mirrorball®
application note

development of a homogenous RSV neutralisation assay on the mirrorball® fluorescence cytometer

introduction

Human respiratory syncytial virus (RSV) is a syncytial virus that can cause serious respiratory tract infections, especially in infants and young children[1]. To date there is no licensed vaccine against RSV infection and the only preventative treatment option is the neutralising anitbody palivizumab, which is given to high risk infants by passive administration[2]. Therefore there is a strong drive to identify novel RSV-neutralising antibodies for therapeutic/ preventative applications.

Here we present the development and implementation of a simple RSV-neutralisation assay on TTP Labtech's mirrorball fluorescence cytometer. Compared to established virus neutralisation assays, this method offers several distinct advantages:

- homogenous, no-wash protocol captures data from adherent and detached cells
- cells and virus remain safely contained within the assay plate with mirrorball's laser scanning approach
- compatible with high-density 96-, 384- and 1536-well plates
- whole-well scanning delivers robust data for uneven cell distribution
- assay readout normalised to total cell count
- mirrorball's *in situ* image-based read preserves cell morphology, highlighting the fused-cell phenotype of infected cells

materials and methods

materials

- HEp-2 cells (2000 cells/20 µL, single cell suspension)
- RSV virus (500 pfu/10 µL)
- neutralising mAB
- cell culture medium
- 384-well assay plate (Corning cat# 3712)
- detection antibody (motavizumab, conjugated to AF488)

methods

day 1

- prepare serial dilutions of the neutralising mAB in cell culture medium
- to each well of the assay plate add 10 µL of RSV virus and 10 µL of the neutralising antibody
- incubate plate for 1 hour at 37°C
- add 20 µL of the HEp-2 cell suspension to plate and incubate for 4 days to allow the infection to proceed

day 4

- add 10 µL of motavizumab-AF488 detection antibody (4 µg/mL, final concentration = 0.8 µg/mL) and Hoechst (2x) to the plate and incubate overnight to stain

day 5

- read the plate on the mirrorball instrument

key benefits

the purpose-built system design of TTP Labtech's mirrorball ensures reliability for

- **no-wash protocol**
- **cells and virus remain safely contained within the assay plate**
- **compatible with high-density assay plates**
- **whole-well scanning delivers robust data for uneven cell distribution**
- **assay readout normalised to total cell count**
- **mirrorball's in situ image-based read preserves cell morphology**

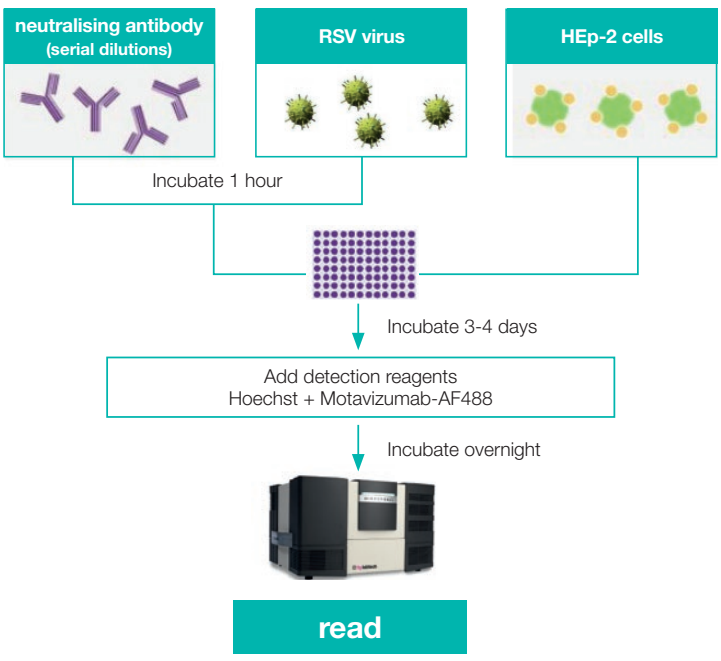

Fig 1. Method outline for the RSV neutralisation assay

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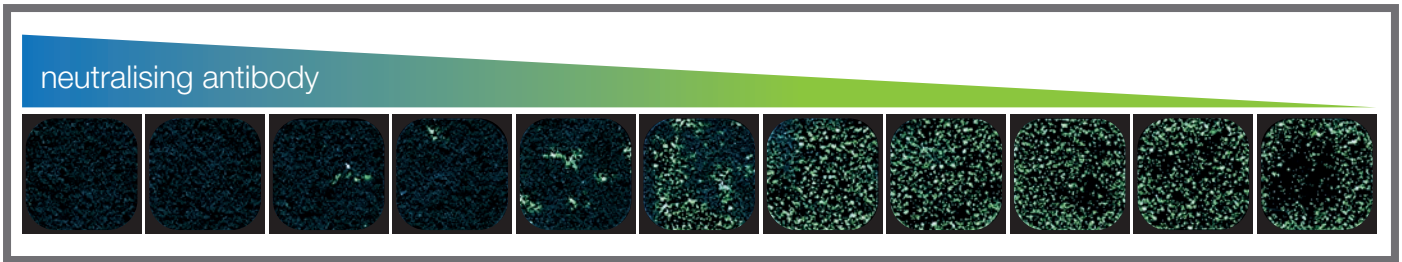

