ACTIVE MOTIF®

Enabling Epigenetics Research

Chariot™

Catalog No. 30025 & 30100

(Version C3)

Revision	Date	Description of Change
C2	July, 2023	The “Sterile H_20” is now described as “Water”. However, the composition has not changed.
C3	October, 2023	The Active Motif beta-galactosidase Staining Kit is no longer available. Prepare reagents for staining as described in the manual or use any commercially available beta-galactosidase staining kit such as the Invitrogen beta-Gal Staining Kit (catalog no. K146501).

Contents

Overview

Chariot™ is a patented* transfection reagent suitable for the delivery of proteins, peptides and antibodies into a variety of cultured mammalian cells. Chariot Kits come in two sizes and can be used with many different plate sizes as indicated in the table below.

Plate	Chariot™	Transfections per Kit (25-reaction Kit)	Transfections per Kit (100-reaction Kit)
96-well	0.5 µL	300	1200
24-well	2 µL	75	300
12-well	3.5 µL	42	171
6-well or 35 mm	6 µL	25	100
60 mm	20 µL	7	30
100 mm	50 µL	3	12

Kit Contents

Component	25 rxns	100 rxns	Composition	Storage
Chariot Transfection Reagent	0.3 mg	1.2 mg		-20°C for up to 6 months
PBS	1 mL	1 mL	1.5 mM KH_2PO_4 150 mM $NaCl_2$ 5 mM Na_2HPO_4	-20°C
β-galactosidase (Positive Control)	25 µg	25 µg		-20°C for up to 6 months
Sterile H_2O	1 mL	1 mL		-20°C

Quality Control

- Chariot Transfection Reagent is functionally tested by delivery of protein and antibody into many different cell lines (including HS-68, NIH 3T3, and HeLa). Please visit our website for a complete list of cell lines that have been tested.
- Chariot Transfection Reagent is tested for the absence of bacterial and fungal contamination in DMEM cell culture media supplemented with 10% FBS.
- Chariot Transfection Reagent is non-cytotoxic at the recommended concentrations.

*Chariot is covered under U.S. Patent No. 6,841,535. Purchase includes the right to use for basic research purposes only. Other use licenses are available, please contact Active Motif Technical Services for additional information.

Introduction

Chariot™ is a revolutionary new transfection reagent capable of efficiently delivering proteins, peptides and antibodies into cultured mammalian cells in less than two hours.

Current transfection techniques include microinjection[1], calcium phosphate coprecipitation[2], cationic liposomes[3], viral vectors[4] and electroporation[5]. These methods are capable of transporting DNA into cells, but the techniques can be cumbersome and even cytotoxic. Once transfection has been completed, the researcher must wait 12-80 hours post-transfection to detect expression of the gene of interest.

