Simplifying Progress

Quantifying *in vitro* complement-dependent cytotoxicity (CDC) using advanced flow cytometry

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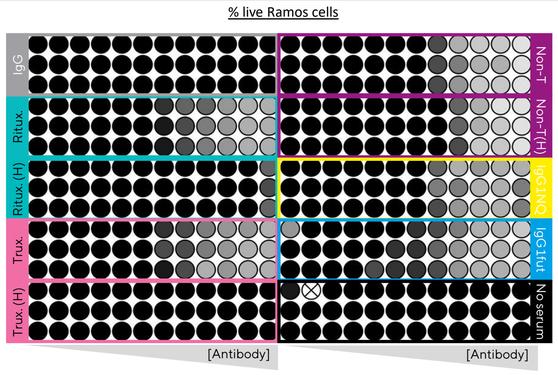
Introduction

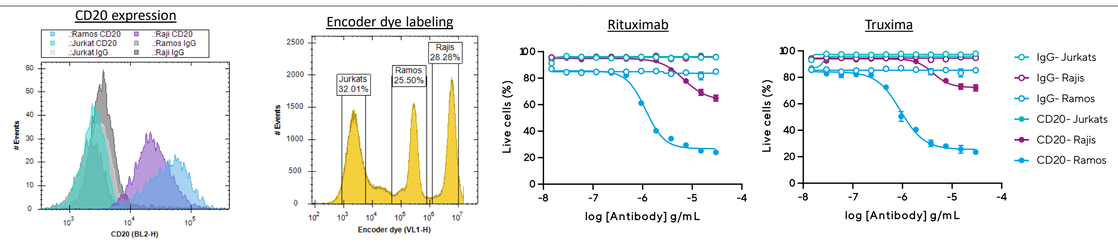
- In recent decades, rapid progression of monoclonal antibody (mAb) development has culminated in approvals for over 100 therapeutics in the United States[1].
- mAbs enhance the immune response through three key Fc-mediated functions: antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC).
- CDC is initiated when a mAb triggers activation of the complement cascade, which ultimately results in tumor cell lysis.
- We present a streamlined assay for measuring CDC using the iQue® Advanced Flow Cytometry Platform.
- Target cells are cultured with mAbs of interest and human serum, then labeled using iQue® Cell Membrane Integrity (R/Red) Dye to quantify cell death.
- Combining the high-throughput of the iQue® with rapid data analysis using the integrated iQue Forecyt® software streamlines antibody characterization and drug discovery processes.

High-throughput comparison of anti-CD20 CDC activity



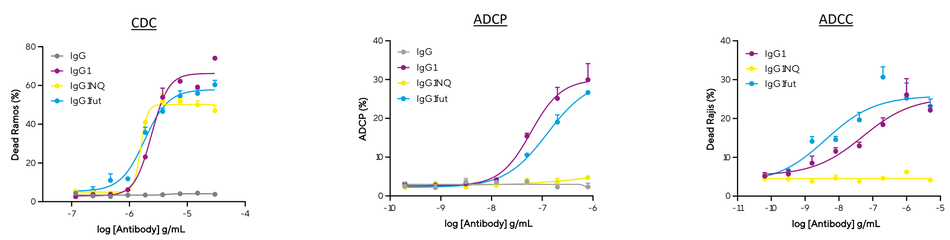
- Anti-CD20-IgG1 mAbs were screened for their CDC inducing activity against high CD20 expressing Ramos cells from a B cell lymphoma line in a 384-well plate format (n=3, 12 concentrations per mAb).
- Three native isotypes (Rituximab, Truxima and a non-therapeutic (non-T) mAb) were analyzed both in their native form and after being heated (H) for 90 minutes at 69°C.
  - Heating abolished CDC activity of the Rituximab and Truxima but not the Non-T mAb.
- Activity of Fc isotype mutants IgG1fut (non-fucosylated) and IgG1NQ (non-glycosylated) was comparable to non-T.

