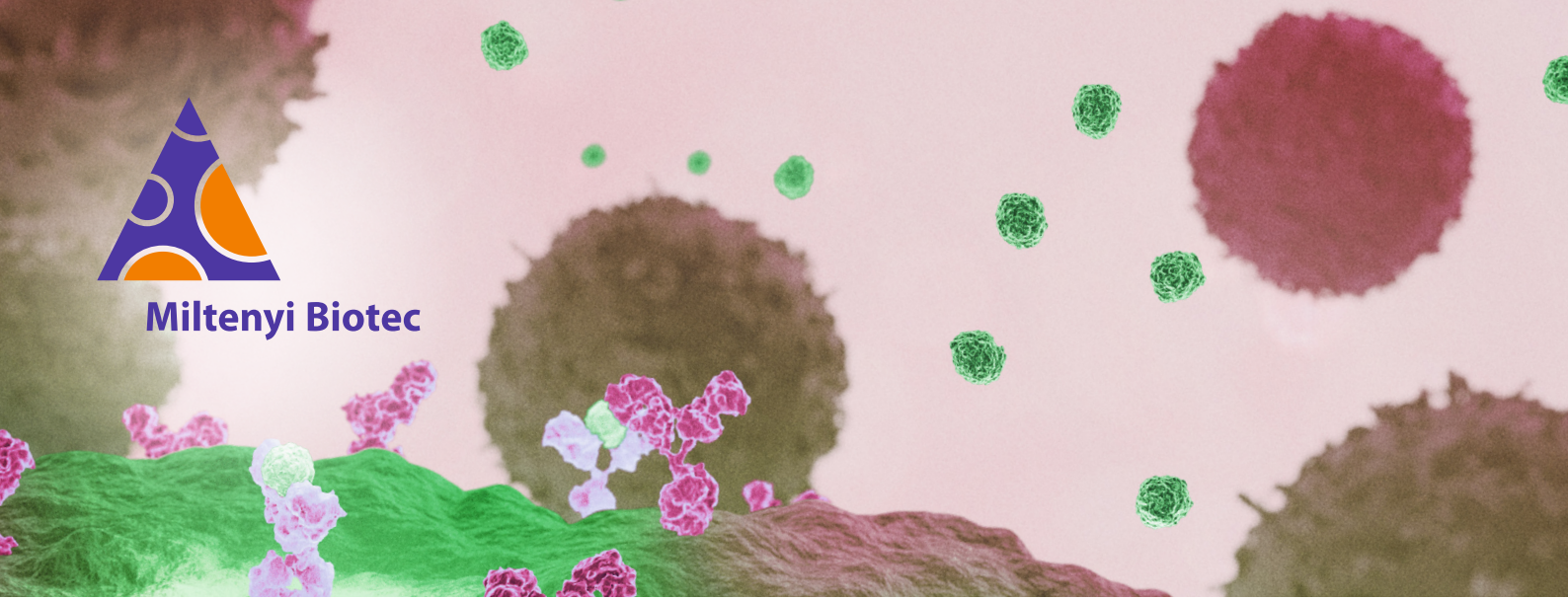

Miltenyi Biotec

How to switch to optimal activity-based cytokine dosing

Background information

Many cell culture protocols use weight as a measure for cytokine dosing. However, the biological activity of recombinant cytokines is variable. It depends on the production process and thus varies between suppliers and even between production lots (fig. 1). This simply means cytokine weight does not equal cytokine activity. Dosing recombinant cytokines based on protein weight might therefore result in adding different amounts of active cytokine and cause variable cell culture conditions. Cytokine dosing based on calibrated activity units (U or IU) minimizes this variation and increases cell culture reproducibility.

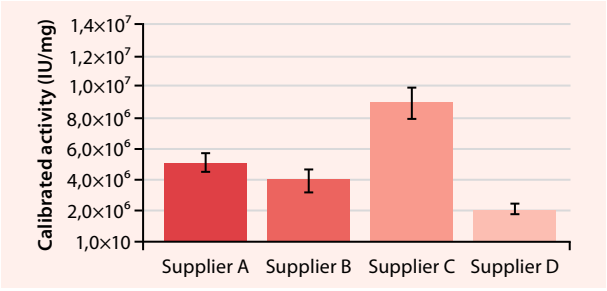

Figure 1: Protein weight does not equal the amount of active cytokine. The same amount of total protein (weight) was used in a side-by-side comparison of IL-2 of four different suppliers. Biological activities were determined and calibrated to the same international standard (NIBSC 86/504). The variations of the depicted calibrated activities (IU/mg) illustrate that the amount of active cytokine does not equal the protein weight.