Fig 2. Whole-well fluorescence images from mirrorball; FL-1 (Hoechst, blue) and FL-2 (motavizumab AF488, green)

results

The neutralisation of RSV before exposure to HEp-2 cells was assessed by a homogenous immunofluorescence assay in 384-well plates. Whole-well images (Fig 2) immediately highlight that the number of RSV-infected green fluorescent cells increases with decreasing concentration of the neutralising antibody. This increase is accompanied by a change in cell morphology: whereas the non-infected cells grow as a disperse monolayer with distinct nuclei for each cell, the infected cells show clear signs of multinucleation (syncytia formation), one of the hallmarks of RSV infection[3].

Several readouts may be used to quantify RSV infection.

By considering the total fluorescence intensity of cells in the green channel (FL-2), the total number of infected cells can be estimated.

The concentration-response curve (Fig 3a) shows a good fit to the datapoints at high concentrations of the neutralising antibody, however, the fit is less representative at lower concentrations. At the lowest concentration of the neutralising antibody there appears to be a decrease in the number of infected cells. In isolation, this result would suggest that the assay was unreliable, however this is not the case. RSV infection is known to inhibit cell proliferation, so a better readout for this assay should consider the proportion of infected cells, represented by the readout "ratio of total green (FL-2) intensity: total blue (FL-1) intensity". The concentration-response curve for this readout (Fig 3b) now shows an excellent fit to the datapoints across the whole range. Finally, by considering the median area of nuclei in the well (Fig 3c), mirrorball can also provide a measure for syncytia formation.

discussion

This application note describes a simple and robust RSV neutralisation assay. The unique optics of TTP Labtech's mirrorball fluorescence cytometer enable a homogenous assay format, thus removing the requirement for wash steps to remove unbound fluorescent detection reagent. This is particularly advantageous in the context of viral infectivity, where infection itself can promote the detachment of cells from the microplate[4] and therefore lead to variable data in washed assay formats. Not only do homogenous assays reduce screening times by eliminating the requirement for wash and incubation steps, but they also minimise biosafety handling concerns, as the cells and virus are contained within the lidded (or sealed) microplate at all times[4].

mirrorball is the first system in its class to offer simultaneous scanning with multiple lasers, allowing direct correlation of fluorescence across lasers. We showed that normalisation of the RSV infection signal [total intensity green (FL-2)] to the cell number eliminates data variability associated changes in cell number. The *in situ* read on the microplate preserves the cells in their culture environment, allowing changes in cell morphology to be measured. For example, by determining the median area of nuclei in the well a measure of syncytia formation was obtained.
The open source whole-well Tiff images generated by mirrorball readout present an avenue for further imaged-based analysis, if desired.

references

[1] McNamara & Smyth: "The pathogenesis of respiratory syncytial virus disease in childhood" British Medical Bulletin (2002) 61: 13-28

[2] Gomez *et al.*: "Respiratory Syncytial Virus: pathology, therapeutic drugs and prophylaxis" Immunol. Lett. (2014) 162: 237-47

[3] Domachowske & Rosenberg: "Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment" Clin Microbiol Rev (1999) 12: 298–309

[4] Rasmussen *et al.*:"Adapting high-throughput screening methods and assays for biocontainment laboratories" Assay Drug Dev Technol (2015) 13: 44-54

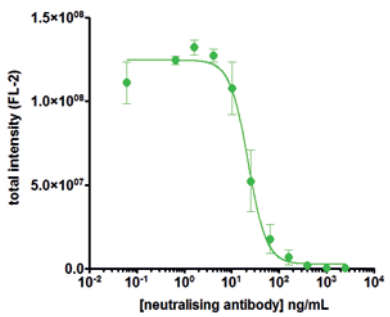

Fig 3a. Concentration-response curve [Total FL-2 intensity]

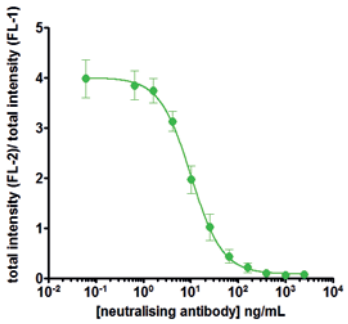

Fig 3b. Concentration-response curve [Total FL-2 intensity/Total FL-1 intensity]

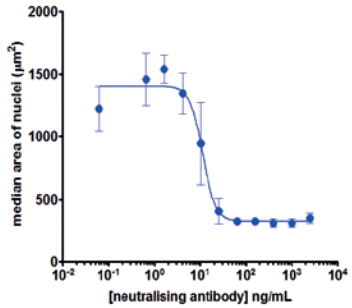

Fig 3c. Concentration-response curve [Median area of nuclei]