abcam

Multiplex immunoassays with FirePlex® particles

Validation, performance and benchmarking data

The multiplex immunoassays based on the FirePlex particle technology features highly customizable analyte panels that use our recombinant monoclonal antibody pairs to ensure highly sensitive assays with a broad dynamic range.

To ensure accurate and reproducible results, we subject the assays to rigorous validation in line with 'fit-for-purpose' biomarker assay development principles[1,2]. We also check correlation with ELISA, Luminex assays, and cytometric bead arrays.

Antibody pairs and protein standards

Antibody pairs are screened, for performance in multiplex panels, from the pool of recombinant rabbit monoclonal pairs developed for, and validated in, our SimpleStep ELISA® products. Protein standards are calibrated to NIBSC standards, where available, or an alternative reference calibrator.

Sensitivity and dynamic range

Assay sensitivity and dynamic range for each analyte are determined within multiplex panels to reflect experimental use.

The limit of detection is determined by measuring background signal in 12 wells, adding twice the standard deviation to the mean signal, and then interpolating on the standard curve.

Standard curves are plotted using the FirePlex analysis software with an error-weighted 4PL algorithm. Curves are optimized to avoid signal saturation and for signal-to-noise. Precision profile plotting ensures that CVs are below 10% across the full standard curve.

Using the standard 1 hour incubation protocol, median assay sensitivity is 0.5 pg/mL with standard curves typically spanning either 1.5–3,333 pg/mL or 4.6–10,000 pg/mL depending on the analyte. Sensitivity can be significantly improved and the standard curve extended (figure 1) by using an alternate overnight incubation.

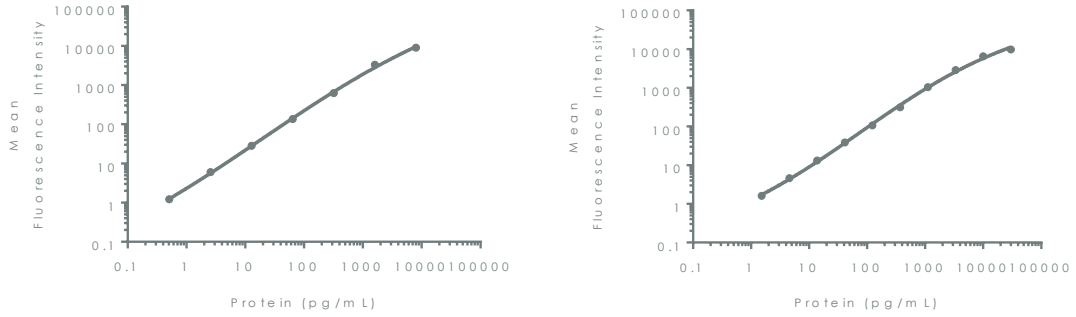


Figure 1. Representative curves for one analyte from a multiplex panel showing the extended ranges of 0.6 to 10,000 pg/mL for mouse IL-13 (left) and 1.5 to 30,000 pg/mL for human TNF alpha (right).

Linearity and recovery

Linearity of dilution/parallelism studies are used (examples in figures 2 and 3) to confirm that the assay and standard curve can be used to determine analyte concentrations across the dynamic range and also to calculate the minimum required dilutions.

Human and mouse analytes are routinely validated in serum, plasma (EDTA, heparin, citrate), cell culture supernatant and urine. Human analytes are also routinely validated in saliva, milk (defatted), bronchoalveolar lavage (BAL), cerebrospinal fluid (CSF) and synovial fluid.

