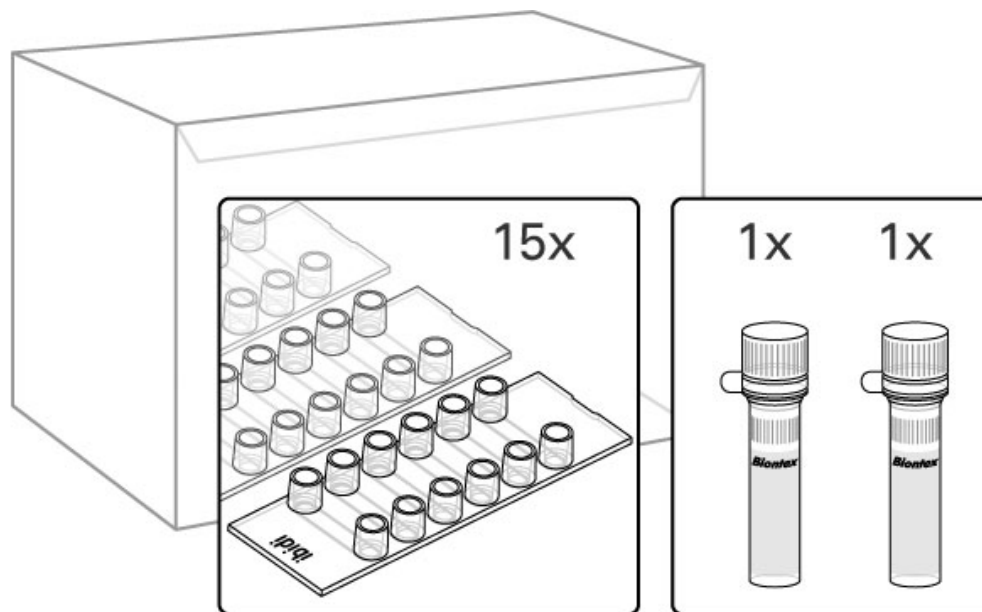


Cell Culture, Proteofection and Microscopy in a Slide

The **μ-Proteofection Kit VI** combines μ-Slides from ibidi GmbH with PROTEOfectene™ from Biontex into a powerful kit that enables the user to deliver proteins into living cells and to observe the subsequent processes with live-cell imaging.

PROTEOfectene™ offers an alternative to nucleic acid transfection and a powerful strategy for functional studies by delivering proteins into living cells. This new and innovative reagent opens new fields of investigation in the rising field of proteomics to elucidate complex molecular mechanisms. PROTEOfectene™ is a lipid-based formulation which only requires mixing with the protein of interest and forms non-covalent complexes with proteins. These proteoplexes are absorbed by cells via endocytosis and the proteins are released into the cytoplasm, retaining their structure and function.

The **μ-Slide VI** is designed for high resolution microscopy and is optimal for both experiments with living cells and for cell fixation and staining of cells. Thanks to the high optical quality of the material fluorescence microscopy is possible at any wavelength and at maximum resolution.



Contents μ-Proteofection Kit VI:

PROTEOfectene™	250 μl	Biontex Laboratories GmbH
R-Phycoerythrin (Positive control; 100 μg/ml in PBS)	100 μl	Biontex Laboratories GmbH
μ-Slide VI, <i>ibiTreat</i>	15 Slides	ibidi GmbH

Shipping: At room temperature

Storage: PROTEOfectene™ 4 °C; do not freeze
 R-Phycoerythrin 4 °C; do not freeze
 μ-Slide VI Room temperature

Stability: Best before: see label

Use: Only for research purposes *in vitro*, not intended for human or animal diagnostic, therapeutic or other clinical uses.

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1 General Information

1.1 Working with ibidi μ-Slides

1.1.1 Material

The μ-Slides consist of a plastic with highest optical quality. The material exhibits extremely low birefringence and autofluorescence, both similar to that of glass. It is not possible to detach the bottom from the slide. The μ-Slides are not autoclavable since they are temperature stable up to 60°C/140°F only. Please note that gas exchange between the channel and incubator's atmosphere occurs partially through the plastic bottom which should not be covered. Thus, it is recommended to place the μ-Slide on an ibidi μ-Slide rack.

1.1.2 Geometry of the μ-Slide VI

The μ-Slide VI provides standard slide format according to ISO 8037/1. The lateral adapter to adapter distance of 9 mm (like 96 well plates) allows using multichannel pipettes.