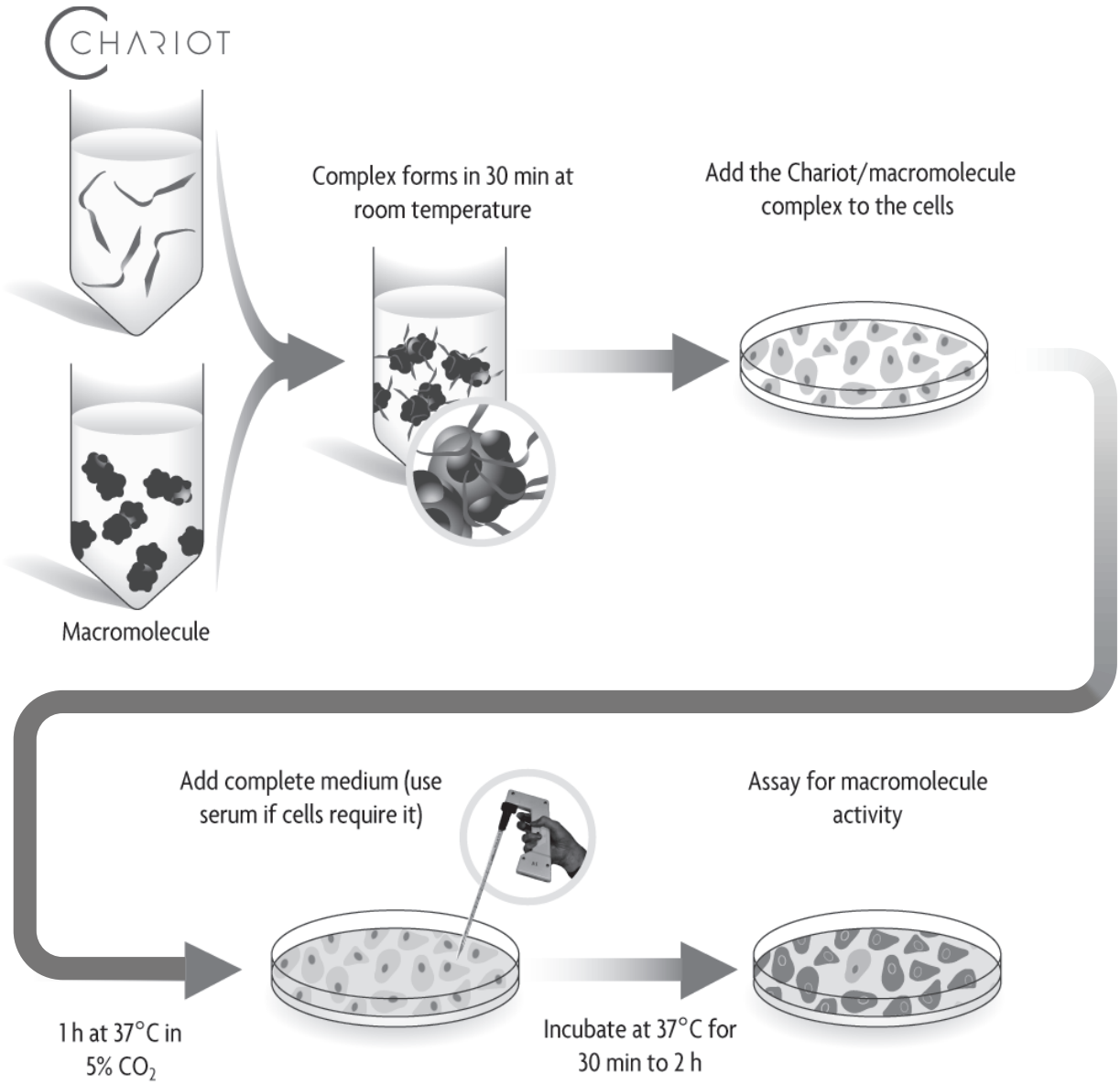
Recently, novel methods to deliver proteins have been reported and commercialized, *i.e.* HIV-1 TAT, *Drosophila* Antennapedia homeotic transcription factor and herpes simplex virus-1 DNA binding protein VP22 (for a recent review, see ref. 6). Transduction via TAT-fusion proteins results in inactivation and denaturation of the protein. To deliver an active protein, correct renaturation is required upon internalization. In addition, TAT must be covalently linked to the compound or macromolecule to be delivered by a chemical reaction.

Penetratin 1 is a 16 amino acid peptide corresponding to the third helix of the homeodomain of Antennapedia protein. Activated Penetratin has an N-terminal pyridyl disulfide that is used to covalently couple it to the macromolecule to be delivered. However, chemical coupling can be cumbersome and requires the macromolecule being delivered to carry a free thiol group.

Another delivery system utilizes the translocation properties of the 38 kDa herpes simplex virus-1 DNA binding protein VP22. VP22 must be fused to the peptide/protein to be delivered and, therefore, requires the construction of a suitable expression vector.

Chariot is a 2843 dalton peptide and unlike these current transfection techniques, Chariot forms a non-covalent complex with the protein, peptide or antibody of interest. This completely bypasses the transcription-translation process associated with gene expression, reducing the time until the cells can be assayed from days to under two hours. The Chariot-macromolecule complex stabilizes the macromolecule and helps to protect it from degradation during the transfection process[7, 8]. Upon internalization, the complex dissociates and the macromolecule is free to proceed to its target organelle. Moreover, efficient delivery is observed at 4ºC, suggesting that the delivery mechanism is independent of the endosomal pathway[8]. Therefore, the macromolecule is not subjected to the conditions of that pathway, which can modify the structure of the macromolecule during internalization. Chariot is non-cytotoxic and serum-independent[8]. It is ideal for *in vivo* studies because fixing is not required.