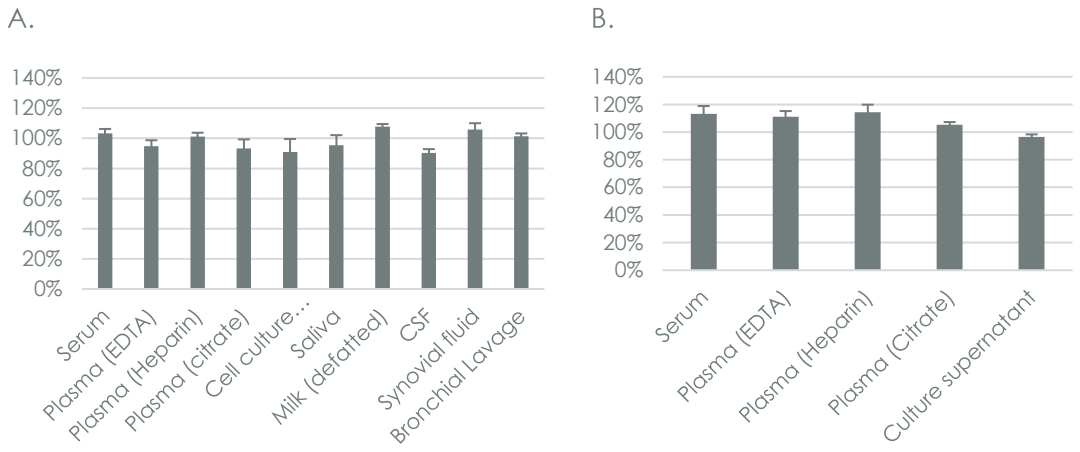


Figure 2. Linearity of dilution in native samples (A) and spike-recovery (B) studies for sTNF RI. Mean recovery and standard error are plotted. A: Each sample was tested at a 1:4 dilution and at six further dilutions for serum and plasma and three for other sample types. A two-fold dilution series was used. Analyte concentrations were interpolated from the standard curve. Percentage recovery is relative to the 1:4 dilution. B: Three different concentrations of protein standard were spiked into three different sample dilutions. Analyte concentration was interpolated from the standard curve. Percentage recovery was calculated after subtraction of the no-spike control value.

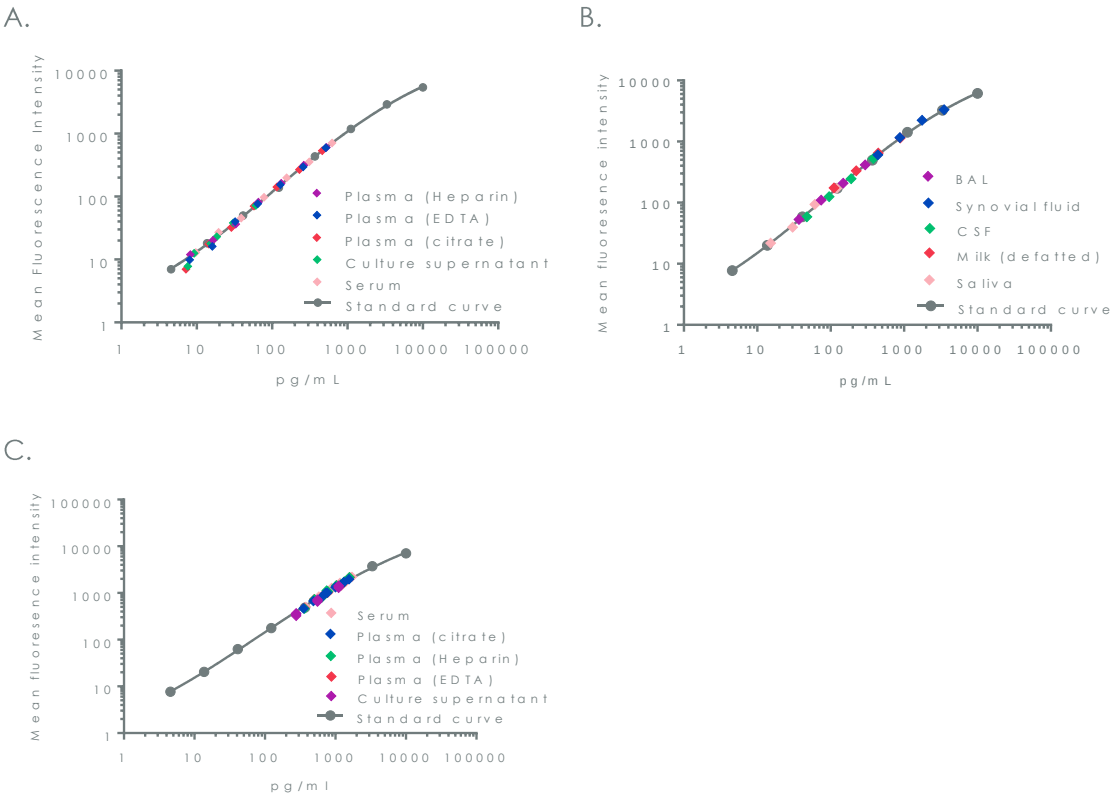


Figure 3. Confirmation of parallelism using the same data as figure 2 for native linearity of dilution (A and B) and spike-recovery (C). A: For each sample, analyte concentration in the 1:4 dilution was calculated by interpolation against the standard curve. The concentration of each subsequent dilution was then calculated and plotted, based on the dilution factor, from the concentration of the 1:4 dilution. B: The spiked concentration after non-spike control subtraction is plotted. The range of concentrations tested by spike-recovery was limited by the level of native protein.

