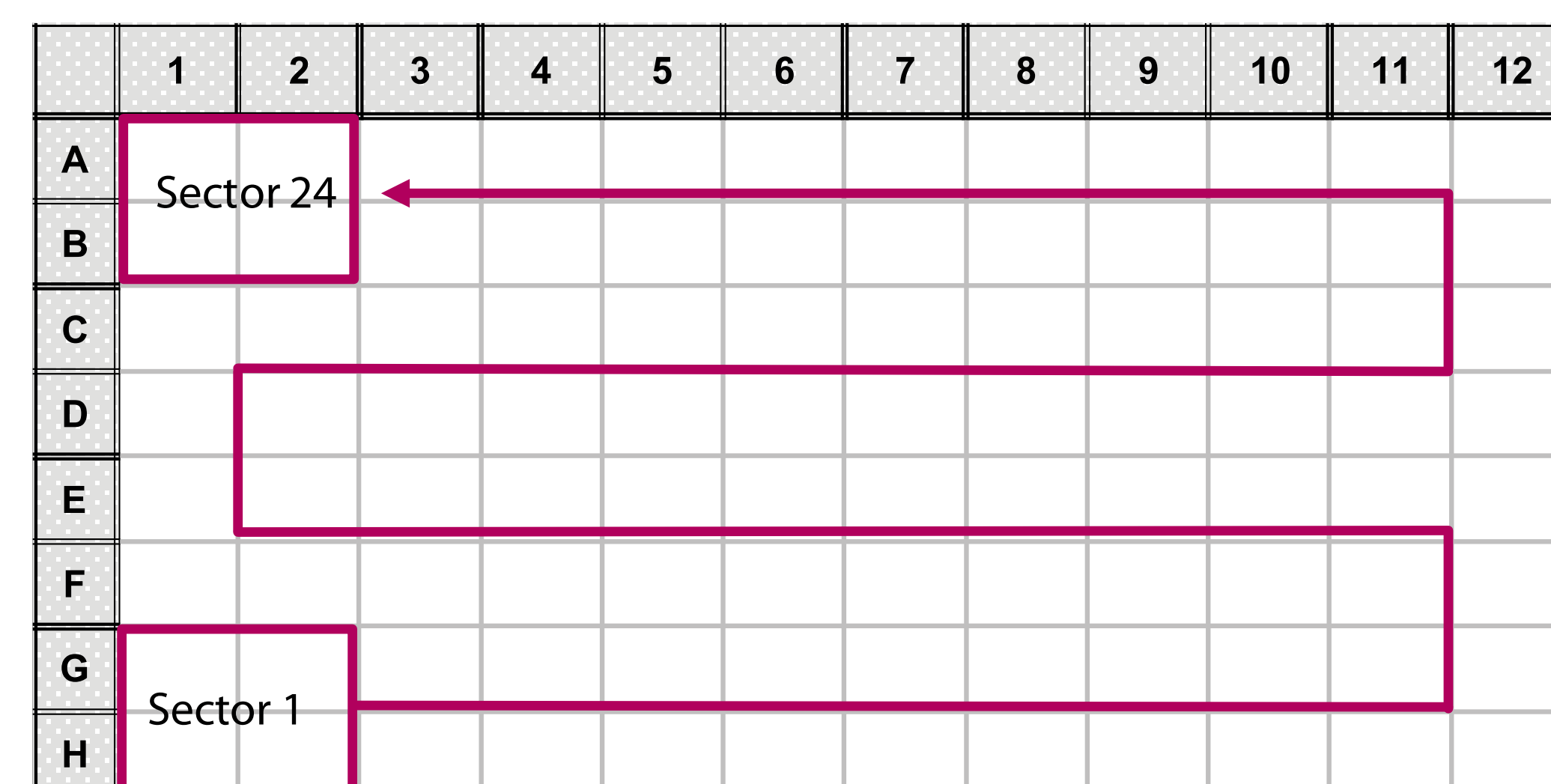


In case of instability, immune complexes dissociate quickly, leading to a signal gradient observed from the left to the right side of the plate (Figure 5A). To face this issue, optimized protocols are developed to assure long term stability of immune complexes (Figure 5B).