Flow Chart of Process



Protocols

Preparation of Chariot

Chariot is provided as a lyophilized powder and arrives at room temperature. Store at -20ºC for long term storage.

25-reaction Kit: 0.3 mg of Chariot is provided. Resuspend the entire contents of the Chariot vial in 150 µL sterile H_2O. Mix gently by tapping tube.

100-reaction Kit: 1.2 mg of Chariot is provided. Resuspend the entire contents of the Chariot vial in 600 µL sterile H_2O. Mix gently by tapping tube.

After resuspension, store at -20°C in small aliquots to avoid repeated freeze/thaw cycles.

Note: If you will be transfecting a peptide or low molecular weight protein (< 10 kDa), you must make an additional 1:10 dilution of the resuspended Chariot solution in sterile H_2O before use. This will improve transfection efficiency by preventing the formation of aggregates.

Note: Diluting Chariot in DMSO has been shown to improve delivery of some antibodies. It has also provided more uniform delivery of some proteins. Please refer to the sections in the Troubleshooting Guide (page 10) for more information.

Preparation of β-galactosidase

β-galactosidase is an enzyme that hydrolyses β-galactosides, such as lactose and the artificial chromogen X-gal. β-galactosidase is composed of four identical subunits. When β-galactosidase hydrolyses X-gal, it produces a blue color that can be visualized under a bright field microscope. A 119 kDa subunit of the β-galactosidase protein is provided in this kit as a positive control for the Chariot Transfection Reagent.

The β-galactosidase positive control protein is provided as a lyophilized powder. Store at -20ºC. 25 µg of protein is provided. Resuspend the entire contents of the β-galactosidase positive control vial in 100 µL sterile H_2O. This makes a 0.25 µg/µL stock. Use 1.0 µg β-galactosidase per transfection reaction. After resuspension, aliquot and store at -20°C to avoid repeated freeze/thaw cycles.

When the β-galactosidase positive control protein is transfected, the cells must be stained to assay for the efficiency of the transfection. A detailed staining protocol and buffer recipes are provided on page 8. Alternatively a commercially available beta-galactosidase Staining Kit such as the Invitrogen beta-Gal Staining Kit (catalog no: K146501) can be used.

Transfection Protocol

These conditions are recommended as guidelines only. Efficient transfection may require optimization of reagent concentration, cell number and exposure time of cells to the Chariot-macromolecule complex. Conditions should also be optimized for each cell line and kept consistent to obtain reproducible results. This procedure has been optimized for the transfection of adherent cells using the β-galactosidase positive control. A protocol for using suspension cells can be found on page 7.

1. Seed cells according to the following table:

Plate	Surface Area	Cells	Growth Medium
96-well	20 mm^2	1.0 x 10^4	0.2 mL
24-well	200 mm^2	0.05 x 10^6	0.5-1.0 mL
12-well	401 mm^2	0.1 x 10^6	1-2 mL
6-well or 35 mm	962 mm^2	0.3 x 10^6	3-5 mL
60 mm	2827 mm^2	0.8 x 10^6	5 mL
100 mm	7854 mm^2	2.2 x 10^6	10 mL

2. Incubate the cells at 37°C in a humidified atmosphere containing 5% CO_2 until the cells are 40-50% confluent.

Note: The transfection efficiency may be sensitive to culture confluency, so it may be necessary to optimize cell density for each cell line.

3. **Preparation of diluted macromolecule:** Dilute your protein, antibody or peptide of interest in the appropriate volume of PBS as indicated in the table below. For antibodies or proteins, we recommend using 0.5-1 µg per transfection reaction. For peptides or proteins that are < 10 kDa, we recommend using 100-500 ng per transfection reaction.

Note: The amount of macromolecule used must be optimized, we recommend performing a titration of the protein/antibody or peptide quantities.

Plate	Final Volume of Macromolecule Dilution in PBS
96-well (or 8-well chamber slide)	10 µL
24-well	50 µL
12-well	100 µL
6-well or 35 mm	100 µL
60 mm	200 µL
100 mm	200 µL

* If you are using 96-well plates, you may wish to reduce the amount of protein or antibody used to 0.2 µg and amount of peptide to 50 ng.