Fortunately, protocols can easily be converted from weight-based to activity-based (unit-based) cytokine dosing following this step-by-step guideline. The guideline furthermore describes how to determine the optimal cytokine dose for a specific protocol and how to prepare convenient cytokine stock solutions. Suitable entry points into the guideline for specific questions are listed in table 1.

Step	Topic	Relevant if …
1	Drawbacks of weight-based protocols	… you need some background information about weight versus unit-based cytokine dosing.
2	Calculation to convert weight-based into activity-based protocol	… you have a weight-based protocol.
3	Titration to identify the optimal cytokine concentration	… you do not know if you are dosing too high or too low.
4	Exact dosing by using the calibrated lot-specific activity of MACS® Premium-Grade Cytokines	… you always want to apply the same amount of active cytokine without lot-to-lot testing.

Table 1: Overview of the most relevant sections or starting points into this step-by-step guideline for specific questions.

Step-by-step guideline

1. Drawbacks of a weight-based protocol

Cell culture protocols that are based on cytokine weight (ng/mL) can result in the application of different amounts of active cytokine (U/mL or IU/mL). Even assay optimization and keeping all other conditions as consistent as possible, are not sufficient to cover the applied variable amounts of active cytokine in different experiments. Consequently, weight-based protocols cause variable cell culture conditions that reduce reliability and reproducibility of the experimental results.

Attention:
Cytokine weight does not equal cytokine activity!

Example for T cell cultivation:
Cells of the T cell line CTLL-2 were incubated for 22 h with 0.3 ng/mL IL-2 IS according to a weight-based protocol (fig. 2A) or with the optimal amount of active IL-2 IS (18 IU/mL) according to the activity-based protocol determined in the following step-by-step guide (fig. 2B). Three different production batches (lot 1, 2 and 3) were tested in independent experiments in a standardized assay and conditions were kept as consistent as possible.

Although the cytokine production process and the assay are optimized to keep lot-to-lot variations to a minimum, cells responded differently when the same amount of total protein (weight) was applied (fig. 2A). Weight-based application of IL-2 IS from production lot 3 resulted in an approximately 2-fold higher growth than IL-2 IS from production lot 1. In contrast, when cytokines were dosed according to the calibrated biological activity (IU), cell responses were highly comparable between the different experiments (fig. 2B). This shows that variations of the cytokine activity caused the differences within the experimental outcomes in Figure 2A, and not inter-assay variations. Thus, using the optimal amount of active cytokine reduces the variability to a minimum compared to weight-based dosing and enables reproducible cell culture conditions.

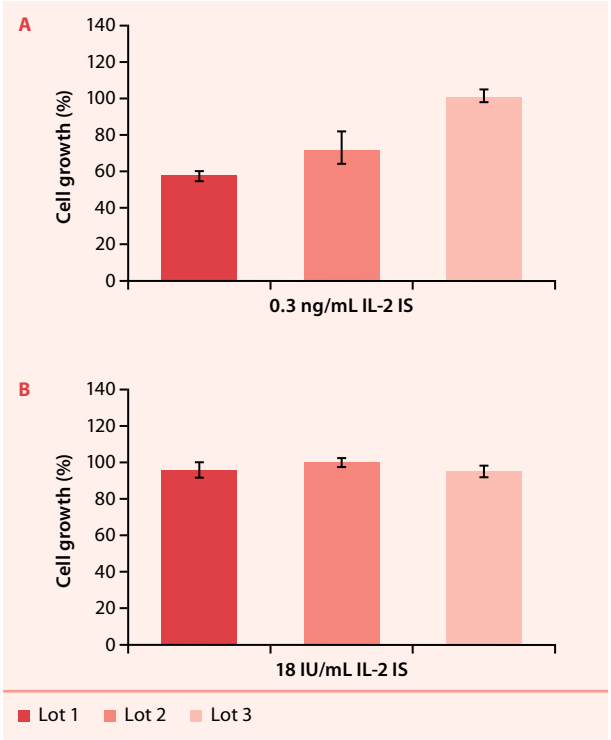

Figure 2: Applying optimal amounts of active cytokine increases cell culture reproducibility compared to weight-based dosing.
CTLL-2 cells were cultivated with human IL-2 IS from three different production batches for 22 h at 37 °C and 5% CO_2. Cytokine was dosed according to A) weight (0.3 ng/mL) and to B) calibrated biological activity (18 IU/mL as determined in the step-by-step guide). Cell growth is depicted and error bars show covariance % within one assay.

