

## Cell Penetration

### Supporting material for product series CPP-C, CPP-P, CPP-A, CPP-K

#### 1 Introduction to Cell Penetration [1-12]

Plasma membranes protect cells from environment. They are commonly permeable only for lipids and small non-polar molecules. To transfect cells with plasmids, genes, micro-RNA, oligonucleotides and mimics of nucleic acids different chemical or mechanical methods are used, such as lipophilic and amphiphilic detergents, liposomes, viruses, microinjection, and electroporation. In many cases these methods give very poor yields of transfected cells and/or require additional selection of clones. Furthermore, these methods are usually not suitable for *in vivo* experiments, animal studies, or drug delivery to patients. Hence, other techniques are required to transport cargo through plasma membranes of live cells.

##### 1.1 Cell Penetrating Peptides (CPPs)

Fairly recently a number of peptides and proteins were shown to have cell penetrating properties. These molecules are termed "Trojan horse peptides", "Protein Transduction Domains" (PTDs) or "Cell penetrating peptides" (CPPs).

Such peptides and proteins with a *membrane transduction domain* are derived as partial sequences from transcription factors, bacterial surface proteins, toxins, amphiphatic helix-forming peptides and from ligands of membrane-bound proteins. They differ strongly in their amino acid sequences, but all contain a transduction domain. The most often used CPPs are penetratin, HIV-Tat, polyArg (R<sub>8</sub>), Pep-1, substituted ligo-peptides, ligands for integrins, for G-protein coupled receptors and for immuno-receptors.

##### 1.2 Cargo

CPPs are used to internalize peptides, proteins, oligonucleotides, nucleic acids, modified nucleic acids like PNAs or morpholino oligonucleotides and plasmids [9-10, 13-28].

##### 1.3 Cells

CPPs were successfully tested on a large number of different cells including mammalian cells [23] and plant cells [29], cell cultures and primary cells. They

are used for intracellular delivery into nerve cells [30] and for penetration of the blood-brain barrier [31].

#### 2 General Experimental Procedure

##### 2.1 Product Selection Guide

"Which CPP or CPP-cocktail shall I use?"

We offer CPPs that form **non-covalent** complexes with cargo. In addition to the classical **single CPPs**, the CPP-cocktails **JBS-Proteoducin** and **JBS-Nucleoducin** were developed as multi-component mixtures for maximum transduction rates and efficiencies. The different components of these cocktails ensure compatibility with various cell types and membrane structures, trigger different transduction mechanisms and are able to form complexes with structurally very different cargos. They exhibit only small cytotoxic effects and facilitate transduction of a large number of cell lines including HeLa, Swiss 3T3, NIH 3T3, COS-7, Jurkat and NB-4 with peptides, proteins and nucleic acids.

**JBS-Proteoducin (CPP-C01)**: This cocktail is developed for internalization of peptides and proteins and has been composed & optimized by using fluorescence-labelled bovine serum albumin (BSA), various primary and secondary antibodies and -galactosidase as model cargos. It is also suitable for transport of cargo coupled with quantum dots into cytosol or into the nucleus [32].

**JBS-Nucleoducin (CPP-C02)**: Internalization of mono- and oligonucleotides into live cells is a prerequisite for many studies to the signal transduction and in search for binding partners but remains a challenge. Current techniques using streptolysine strongly reduce cell viability and allow measurements of up to few minutes only [33]. **JBS-Nucleoducin** is a cocktail that is optimized for internalization of nucleotides into a broad variety of live cells with good transduction rates and with only marginal reduction of the cell viability.

The amphiphilic CPPs **MPG**, **MPG** and **CAD-2** are designed for formation of non-covalent complexes with peptides, proteins, plasmids and nucleic acids and their mimics. These CPPs differ strongly in their

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polarities. The most hydrophobic one is CAD-2, the most hydrophilic is MPG. Due to their specific charge distributions they have different preferences for cell membranes with different polarities allowing cell-line-specific experimental set ups.

**Penetratin** and **HIV-Tat** are classical CPPs that are widely used for forming conjugates or fusion proteins. Addition of a 10- to 20-fold excess of free peptide enhances the internalization strongly. In some cases also the internalization of non-covalent complexes with nucleic acids were described. We obtained good results with non-covalent complexes with nucleotides and satisfactory results with proteins.