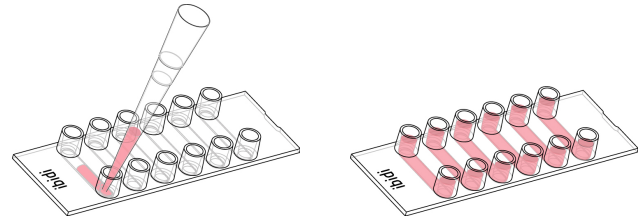
Geometry	
Number of channels	6
Channel volume	30 μl
Channel length	17 mm
Channel width	3.8 mm
Channel height	0.4 mm
Adapters	female Luer
Volume per reservoir	60 μl
Growth area	0.6 cm ² per channel
Coating area using 30 μl	1.2 cm ² per channel
Bottom matches coverslip	No. 1.5

1.1.3 Preparation for Cell Microscopy

To analyze your cells no special preparations are necessary. Cells can be observed live or fixed directly in the μ-Slide on an inverted microscope. You can use any fixative of your choice. The μ-Slide material is compatible with a variety of chemicals, e.g. Acetone or Methanol. Further specifications can be found at www.ibidi.com. Due to the thin bottom of only 180 μm, high resolution microscopy is possible.

1.1.4 Filling of the Slides

Apply 30 μl of cell suspension per channel of the μ-Slide-VI with a pipet. To avoid air bubbles put the pipet tip right on the channel inlet and point at the channel as shown in the picture below.



After cell attachment (approx. 4 – 6 hours) fill the reservoirs with 60 μl medium each.

1.1.5 Immersion Oil

When using oil immersion objectives, only the immersion oils specified in the table may be used. The use of different oil can lead to damages of the objective.

Company	Product	Ordering number
Cargille	type DF, Formula Code: (Cargille) 1261	16242
Zeiss	518 F	(Zeiss) 444960
Olympus	50CC	(Olympus) 35506
Nikon	50 CCM DF	(Nikon) MXA 20351
Leica	immersion oil, low fluorescence	(Leica) 11513859

Tip:

The day before seeding the cells we recommend to place the cell medium and the μ-Slide into the incubator for equilibration. This will prevent the liquid inside the channel from emerging air bubbles over the incubation time.

Trapped air bubbles can be removed from the channel by inclining the μ-Slide and knocking at one edge.

1.2 Working with PROTEOfectene™

1.2.1 Specifications

Application	Delivery of proteins into living eukaryotic cells in μ-Slides VI
Sterility	tested
Cell culture	tested
Stability	1 year
Storage	4 °C

R-Phycoerythrin (positive control) is a fluorescent protein with a molecular weight of approx. 240 kDa.

Excitation maxima: 480, 545 and 565 nm.

Emission maximum: 578 nm (visible red).

1.2.2 Quality Control

To assure the performance of each batch of PROTEOfectene™ using rigorous standards, the following assays are conducted:

Purity:	Silica Gel TLC assay
Sterility:	Thioglycolate assay
Biological Activity:	Delivery of R-Phycoerythrin in NIH3T3 cells monitored by cytofluorometry and fluorescence microscopy.

1.2.3 Explanatory Remarks

Important criteria for efficient protein delivery into cells

Proteins differ from one another in term of size, structure, composition and biophysical properties. Unlike different nucleic acids which have very similar biophysical properties, the interaction of proteins with a reagent such as PROTEOfectene™ is highly variable. Thus, optimal delivery conditions for one particular protein cannot be transferred to another type of protein, and delivery efficiencies may vary from one cell line to another.

Due to their specific properties some proteins might not be efficiently delivered with PROTEOfectene™. For instance, highly alkaline proteins that are positively charged under physiological conditions are very difficult to deliver into cells.

However, there are no rules to determine whether a specific protein can be delivered or not.

Important Parameter: Protein purity

Any impurities, contaminants or additives present in the protein solution of your protein of interest may affect delivery efficiency. For this reason, the protein should be as pure as possible.

Stabilizers such as detergents or BSA can inhibit the delivery if present excessively in relation to the protein of interest. Stabilizers such as glycerol or other similar additives do not interfere with the protein delivery.


Preservatives such as sodium azide could hypothetically lead to cytotoxicity if present in high concentrations. They can be removed by dialysis if necessary.

2 Standard protocol

2.1 General Considerations

The instructions given below represent a standard protocol that was applied successfully on a variety of cell types. It is best to start by following the standard protocol as a general guideline. Optimal conditions and parameters do vary from protein to protein and from cell line to cell line and have to be found for each new setup, as described in chapter 3.