**Figure 5. Stability Assessment of Immune Complexes in Read Buffer.**
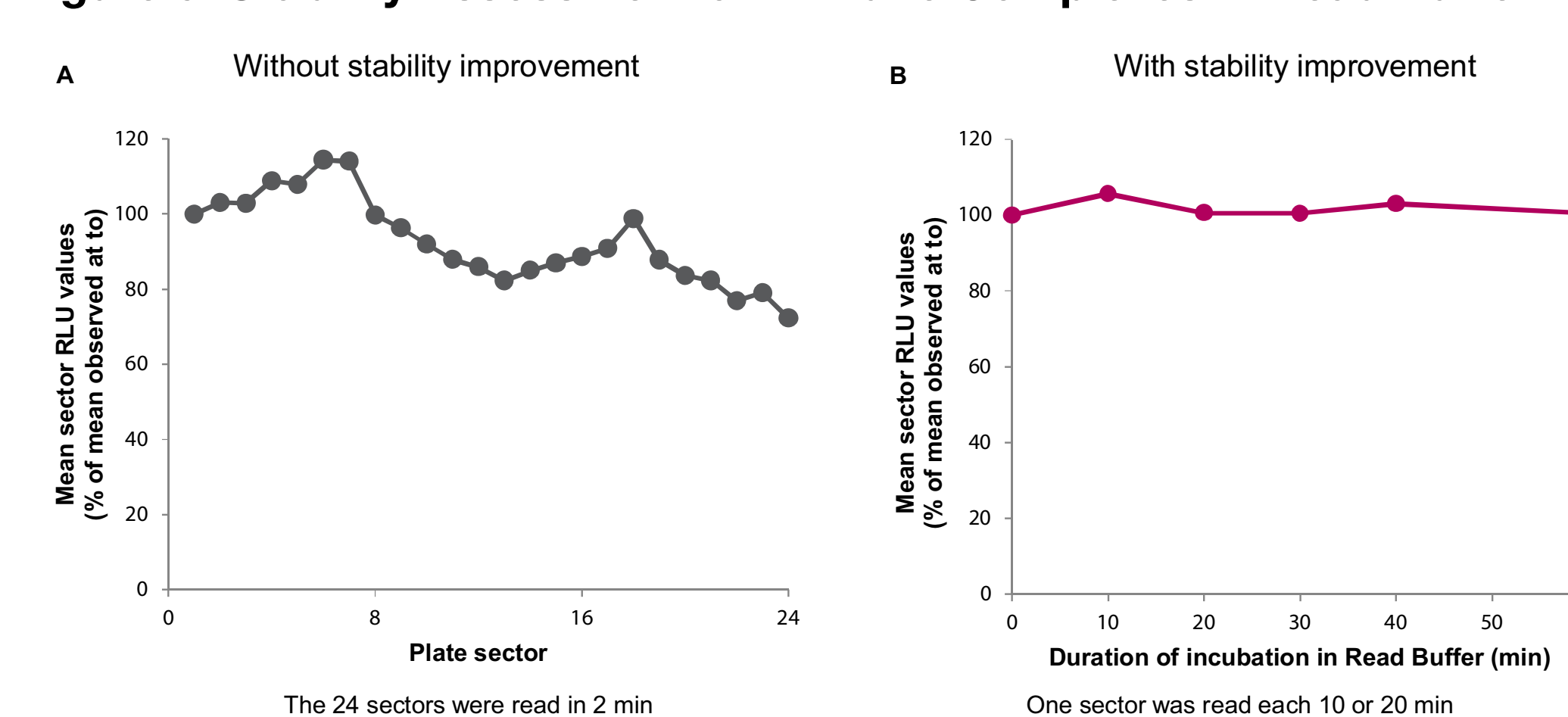


Stability improvement showed positive impacts on:

- Smooth scheduling without bottleneck
- Data reliability
- Plate homogeneity
- Easiest selection of plate lots

### Case Study: Comparison of Manual vs Automated Methods (ELISA)

A newly automated method for an existing manual immunogenicity assay had been developed which introduced an analytical delay of up to 3 hr from the time samples were introduced to the coated plate until they were incubated with detection reagent. This delay needed to be assessed and the stability of the samples on the coated plate needed to be confirmed, as compared to the existing, faster but less efficient manual method.