The transduction activity of the BAX-inhibitory peptide **CPPP-2** is lower compared to the other offered CPPs however, the complex enters the cell by a yet unidentified mechanism not requiring interaction with proteoglycans. CPPP-2 is a cytoprotective peptide and improves cell viability. It can be used for cell protection and therefore combined with other CPPs or therapeutics, which are toxic or used in toxic concentrations. Complex formation requires a 100-fold molar excess over cargo

## 2.2 Basic Experiments

### 2.2.1 Planning and Considerations

*“What do I need for my experiments?”*

- Facilities for cultivation of adherent or suspension cells
- Equipment to detect internalized cargo such as by selective staining, fluorescence microscopy or by cell lysis followed by PAGE-electrophoresis and/or blotting
- Cocktail, single CPP or set of CPPs
- Reference cargo as positive control (e.g. **CPP-A08**, **NU-803-488**). Please note that the kits **CPP-K01** and **CPP-K02** already contain reference cargo

### 2.2.2 Preparation of Cells

#### General

- Cultivate cells under the commonly used optimum conditions
- Use cells only to the 3<sup>rd</sup> passage

- Add antibiotics (such as penicillin and streptomycin) to prevent growth of bacteria during incubation
- Check cells microscopically for their vital shape and the absence of bacteria
- Check for the absence of mycoplasma (e.g. using kit **PP-401**).

#### Adherent cell lines

- Seed  $0.3 \times 10^6$  cells per well in 2 ml complete growth medium (e.g. DMEM with 10% FCS for HeLa-cells)
- Incubate cells at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> until cells are 50-75% confluent
- Control the cell density microscopically

#### Suspension cells

- Seed  $0.3 \times 10^6$  cells per well in 2 ml complete growth medium. Cells are cultured in standard medium
- Harvest cells by centrifugation (400 g, 5 min)
- Remove supernatant and wash cells additionally 3x with PBS.

### 2.2.3 Complex Formation

#### General

*Complex formation using single CPPs:*

The herein described internalization by complexation of a cargo molecule with CPPs is the most convenient method requiring only mixing the components with subsequent formation of non-covalent aggregates. Avoid presence of high concentrations of inorganic salts, peptides and other proteins during complex formation since these may inhibit formation of non-covalent bonds. Dissolution of some CPPs requires a thorough disaggregating procedure.

For transduction of peptides and proteins a ten- to twenty-fold excess of CPP (molar ratio) is necessary while negatively charged nucleotides, nucleic acids and plasmid DNA require a ten-fold excess of positive charges from CPP.

*Complex formation using cocktails:*

Internalization of cargo into live cells depends on many parameters such as cell-type, state of the cell, used CPP, cargo, temperature and concentrations of all used components **[34-42]**. When using single

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CPPs, many of these parameters may have to be individually optimized.

With the cocktails **JBS-Proteoducin (CPP-C01)** and **JBS-Nucleoducin (CPP-C02)** we provide a universal approach for internalization of cargo through compatibility with numerous cell types with various membrane structures, triggering different mechanisms of transduction and allowing complexation with structurally different cargos. Generally, the cocktails show higher transduction rates and efficiencies compared to single CPPs alongside with convenience in handling.

#### Stock solutions

1. Prepare stock solutions of CPPs, JBS-Proteoducin or JBS-Nucleoducin according to the respective data sheets. Use sterile and oxygen free water (bubble helium or argon through the water).
2. Vortex and repeat 3x freezing to -80 °C and thawing.
3. Sonicate for 5 minutes.
4. Use stock solution immediately or aliquot and store aliquots at -20 °C to -80 °C for up to two months.

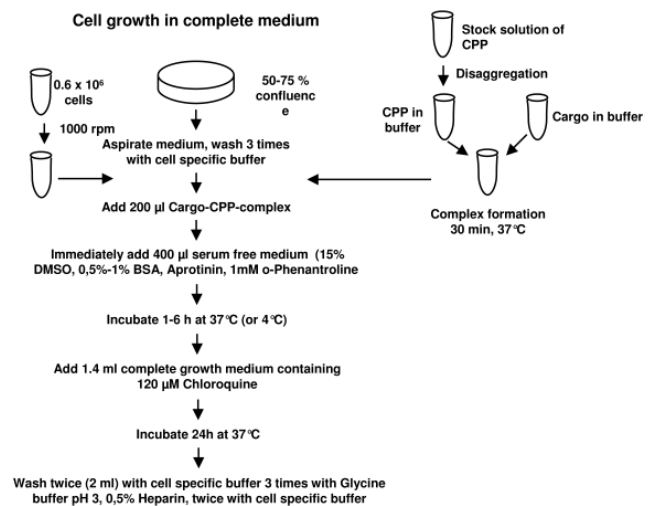
#### General procedure for complex formation