PROTEOfectene™ is provided with R-Phycoerythrin as a positive control. Use it with a protein:reagent ratio of 1:2 (for 1 μg of protein 2 μl of PROTEOfectene™ are needed). This control protein is provided to help you set up your experiment and should be used for each new cell line with which you experiment (see chapter 4.1).

 Note: The purity of the protein and the presence or absence of additives and contaminants have a high impact on the delivery efficiency.

The following instructions refer to one channel of a μ-Slide VI.

2.2 Preparing the Reagents

Prior to transfection, bring PROTEOfectene™ and the protein to room temperature. Mix each solution gently.


2.3 Preparing the Cells

Seed the cells on the day before the protein delivery experiment. The suitable cell density will depend on the growth rate and the condition of the cells. Cells should be 50 – 70% confluent (percentage of growth surface covered with cells) at the time of experiment.


1. Prepare a cell suspension with a concentration of $3 - 4 \cdot 10^5$ cells/ml in complete culture medium.
2. Fill 30 μl of the cell suspension into one channel of the μ-Slide VI.
3. When the cells have adhered (approx. 2 – 6 h after seeding) fill each reservoir of the channel with 60 μl of complete culture medium.

2.4 Preparing the Proteoplex

4. Dilute the protein in 1x PBS to 100 μg/ml.

 Note: While a small concentration of glycerol (1 – 5%) in the protein solution is acceptable, BSA can completely inhibit the protein delivery.

5. Pipet 0.4 μg protein (4 μl; 100 μg/ml) into a microtube.
6. Add 0.8 μl PROTEOfectene™. Mix by gently pipetting up and down the whole volume.

 Note: Do not dilute PROTEOfectene™. If pipeting of small quantities is required, prepare a greater amount of proteoplex.

7. Incubate for 10 – 15 min at room temperature.
8. Meanwhile remove the culture medium from the reservoirs of the cell-filled channel. Keep the channel itself filled.
9. After the incubation period, add 30 μl of complete cell culture medium to the microtube containing the proteoplex.

2.5 Proteofection

10. Now swap the culture medium present in the channel with the proteoplex solution in the microtube. To do so, tilt the slide approximately 45 degree over its longitudinal axis and pipet 30 μl of the proteoplex from the microtube into the upper reservoir. The proteoplex now displaces the culture medium from the channel. The geometrical conditions given within the channel prevent the two solutions from mixing significantly. Remove the 30 μl of the displaced culture medium from the lower reservoir.
11. Incubate the slide under standard conditions for the cells.
12. 6 h later, fill both reservoirs with 60 μl of complete culture medium, respectively.
13. The culture medium has to be changed daily in assays of a total duration longer than two days.

3 Optimization Protocol

3.1 Protein:Reagent Ratio

Start by optimizing the protein:reagent ratio for the used protein and particular cell type used. To do this, take the given starting-amount of 0.4 μg of protein and vary the protein:reagent ratio from 1:0.5 to 1:5. That means for 0.4 μg of protein 0.2 μl to 2 μl PROTEOfectene™ are used.

3.2 Amount of Protein

Then increase the amount of protein to be delivered while maintaining the previously determined ratio of protein to PROTEOfectene™. 0.2 μg to 0.8 μg per channel are protein amounts that have worked well in the past. However, these values can be exceeded or undershot in individual cases.

3.3 Alternative Protocol

If the amount of protein delivered into cells is low despite having optimized the protein:reagent-ratio and the amount of protein, the period of time between seeding the cells and adding the proteoplex can be shortened. This can – depending on the used cell line – increase the amount of protein delivered:

Seed the cells; incubate for 6 h; add proteoplex; incubate overnight; fill reservoirs with complete cell culture medium; incubate until evaluation.

3.4 Other Parameters

After having identified the optimal protein:reagent ratio and protein amount, you may continue to optimize if desired by varying other parameters, as listed below.

3.4.1 Cell Density

Best results are reached when cells are 50 – 70% confluent (percentage of growth surface covered with cells) at the delivery time.

3.4.2 Dilution Buffer of the Protein

1x PBS is recommended but other buffers (e.g. TRIS, HEPES, HBS) may be more appropriate depending on proteins.

3.4.3 Incubation Time

The optimal space of time between delivery and evaluation varies with cells, type of protein, biological function of the protein, etc.

Perform a time-course experiment to set up the optimal incubation time which will vary with – for example – protein activity or the half-life of the protein.

The delivery efficiency can be determined after 6 to 96 h.