4. **Preparation of diluted Chariot:** In a separate tube, dilute the appropriate volume of Chariot into sterile water as indicated in the table below. For low molecular weight proteins and peptides, a 1/10 dilution of the resuspended Chariot must be made before use to ensure optimal transfection.

Plate	**Sterile H_20 for Chariot Dilution**	Protein/Antibody* **Volume of Chariot**	Peptide/LMW Proteins** **Volume of a 1/10 Dilution of Chariot**
96-well	10 µL	0.5 µL	0.5 µL
24-well	50 µL	2 µL	2 µL
12-well	100 µL	3.5 µL	3.5 µL
6-well or 35 mm	100 µL	6 µL	6 µL
60 mm	200 µL	20 µL	20 µL
100 mm	200 µL	50 µL	50 µL

For multiple transfections, do not use a master mix that exceeds the volume required for 4 transfections (6-well plate size) per tube, as this may cause aggregation. (**Note:** This does not apply to peptide transfections)

*Detection of fluorescent antibodies in cells requires a large amount of antibody to be delivered. It may be necessary to increase the amount of Chariot and antibody used.

**Chariot interacts via hydrophobic interactions. Each peptide or protein will have a different hydrophobicity, which is more apparent with lower molecular weight molecules. The amount of Chariot may need to be titrated to determine the optimal amount.

5. **Preparation of Chariot-macromolecule complex:** Add the diluted macromolecule from Step 3 to the diluted Chariot from Step 4 and incubate at room temperature for 30 minutes to allow the Chariot-macromolecule complex to form.
6. **Transfection of cells:** Aspirate medium from cells to be transfected and wash cells once with PBS.
7. Overlay the cells with the Chariot-macromolecule complex. Next, add the appropriate amount of serum-free medium to the plate as indicated in the table below. Gently rock the plate to ensure even delivery.

Plate	**Volume of Chariot-Macromolecule Complex**	**Serum-Free Medium**	**Final Transfection Volume**
96-well	20 µL	80 µL	100 µL
24-well	100 µL	100 µL	200 µL
12-well	200 µL	150 µL	350 µL
6-well or 35 mm	200 µL	400 µL	600 µL
60 mm	400 µL	1600 µL	2000 µL
100 mm	400 µL	4600 µL	5000 µL

8. Incubate at 37°C in a humidified atmosphere containing 5% CO_2 for one hour.
9. Continue to incubate at 37°C in a humidified atmosphere containing 5% CO_2 for 30 minutes to 2 hours.

   Optional: If your cells are sensitive to serum starvation you can choose to add complete growth medium to the cells. **Do not** remove the Chariot-macromolecule complex. We suggest adding the minimum amount of complete growth medium needed as the presence of serum can interfere with Chariot transfection efficiency.

   Peptide: 0.5-1 hour

   Protein: 1-2 hours

   Antibody: 2 hours

10. Process the cells for observation or detection assays. Cells may be fixed or observed directly.

Transfection Protocol for Suspension Cells

These conditions are recommended as guidelines only. Efficient transfection may require optimization of reagent concentration, cell number and exposure time of cells to the Chariot-macromolecule complex. Conditions should also be optimized for each cell line and kept consistent to obtain reproducible results.

1. Seed cells according to the following table:

Plate	Surface Area	Cells	Growth Medium
96-well	20 mm^2	1.0 x 10^4	0.2 mL
24-well	200 mm^2	0.05 x 10^6	0.5-1.0 mL
12-well	401 mm^2	0.1 x 10^6	1-2 mL
6-well or 35 mm	962 mm^2	0.3 x 10^6	3-5 mL
60 mm	2827 mm^2	0.8 x 10^6	5 mL
100 mm	7854 mm^2	2.2 x 10^6	10 mL

2. The Chariot-macromolecule complex is assembled in the same manner as described for adherent cells. See steps 3-5 on pages 5-6.
3. Collect the suspension cells by centrifugation at 200-400 x *g* for 5 minutes. Remove the supernatant.
4. Wash the cells twice with 1X PBS.
5. Centrifuge at 200-400 x *g* for 5 minutes to pellet the cells. Remove the supernatant.
6. Resuspend the cell pellet in the Chariot-macromolecule complex. Add serum-free medium to achieve the Final Transfection Volume. (See step 7 page 6)
7. Incubate at 37°C in a humidified atmosphere containing 5% CO_2 for one hour.