1. Dissolve 0.1 to 20 µg of cargo (peptide, protein or nucleotide), depending on sensitivity of detection or functionally necessary amount in 100 µl of phosphate buffer (e.g. Dulbecco's PBS 1x, without Ca and Mg, pH 7.0 - 7.5).
2. Dissolve CPP stock or cocktail (prepared according to data sheets) in 100 µl of the same phosphate buffer, use
  - for proteins: 1 µl CPP stock per µg of a protein with MW of 100 kDa (representing a molar ratio of 1:10); for MWs higher/lower than 100 kDa proportionally decrease/increase amount of CPP, respectively,
  - for nucleotides: 3-5 µl CPP stock per µg of nucleotide (MW ca. 500 Da, containing 3-4 negative charges) representing a molar ratio of 1:10; for lower/higher negative charges proportionally decrease/increase amount of CPP, respectively,
  - for plasmid DNA: Use 2.5 µl of JBS-Nucleoducin stock solution per µg of plasmid.

When using single CPPs use a ten-fold excess of positive charges from CPP (see data sheet) over negative charges from DNA. Consider plasmid DNA as a polymeric base pair.

3. Mix both solutions thoroughly by repeated pipetting (6x) **Important: Do not vortex or sonicate!**
4. Incubate mixture for 30 min. at 37 °C to achieve complex formation.

#### 2.2.4 Internalization



**Figure 1**

*Transduction of adherent and suspension cells in 6 well (35 mm) plates or tubes*

#### Volumes

The transfection protocol is calculated for 6-well / 35 mm culture plates. For other vessels please adjust volumes of media accordingly.

#### Removal of adsorbed cargo-complex

To remove adsorbed complex from the outside of the membranes a thorough washing of the cells with a glycine buffer of pH 3.0 [43] and/or with a heparin containing buffer [44] is recommended.

#### Adherent cell lines

1. Aspirate medium from prepared cells thoroughly and wash 3x with PBS at 37 °C.

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2. Add 200 µl of complex solution (read above) followed by 400 µl of serum free medium.
3. Mix gently and incubate for 1 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.
4. Add 1 ml of complete growth medium.
5. Continue incubation at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> for approx. 20 h.
6. Wash cells 2x with PBS, 3x with glycine buffer (pH 3, **CPP-A07**) and 2x with PBS (2 ml each).

#### Suspension cells

The cargo-complex with CPPs, JBS-Proteoducin or JBS-Nucleoducin is formed as described for adherent cells.

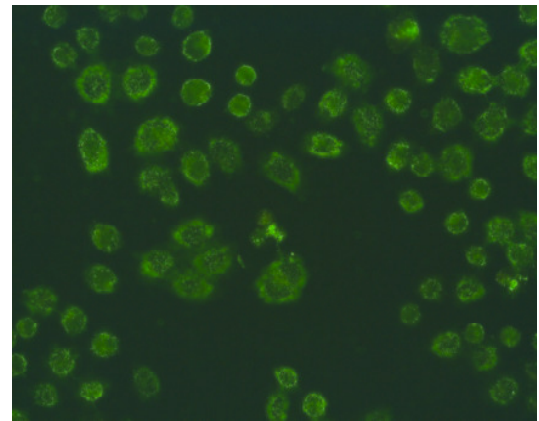
1. Suspend the thoroughly washed cell pellet in 200 µl of complex solution followed by 400 µl of serum free medium.
2. Mix gently and incubate for 1 hr at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.
3. Add 1 ml of complete growth medium.
4. Continue incubation at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> for approx. 20 h.
5. Wash/spin down cells twice with PBS, three times with glycine buffer (pH 3, **CPP-A07**) and twice with PBS (2 ml each). Process the cells for microscopic observation or for detection assays.

### 3 Detection of internalized cargo

#### 3.1 General

##### Transduction Rate

The transduction rate is calculated from the number of transduced cells over total number of cells in a transduction experiment. It depends highly on the amount of complex CPP:cargo used for transduction. Excess of CPP over cargo (more than 20:1) usually does not improve transduction rate but leads to formation of aggregates resulting in undesired adhesion of cargo to the cell membrane. JBS-Proteoducin can achieve transduction rates of nearly 100% (Fig. 2).