3.4.4 Presence/Absence of Serum

PROTEOfectene™ can be used on cells in serum-free medium. In this case, replace the complete culture medium with serum-free medium. This procedure may be more efficient at delivering certain proteins in some cells. Add some serum-containing medium after 6 h, if further incubation time is needed.

4 Troubleshooting

4.1 Positive Control

If the evaluation of the test shows no protein delivery, a positive control within the test can indicate possible causes for the lack of protein delivery.

If the positive control shows protein delivery into cells, but the sample with your protein does not, it is probable that cell condition and density as well as general handling were in order. The error search should primarily focus on parameters affecting the lipoplex formation (protein:reagent-ratio; type and pH-value of the buffer; type, charge and purity of the protein).

If both, the positive control and the sample, show no protein delivery, further experiments should be conducted using the positive control only, before continuing tests with your own protein. R-Phycoerythrin has been delivered into many different cells, the probability of a successful transport is therefore high. The error search should therefore initially be focused on condition, health and type of the used cells.

If cytotoxicity is a problem, the positive control enables the user to determine whether the delivered protein is influencing cell viability.

4.2 Low Delivery Efficiency

4.2.1 Protein Purity

Make sure that the protein is highly pure and devoid of additives such as BSA or detergents.

4.2.2 Cell Density

A non-optimal cell density at the time of protein delivery can lead to insufficient uptake of the proteoplex. The optimal confluence (percentage of growth area covered by cells) ranges from 50 to 70%.

4.2.3 Cell Condition

Cells that have been in culture for a long time (> 8 weeks) may become harder to proteofect. Use freshly thawed cells that have been passaged at least once.

Cells should be healthy and proliferating well during the assay. The presence of contaminants (e.g. mycoplasma) diminishes the delivery efficiency considerably.

Biontex offers MycoSPY™ und MycoRAZOR™ for fast reliable detection and efficient removal of mycoplasmal contamination.

4.2.4 Medium used for Preparing the Proteoplex

Change the protein dilution buffer or the pH to improve the delivery. Highly alkaline proteins are difficult to deliver due to the presence of positive charges. This can be compensated in part by the hydrophobic properties of the protein.

The charge of the protein can be modified by means of the pH.

4.2.5 Old Proteoplexes

Proteoplexes have to be freshly prepared every time. Proteoplexes prepared and stored for more than 1 h aggregate, which leads to delivery of inactive clusters. Add proteoplexes immediately after their formation.

4.2.6 PROTEOfectene™ Temperature

The protein solutions and the reagent should be used at room temperature and should be vortexed prior to use.

4.2.7 PROTEOfectene™ Storage

Delivery efficiency can decrease if PROTEOfectene™ is kept at room temperature for more than one week.

4.3 Cellular Toxicity

4.3.1 Concentration of Proteoplexes is too High

To decrease the amount of proteoplexes, lower the amount of protein during complex formation while keeping the protein:reagent ratio constant.

Complex aggregation can cause toxicity; prepare complexes freshly and adjust the ratio as outlined in chapter 3.

4.3.2 Unhealthy Cells

- Check cells for contamination (e.g. for mycoplasma with MycoSPY™ from Biontex).
- Use freshly thawed cells, passaged at least once.
- Ensure optimal culture medium conditions (e.g. pH or type of medium used).

- Make sure cells are not too confluent or cell density is not too low. Cells should be 50 to 70% confluent.

4.3.3 Protein is Cytotoxic

Use suitable controls such as untreated cells and a positive control with R-Phycoerythrin.

4.3.4 Incubation Time

Reduce the incubation time of cells with complexes. The delivery medium can be replaced by fresh medium after 6 to 24 h if necessary.

4.3.5 Protein Quality

Use highly pure protein as impurities can lead to cell death.

4.3.6 Key Protein Delivery

The delivered protein itself can have an impact on cell vitality if it influences key points of the cells' metabolism.

5 Miscellaneous

5.1 Important Information

Biontex μ-Proteofection Kit VI and μ-Proteofection Kit VI AB are developed and sold for research purposes and *in-vitro* use only. It is not intended for human therapeutic or diagnostic purposes. Appropriate care should be exercised when handling many of the reagents described in this publication.

5.2 Warranty

Biontex guarantees the performance of PROTEOfectene[®] and PROTEOfectene[®] AB, when used in accordance with the information given in this publication, for a period of 12 months from the date of purchase. If you are not satisfied with the performance of the product please contact Biontex or one of its authorized distributors.

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