Control samples were used for the comparison. However, as assay response varies from run to run direct comparison of the OD response of control samples was inappropriate. Instead, the response of each control sample was normalized against the run-specific cut-point (rCP) and the mean values from a number of runs were compared. This comparison was repeated for each of the assay steps, screening, confirmatory and titer. An acceptance criteria of +/-30% mean bias between normalized results was set (Table 1).

**Table 1. Comparison of Manual vs Automated Liquid Handling for Immunogenicity.**

| | Manual Screening | | | Automated Screening | | |
|---|---|---|---|---|---|---|
| | LPC/rCP | HPC/rCP | LPC/HPC | LPC/rCP | HPC/rCP | LPC/HPC |
| Mean | 2.88 | 4.87 | 0.59 | 3.10 | 5.77 | 0.54 |
| SD | 0.536 | 1.368 | 0.113 | 0.268 | 0.563 | 0.017 |
| %CV | 18.6 | 28.1 | 18.3 | 8.6 | 9.8 | 3.1 |
| n | 26 | 26 | 26 | 24 | 24 | 24 |
| %Bias | | | | 7.7 | 18.4 | |

| | Manual Confirmatory | | | Automated Confirmatory | | |
|---|---|---|---|---|---|---|
| | LPC/rCP | HPC/rCP | LPC/HPC | LPC/rCP | HPC/rCP | LPC/HPC |
| Mean | 2.97 | 5.50 | 0.54 | 2.34 | 4.29 | 0.54 |
| SD | 0.506 | 0.822 | 0.067 | 0.258 | 0.418 | 0.031 |
| %CV | 17.0 | 15.0 | 12.3 | 11.1 | 9.7 | 5.7 |
| n | 55 | 55 | 55 | 60 | 60 | 60 |
| %Bias | | | | -21.4 | -22.0 | |

| | Manual Titer | | | Automated Titer | | |
|---|---|---|---|---|---|---|
| | LPC/rCP | HPC/rCP | LPC/HPC | LPC/rCP | HPC/rCP | LPC/HPC |
| Mean | 3.20 | 6.14 | 0.52 | 2.57 | 4.86 | 0.53 |
| SD | 0.415 | 0.686 | 0.036 | 0.242 | 0.441 | 0.028 |
| %CV | 13.0 | 11.2 | 7.0 | 9.4 | 9.1 | 5.4 |
| n | 146 | 146 | 146 | 31 | 32 | 31 |
| %Bias | | | | -19.8 | -20.9 | |

In all cases the acceptance criteria was met, showing that the response from the automated method was comparable to the manual method, and that stability of the samples and the coated plates had not been affected. Additionally, the %CV of the normalized responses showed a marked increase in precision for the automated method. The ratio between the low and high positive controls also gave a more consistent result with significantly lower %CV values, showing there was less variation between low and high control responses per plate.

## Discussion and Conclusion

In the presented work, a strategy to develop new ligand binding assays for automation was discussed as well as practical assessments regarding stability. A comparison of quasi-quantitative data obtained in an immunogenicity assay with manual and automated methods was used to illustrate the advantages of automation.

The conclusions are:

- Benefits of automation require well-defined strategies to develop new methods
- Smooth transition from method development to validation is assured when automation is clearly in perspective
- Stability of reagents as well as method robustness are critical parameters to assess in the perspective of automation
- For immunogenicity assays, the comparison of response ratio offers a suitable tool for manual vs automated method evaluation

## References

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