**Figure 2**

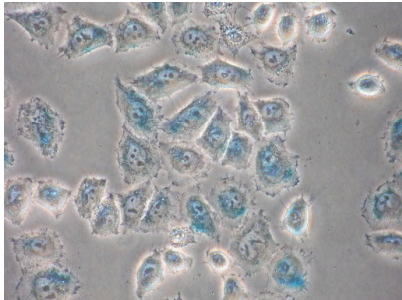
*Nearly 100% transduction of HeLa-cells with a fluorescent antibody using JBS-Proteoducin*

##### Transduction Efficiency

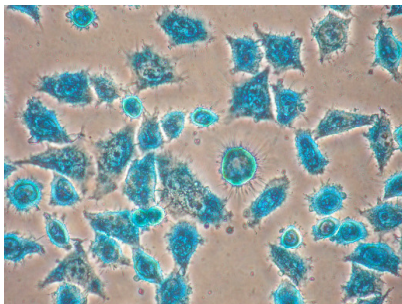
While transduction rate reflects only the number of cells penetrated with cargo, the term transduction efficiency is used for reflecting the amount of cargo actually internalized. In direct comparison to other commercially available transduction reagents based on CPPs, JBS-Proteoducin shows significant higher transduction efficiencies (Fig. 3).

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Competitor "Ch"



JBS-Proteoducin

**Figure 3**

Transduction efficiency with  $\beta$ -galactosidase as cargo is higher for JBS-Proteoducin compared to other commercially available CPP-products

### 3.2 Positive controls

Detection of internalized cargo is cargo-specific and typically requires microscopy combined with a selective staining reaction, a fluorescence reader or a specific electrophoretic/blotting detection. If possible characterize the internalized bioactive cargo functionally.

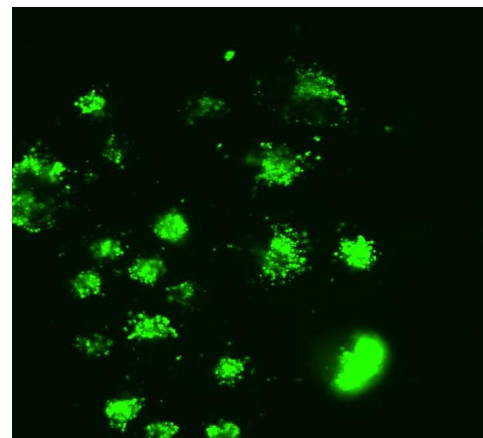
As positive control we offer a fluorescent protein (Atto-BSA [CPP-A08](#)) and a fluorescent nucleotide (Atto-dUTP [NU-803-488](#)).

#### Internalization of Atto-BSA ([CPP-A08](#))

Please refer to section 2.2.3. 0.5-2  $\mu$ g of Atto488-BSA (MW 68 kDa) in complex with 0.7-1.4  $\mu$ l of CPP stock are usually sufficient. Subsequently, internalize complex into adherent or suspension cells as described in section 2.2.4.

#### Internalization of fluorescent nucleotide Atto488-dUTP ([NU-803-488](#))

Prepare the complex according to section 2.2.3 from 0.2-2  $\mu$ l Atto488-dUTP (MW = 1092, 1mM) in 100  $\mu$ l PBS and 3-10  $\mu$ l stock solution of CPPs in 100  $\mu$ l PBS and transduce (section 2.2.4).



**Figure 4**

Internalization of Atto488-dUTP into Hela cells with JBS-Nucleoducin

#### Fluorescence microscopy

- Grow cells on a cover slip. Use cover slips with modified surface (poly-L-Lysine) for adhesion of suspension cells. Give the slip into a well before growing the adherent cells or before transferring transduced suspension cells.
- After incubation place cover slip on a slide and observe fluorescence directly without fixation. Use a magnification of > 100.
- Use fluorescence microscopy to visualize cell shape (viability), to estimate transduction rate and transduction efficiency and check intracellular distribution of cargo (vesicle, nucleus).

#### FACS-scan

- Wash cells thoroughly with PBS, and twice with acidic glycine buffer (pH 3.0, [CPP-A07](#)).
- Incubate briefly with 250  $\mu$ l PBS containing trypsin (0.1 mg/ml) and EDTA (0.22 mg/ml).
- Add 250  $\mu$ l of PBS.
- Transfer into tubes and centrifuge 5 min/1000 g.

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- Remove supernatant, suspend pellet in 100 µl of water and perform FACS-measurements.
- Use the FACS-analysis to estimate the percentage of transduced cells and differentiate with propidium iodide between live and dead cells.