Induction of CDC correlates with target antigen expression



- Three cell types (Ramos, Raji and Jurkat) were profiled for their CD20 expression.
- Cells were distinguished using iQue® Cell Proliferation and Encoding (V/Blue) Dye when combined (5K/well) in a CDC assay.
- Induction of CDC by Rituximab (anti-CD20-IgG1) and Truxima (a Rituximab biosimilar) varied depending on cell type.
- Ramos expressed most CD20 and were more susceptible to CDC, whilst Raji cells showed mid-level CD20 expression and CDC. No death of the antigen negative Jurkat cells was observed.

Fc function differs between isotype mutants



anti-CD20 isotypes: EC_{50} values

	CDC		ADCP		ADCC	
Isotype	Predicted[2]	EC_{50} (µg/mL)	Predicted[2]	EC_{50} (ng/mL)	Predicted[2]	EC_{50} (ng/mL)
IgG1	++	2.41	+++	59.5	++	51.0
IgG1NQ	+/-	1.56	-	EC_{50} > max	-	EC_{50} > max
IgG1fut	++	1.82	+++	126.6	++++	4.1

- Fc functions of an anti-CD20-IgG1 Rituximab-based antibody were compared with two Fc isotype mutants: an IgG1fut (non-fucosylated) and IgG1NQ (non-glycosylated).
- ADCP was measured in a PBMC and Ramos cell co-culture assay, with analysis performed using the iQue® Human ADCP kit. ADCC was quantified from a co-culture of natural killer (NK) cells with Raji targets using the iQue® Human NK Cell Killing Kit.
- CDC was comparable between the three mAbs. IgG1NQ showed neither ADCP nor ADCC activity. Native IgG1 displayed slightly enhanced ADCP relative to the IgG1fut, whilst the IgG1fut showed greater ADCC activity. Responses were in line with predictions for each mutant.

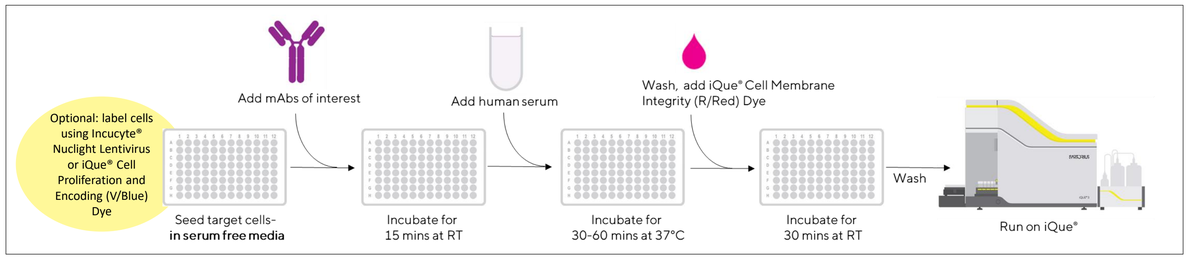
iQue® System

iQue® Advanced Flow Cytometer
Adopts a patented sampling method allowing for rapid sample acquisition to deliver fast actionable results. Capable of handling 96- and 384-well plates.

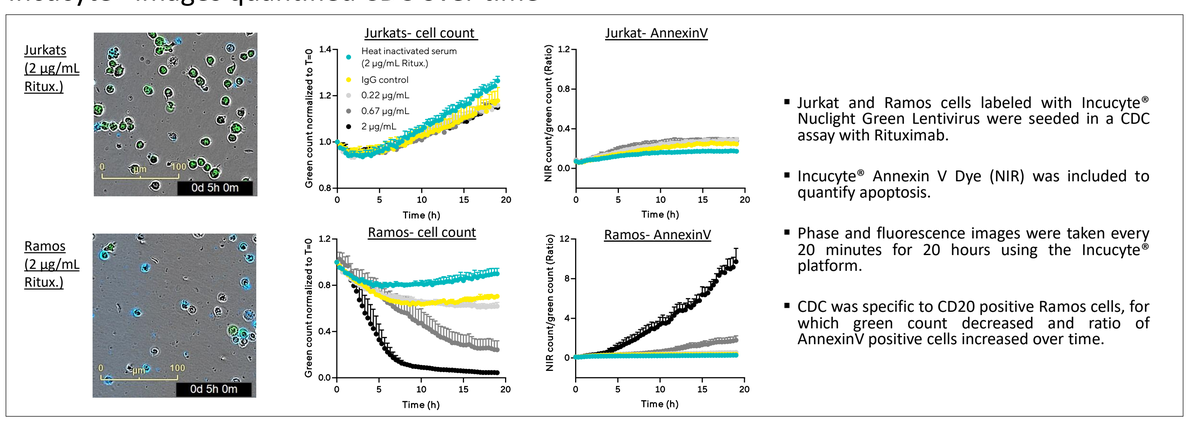
Sartorius Reagents and Consumables
A suite of reagents, kits and protocols for cell health and function screening.