Specificity in multiplex

Combinatorial testing confirms that individual FirePlex immunoassays retain specificity in multiplex with other assays and further confirms the specificity of the antibody pair against the analyte of interest.

Each FirePlex capture antibody-particle conjugate is tested with its own detector antibody, and with unique pools of other detector antibodies, both with and without different combinations of protein standards.

For >95% of all possible custom multiplex panels, non-specific signals are either insignificant or <1% of the true signal. Incompatible combinations are checked during panel design.

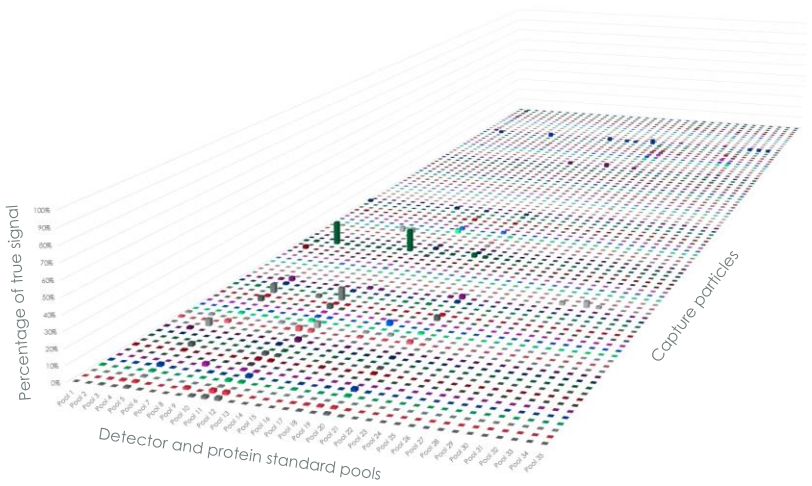


Figure 4. Example of combinatorial testing using unique detector antibody / protein standard pools. Over the 88 analytes and 3.8×10^{22} possible custom panels tested, only one combination (Eotaxin and IP-10) was identified as incompatible. The two tallest bars represent two pools tested against the IP-10 capture particle; both contain Eotaxin assay components, but have no other analyte in common.

Consistency between singleplex and multiplex

As an additional check for assay specificity in multiplex, we test FirePlex immunoassays as both singleplex and multiplex assays to confirm alignment between the standard curves (figure 5) and for consistent results in biological samples.