2. Converting a weight-based protocol into an activity-based protocol
A weight-based protocol (ng/mL) can easily be converted into an activity-based protocol (U/mL or IU/mL) if the biological activity of the applied cytokine is known. For this, the following formula can be used:

$$\text{Protocol concentration in IU/mL} = \frac{\text{Biological activity (IU/mg)} \times \text{Protocol concentration (ng/mL)}}{1\times10^{6}}$$

Example for T cell cultivation:
- Minimal biological activity of IL-2 IS: ≥ 5×10^{6} IU/mg (calibrated to NIBSC 86/504)
- Cytokine concentration used in the weight-based protocol: 0.3 ng/mL

$$\text{Protocol concentration in IU/mL} = \frac{5\times10^{6}\ \text{IU/mg} \times 0.3\ \text{ng/mL}}{1\times10^{6}} = 1.5\ \text{IU/mL}$$

This standard protocol uses a minimal concentration of 1.5 IU/mL for cultivation of CTLL-2 cells.

Please note, most suppliers – and also MACS Research-Grade Cytokines – only state the minimal biological activity (or a range of the activity) of a given product. Based on this information, you can only calculate the minimal cytokine concentration (U/mL or IU/mL) previously used in your protocol. The actual amount of active cytokine applied will remain unknown.

Attention:
Most suppliers only state the minimal biological activity, as opposed to the actual activity, of a cytokine batch.

Further note, that the biological activity is a relative value that depends on the applied assay and on the assay conditions. Even if the same assay is used to determine the biological activity of one cytokine, values derived from different experiments can only be compared when calibrated to the same standard. This is why Miltenyi Biotec's MACS Premium-Grade Cytokines are calibrated whenever possible to standards provided by the National Institute for Biological Standards and Control (NIBSC), enabling reliable activity-based dosing of cytokines according to calibrated units.

3. Titrating the optimal cytokine concentration
In order to achieve reliable and reproducible results, the optimal amount of active cytokine should be applied, preventing both, too low cytokine levels and cytokine oversaturation. This optimal cytokine concentration can be determined by titration.

The minimal cytokine concentration (U/mL or IU/mL) calculated in **step 2** can be used as a reference point. The titration experiment should include different cytokine concentrations, covering at least the range of 0.1×–10× of the calculated minimal cytokine concentration. Follow your protocol using the different selected cytokine concentrations. Ideally, the experiment should be conducted in triplicates. Finally, generate a titration curve and identify the optimal concentration. The start of the plateau is the concentration at which the maximum response in your assay is achieved.

Example for T cell cultivation:
The calculated minimal concentration of 1.5 IU/mL was used as reference point. Cells were cultivated under the same conditions for the same time interval but using different IL-2 IS concentrations. The resulting titration curve shows a maximum response at concentrations above 10 IU/mL (fig. 3). To ensure that the cytokine concentration reaches saturation and thus results in a maximal response, the second value of the plateau, 18 IU/mL, would be chosen as optimal concentration for this protocol. In contrast, a concentration of 1.5 IU/mL, which was calculated based on the previously used cytokine weight, is not sufficient to achieve the maximum cell response.

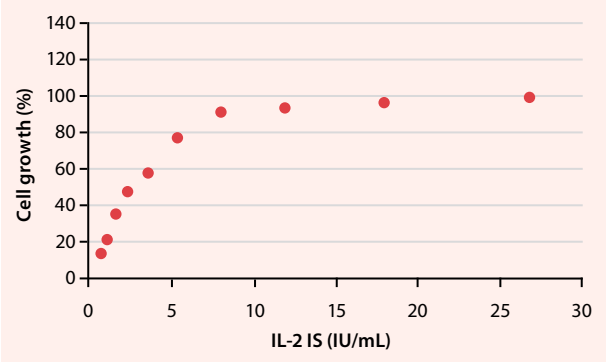

Figure 3: Titration of IL-2 IS on CTLL-2 cells. To determine optimal IL-2 IS dosing, CTLL-2 cells were cultivated with different unit amounts of IL-2 IS and cell growth was determined.

Please note, as the biological activity of cytokines varies between different production batches, the optimal cytokine concentration needs to be determined via titration for every batch (lot-to-lot testing). However, if a calibrated lot-specific cytokine activity is given (as it is for MACS Premium-Grade Cytokines), the optimal cytokine concentration only needs to be titrated once.

Attention:
Without knowing the exact calibrated activity of the used cytokine batch, the optimal cytokine concentration needs to be determined via titration for every production batch (lot-to-lot testing).

4. Skip lot-to-lot testing with MACS Premium-Grade Cytokines
For MACS Premium-Grade Cytokines, the exact calibrated biological activity of the respective production lot is stated in the Certificate of Analysis (miltenyibiotec.com/certificates). Based on this known lot-specific biological activity, you can directly apply the optimal amount of active cytokine without further lot testing once the optimal cytokine concentration for your protocol has been determined (as described in **step 3**).

Benefit:
Save time and money otherwise invested in tedious lot-to-lot testing.