iQue Forecyt® Software
Fully integrated, easy-to-use data analysis software combines plate level analytics with novel data visualization tools. Import pre-set gating templates for instantaneous readouts.

Assay Workflow



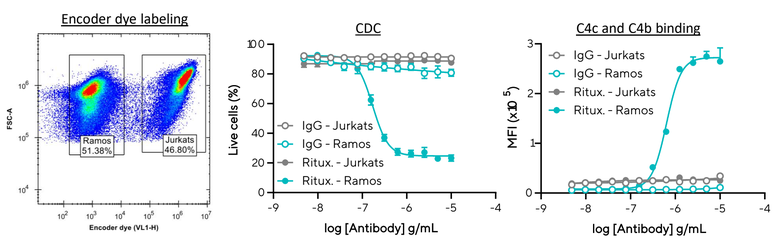
Incucyte® images quantified CDC over time



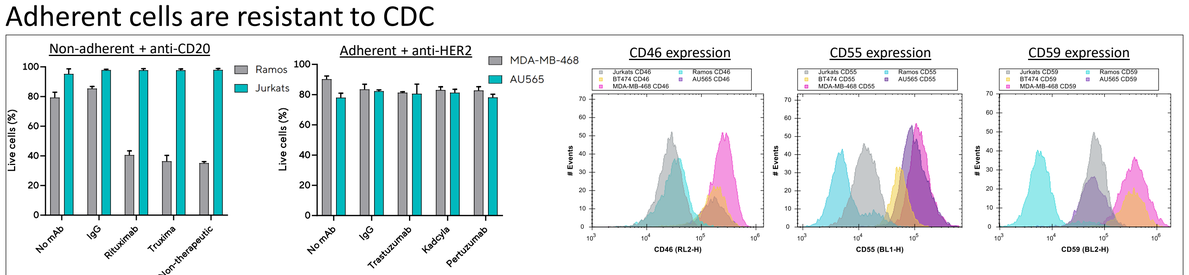
- Jurkat and Ramos cells labeled with Incucyte® Nuclight Green Lentivirus were seeded in a CDC assay with Rituximab.
- Incucyte® Annexin V Dye (NIR) was included to quantify apoptosis.
- Phase and fluorescence images were taken every 20 minutes for 20 hours using the Incucyte® platform.
- CDC was specific to CD20 positive Ramos cells, for which green count decreased and ratio of AnnexinV positive cells increased over time.

Complement protein binding during CDC

- Ramos and Jurkat cells were mixed and seeded with Rituximab or an IgG control antibody.
- An anti-C4c+C4b-FITC antibody quantified the increase in C4c+C4b binding (proteins in the complement cascade) which accompanied the Rituximab concentration dependent increase in CDC (cell death).
- The EC_{50} value for CDC activity was 0.17 µg/mL whilst the EC_{50} for C4c and C4b binding was 0.64 µg/mL.



Adherent cells are resistant to CDC



- Non-adherent cells (CD20 positive Ramos and CD20 negative Jurkats) or adherent cells (HER2 positive AU565 or HER2 negative MDA-MB-468) were combined in CDC assays.
- Anti-CD20 mAbs induced an antigen positive cell type-specific increase in CDC. None of the anti-HER2 antibodies impacted CDC.
- A range of adherent (inc. BT474) and non-adherent cells were profiled for their expression of complement regulatory markers e.g., CD46, CD55 and CD59. The enhanced expression of these markers on adherent cells may confer resistance to CDC.

[1] Kaplon, H., et al., 2022. Antibodies to watch in 2022. mAbs, 14(1).
[2] Invivogen: Reagents And Tools For Cell Biology Research. [online]