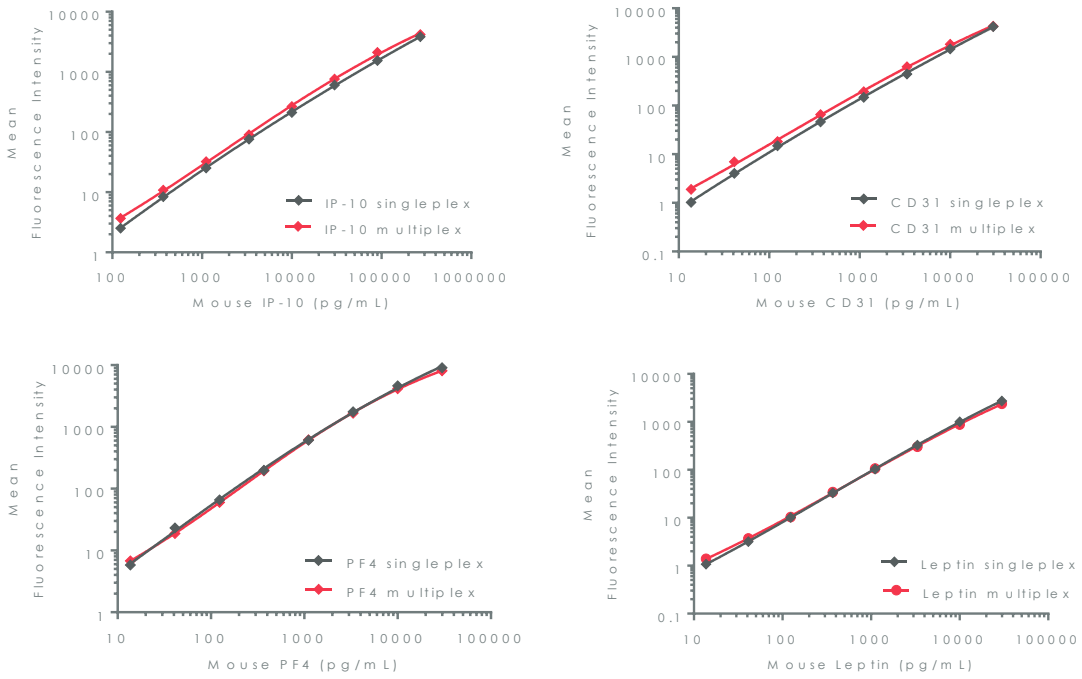


Figure 5. Representative examples of standard curve alignment testing in singleplex and in multiplex. Leptin and PF-4 were tested in an 18 analyte multiplex panel; CD31 and IP-10 in a 15 and 10 analyte panel respectively. Testing of mouse serum samples in the same experiments resulted in an average CV of 3% between singleplex and multiplex assays.

Biologically relevant quantification

Throughout assay development, we test assays and panels in clinically-relevant biological samples (figure 6) to confirm that results are consistent with those in the literature.

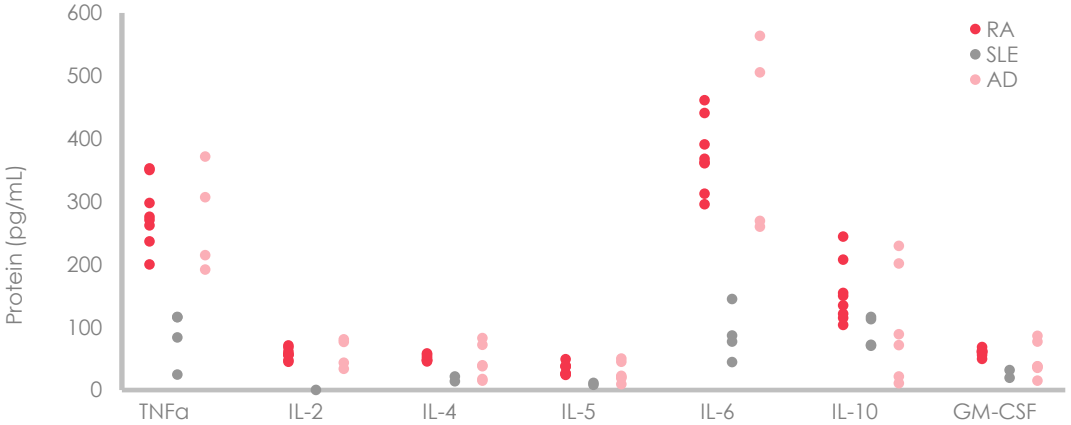


Figure 6. Seven cytokines were quantified in normal serum and serum from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and atopic dermatitis (AD). Each dot represents an individual patient. Data was consistent with relevant publications[3-5].

Reproducibility

Consistent results between assays is extremely important, and the typical inter-assay and intra-assay CVs are <15% and <10% respectively (figure 7).

Accelerated and real-time time course stability studies are used to confirm reagent stability.

The original lot of each assay, and subsequent lots, are retained as reference lots for each subsequent production. Using antibody pairs composed of two recombinant monoclonal antibodies ensures that the specificity and affinity of the antibody pair do not vary between lots.