4.1 How to reconstitute MACS Premium-Grade Cytokines
For optimal reconstitution of MACS Premium-Grade Cytokines, the final cytokine content (protein concentration in mg/mL) and the lot-specific cytokine activity (U/mL or IU/mL) should be taken into consideration:

- In order to achieve optimal stability, cytokines should be reconstituted in deionized, sterile-filtered water to a final protein concentration of 0.1–1.0 mg/mL in a minimal volume of 100 µL. Unless stated differently in the respective data sheet.
- To reconstitute the cytokines according to their biological activity, look up the lot-specific biological activity of your MACS Premium-Grade Cytokine production lot in the respective CoA (miltenyibiotec.com/certificates).

Decide on a convenient stock concentration (U/mL or IU/mL) for your experiments and calculate the needed volume using the formula below:

$$\text{Volume for reconstitution} = \frac{\text{Lot-specific activity (IU/mg)} \times \text{Cytokine weight (mg)}}{\text{Desired stock solution concentration (IU/mL)}}$$

Check if the calculated volume for reconstitution is in the range of the suggested final protein concentration. If yes, proceed with reconstitution, aliquot stock, and freeze it at –20 °C.
If not, please adjust stock concentration until volume and respective protein concentration fit the recommendations.

Benefit:
Have stable cytokine stocks available in your freezer with a convenient concentration.

Example for T cell cultivation:

- Amount of Human IL-2 IS, premium grade: 50 µg
- Order number: 130-097-745
- Lot number: 5200308890
- Biological activity of IL-2 IS: 6.9×10^6 IU/mg (calibrated to NIBSC 86/504)

To obtain a protein concentration between 0.1–1.0 mg/mL, 50 µg IL-2 IS should be reconstituted in 50–500 µL deionized, sterile-filtered water.

A convenient stock solution would, for example, be 1×10^6 IU/mL, to facilitate further applications or dilutions:

$$\text{Volume for reconstitution} = \frac{6.9\times10^6 \text{ IU/mg} \times 0.05 \text{ mg}}{1\times10^6 \text{ IU/mL}} = 345 \text{ µL}$$

Resuspend 50 µg IL-2 IS in 345 µL deionized, sterile-filtered water to get a stock concentration of 1×10^6 IU/mL and a protein concentration of 0.15 mg/mL.

5. Calculating how much stock solution should be applied for activity-based cytokine dosing

Knowing the biological activity of a reconstituted stock (U/mL or IU/mL) and the needed amount of active cytokine, you can easily define the volume of stock solution that needs to be applied in your activity-based protocol with the following formula:

$$\text{Volume of stock solution for one assay} = \frac{\text{Units needed for one assay (IU)}}{\text{Concentration of stock solution (IU/mL)}}$$

Benefit:
Save time otherwise needed for experimental planning.

Example for T cell cultivation:

For one assay, 150 mL cell culture medium needs to be supplemented with 18 IU/mL IL-2. The reconstituted IL-2 IS has a stock concentration of 1×10^6 IU/mL.

$$\text{Volume of stock solution for one assay} = \frac{18 \text{ IU/mL} \times 150 \text{ mL}}{1\times10^6 \text{ IU/mL}} = 2.7 \text{ µL}$$

Add 2.7 µL of the stock solution per assay to apply the determined optimal cytokine concentration of 18 IU/mL.

6. Advantages of activity-based cytokine dosing

Activity-based cytokine dosing according to calibrated units allows you to always apply the same amount of active cytokine to your experiments. Furthermore, you can determine the optimal amount of active cytokine for your assay and increase the reproducibility of your cell culture experiments as well as the comparability of your experimental outcomes.

Benefit:
Increase cell culture reproducibility and get reliable results.

Conclusion

- Cell culture protocols based on the calibrated cytokine activity facilitate optimal and reproducible conditions and thus reliable results.
- Optimal activity-based cytokine dosing according to calibrated units prevents cytokine under- and oversaturation, and associated drawbacks such as elevated reagent costs or artificial effects.
- Using MACS Premium-Grade Cytokines with known calibrated lot-specific cytokine activity saves time otherwise needed for validation experiments.

Glossary

Biological activity: The biological activity of a cytokine is the effect on cells after incubation with the cytokine.

International units (IU): The biological activity of a cytokine is determined by a standardized assay that measures its effect on cytokine-dependent cell lines or primary cells. If the bioassay is calibrated with a protein certified by the NIBSC (National Institute for Biological Standards and Control) international standard, the activity is expressed in international units/mg (IU/mg).

Units (U): The biological activity of a cytokine is determined by a standardized assay that measures its effect on cytokine-dependent cell lines or primary cells. Biological activity is usually expressed as ED50 (ng/mL) or units (U/mg).

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