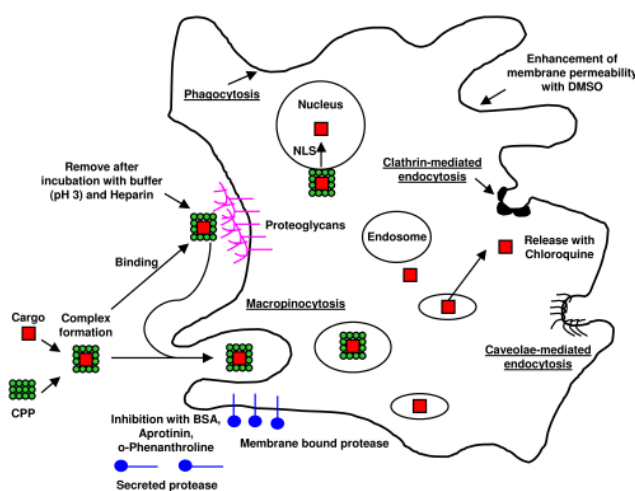
#### Fixation

Fixation may evoke internalization and can thereby lead to wrong results. If possible avoid fixation of cells or perform fixation only after finishing internalization and thorough washing (e.g. after finishing the staining procedure for  $\beta$ -galactosidase). Control/verify without fixation.

- Incubate cells on cover slips for 1h in PBS containing 4% formaldehyde.
- Aspirate the solution.
- Wash three times for 20 min with PBS.
- Wash twice with H<sub>2</sub>O.
- Embed cells with Mowiol-solution.

## 4 Improving Transduction

Efficient delivery of cargo into live cells heavily depends on cell line, cell number used, exposure & processing times, temperatures, and concentrations of cargo:CPP complex and auxiliary reagents. Conditions may have to be optimized for each cell line.



**Figure 5**

*Internalization of cargo into mammalian cells*

### 4.1 Time, Temperature & Concentrations

Since membrane permeability is temperature dependent, often large differences in internalization are observed at 4°C versus 37°C [45]. Generally, internalization can be improved by prolonged incubation with FCS-free medium, however, this may negatively affect cell viability. The higher the concentration of cargo:CPP complex usually the better the internalization. Hence, the highest complex concentration should be preferred as long as not decreasing viability and membrane integrity.

### 4.2 Additives

#### 4.2.1 Permeability Enhancers

Dependent on the membrane composition of cells addition of DMSO may enhance penetration. The highest possible concentration of DMSO should be estimated for each cell type testing the influence on cell viability and membrane integrity. We recommend the use of 10% DMSO in FCS-free transduction medium.

- Add 60 µl DMSO (CPP-A01) to 340 µl serum free medium (15%) and combine this solution with the cells incubated in 200 µl complex in PBS.

#### 4.2.2 Protease Inhibitors

Membrane bound and into the medium secreted proteases may inactivate CPPs. For example, the half live of penetratin in contact with CHO-cells was estimated to 5 minutes [46] indicating a need for using protease inhibitors. We recommend addition of 0.5-1% of BSA (CPP-A02) to the transduction medium. BSA can act as a co-substrate. Additionally, the protease inhibitors aprotinin (CPP-A05) and o-phenanthroline (CPP-A06) may be used. Both inhibitors may prolong the half live of CPPs significantly. To avoid internalization of inhibitors they should be added after complex formation. For cells with high activity of membrane bound and secreted proteases we recommend to add an inhibitor cocktail, however, cocktails with acid chlorides or fluorides must be avoided (reaction with SH-groups).

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- Incubate cells with 200 µl cargo-complex formed in PBS.
- Add BSA (0.5-1%, CPP-A02) and protease inhibitors, e.g. aprotinin (50 µg/ml, CPP-A05) and o-phenanthroline (1 mM, CPP-A06) to 400 µl serum free incubation medium.
- Immediately add this solution to the cells.

#### 4.2.3 Vesicle Destabilizers

Some mechanisms of internalization lead to formation of intracellular vesicles. For release of cargo from these vesicles they must be destabilized. For this purpose chloroquine (CPP-A03) [47], Ca<sup>2+</sup> [47] or wortmannin (INH-001) [34,35,39] have been proven effective when added to the medium.

- Incubate cells with 200 µl cargo-complex in PBS and 400 µl serum free medium for 1 hr.
- Add 1 ml serum containing medium completed with either chloroquine (150 µM, CPP-A03), wortmannin (100 µM, INH-001) or Ca<sup>2+</sup> (6 mM).

#### 4.3 Internalization of Large Cargo Amounts

In cases where the amount of internalized cargo needs to be increased the following strategies should be attempted:

- Prefer JBS-Proteoducin or JBS-Nucleoducin over single CPPs.
- Increase concentration of cargo:CPP complex but avoid changing the ratio of cocktail/ CPP and cargo since this mainly leads to formation of aggregates.
- Use additives as outlined in chapter 4.2.
- Proof cell viability [48] and membrane integrity [49,50,51] by application of enhanced complex concentrations. If necessary try to improve both cell parameters by addition of CPP-2 [52].

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