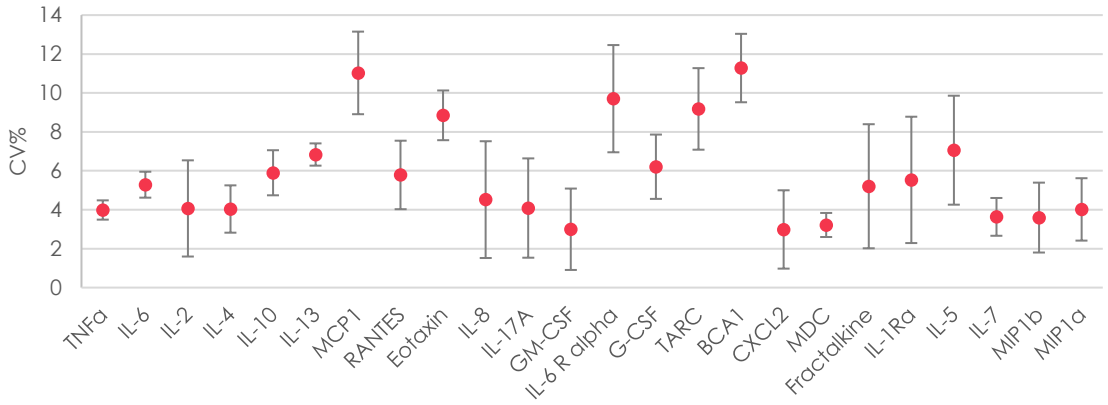


Figure 7. Inter-assay CV% values. To determine intra-assay CVs, a single biological sample is tested at three sample dilutions with four replicates of each dilution. This experiment is performed three times on different days to determine inter-assay CVs.

Benchmarking

Finally, we examine how FirePlex immunoassays perform against other prominent immunoassays. We find an excellent correlation between ELISA, Luminex assays and cytometric bead arrays (figures 8, 9 and 10).

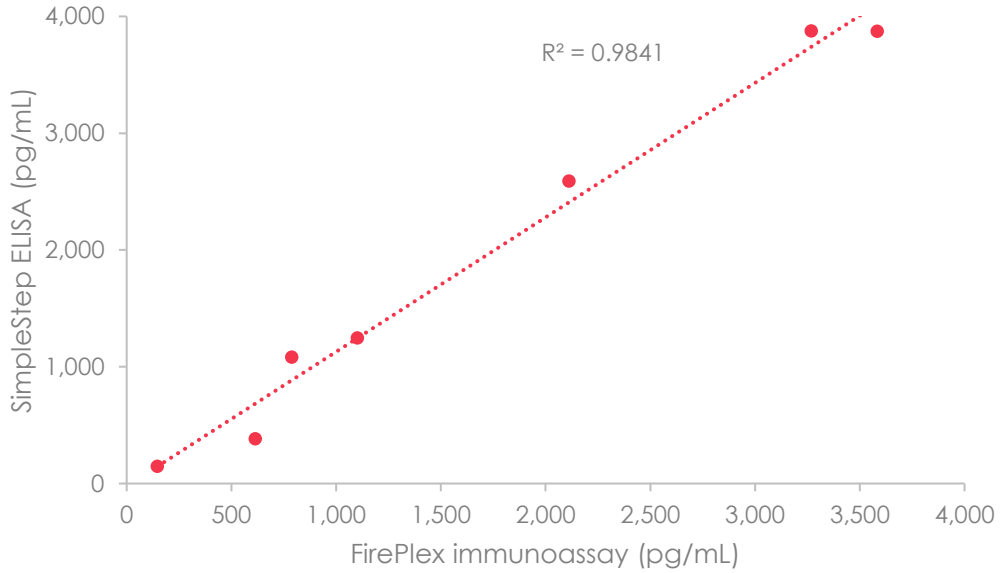


Figure 8. SimpleStep ELISA® kits and a FirePlex immunoassay was used to determine human BCA1, IL-17A, GM-CSF, G-CSF, TARC, IL-10 and RANTES concentrations in supernatant from a PBMC cell culture which had been stimulated with 1.5% PHA-M for 24 hours.

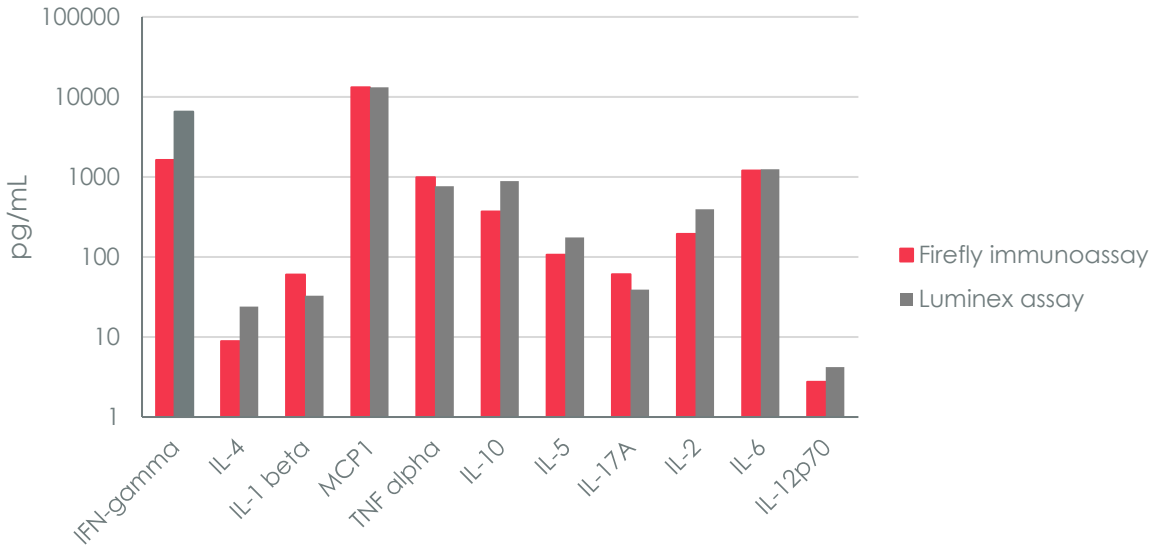
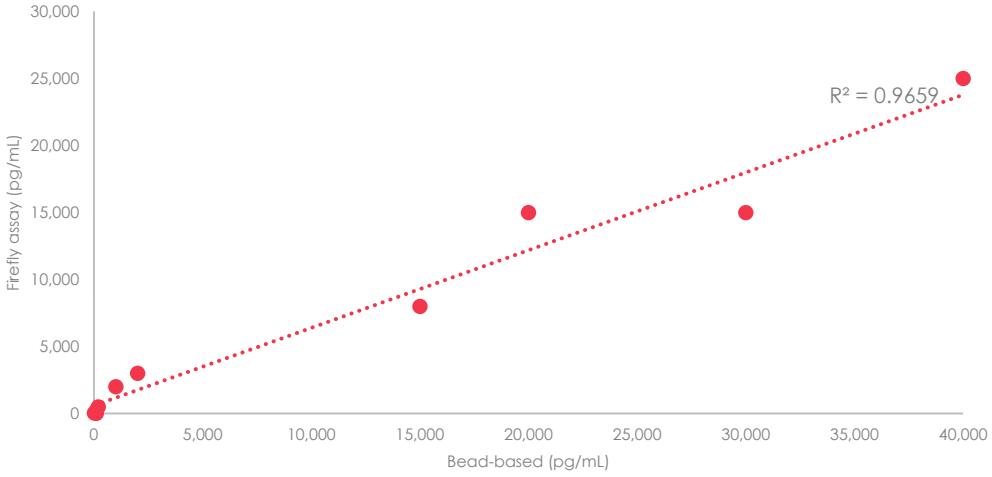


Figure 9. Eleven human cytokines (IFN-gamma, IL-4, IL-1 beta, MCP1, TNF-alpha, IL-10, IL-5, IL-17A, IL-2, IL-6, IL-12p70) in stimulated PBMC cell culture supernatants were analyzed with both Luminex



assays (Millipore catalog# HCytoMAG-60K, tested by Boston University Analytical Instrumentation Core) and a FirePlex immunoassay panel.

Figure 10. FirePlex assays and bead-based multiplex assays for flow cytometers were used to determine the concentrations of eleven human cytokines in PHA stimulated PBMC supernatants.

Sensitive, flexible, convenient

FirePlex immunoassays are developed and validated to enable sensitive and accurate multiplexing across a broad dynamic range. Multiplex panels are fully customizable with a broad choice of analytes, and assays can be analyzed on a flow cytometer, with no specialized equipment required, or samples can be sent to our service center.

Learn more at www.abcam.com/FirePlexImmunoassays

References

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3. Brandt, E. B. & Sivaprasad, U. Th2 Cytokines and Atopic Dermatitis. *J Clin Cell Imunol* **2,** 1–25 (2011).
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5. McInnes, I. B. & Schett, G. Cytokines in the pathogenesis of rheumatoid arthritis. *Nat. Rev. Immunol.* **7,** 429–442 (2007).