8. Continue to incubate at 37°C in a humidified atmosphere containing 5% CO_2 for 30 minutes to 2 hours.

   Optional: If your cells are sensitive to serum starvation you can choose to add complete growth medium to the cells. Do not remove the Chariot-macromolecule complex. We suggest adding the minimum amount of complete growth medium needed as the presence of serum can interfere with Chariot transfection efficiency.

9. Process the cells for observation or detection assays. Cells may be fixed or observed directly.

β-galactosidase Staining Protocol

The solutions below can be used to stain and determine the percentage of cells that are successfully transfected with the beta-galactosidae protein. Alternatively a commercially available beta-galactosidase Staining Kit such as the Invitrogen beta-Gal Staining Kit (catalog no: K146501) can be used.

Make Stock Solutions

Solution	Storage
400 mM potassium ferricyanide	-20°C
400 mM potassium ferrocyanide	-20°C
200 mM magnesium chloride	-20°C
20 mg/mL X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside resuspended in DMF (N, N-Dimethyl-formamide)	-20°C in the dark
10X PBS	-20°C
10X Fixing Solution 20% formaldehyde 2% glutaraldehyde in 10X PBS	-20°C

Staining Protocol

Note: Use volumes appropriate for the size of the plate or dish. Ensure that the Fixing and Staining Solutions cover the cells.

1. Remove the growth medium from the transfected cells and rinse 3 times with 1X PBS.
2. Add 1X Fixing Solution. (Dilute the 10X stock in sterile water to make a 1X solution.) Incubate at room temperature for 5-10 minutes.

3. Prepare the Staining Solution. **Staining Solution should be made fresh each time.**

Solution	Final Volume
250 µL 400 mM potassium ferricyanide	4 mM
250 µL 400 mM potassium ferrocyanide	4 mM
250 µL 200 mM magnesium chloride	2 mM
1.25 mL X-gal (20 mg/mL in DMF)	1mg/mL
23 mL 1X PBS	23 mL
Total Volume	**25 mL**

4. Rinse the cells twice with 1X PBS.
5. Add the Staining Solution to the cells and incubate at 37°C for 30 minutes to 2 hours.
6. Check the cells under a microscope. Calculate the percent of cells transfected with β-galactosidase.

$$\frac{\text{Total no. of blue cells}}{\text{Total no. of cells}} \times 100 = \%\ \text{transfection}$$

References

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5. Neumann, E. *et al.* (1982) *EMBO J.* 7: 841-845.
6. Schwarze, S.L. *et al.* (2000) *Trends Cell Biol.* 10: 290-295.
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Troubleshooting Guide

Problem	Possible Cause	Recommendation
Low Transfection Efficiency	Cell density	Use adherent cells at a confluency of 40-50%
	Chariot or macromolecule dilutions were performed in larger volumes than recommended	Dilute the macromolecule into the final volume of PBS as recommended. Dilute Chariot in the volume of sterile water recommended. It is necessary to make the Chariot macromolecule complex in a concentrated solution. For multiple transfections, do not use a master mix that exceeds the volume required for 4 transfections (6-well plate size) per tube, as this may cause aggregation.
	Chariot and macromolecule were not complexed for 30 minutes	Combine the Chariot dilution and the macromolecule dilution together and allow the complex to form at room temperature for 30 minutes.
	Improper storage of Chariot	Chariot may form aggregates over time if not stored properly. You may need to sonicate the Chariot dilution just before complex formation. We recommend placing the Chariot dilution in a water bath and sonicating the water at close proximity with an ultrasonic processor. At Active Motif, we routinely use Sonics Vibra-cell™ for 1 minute at an amplitude of 30 and a 1-second pulse.
	Medium that contained serum was used to dilute the Chariot-macromolecule complex	Repeat the transfection using serum-free medium to dilute the Chariot-macromolecule complex to the Final Transfection Volume.
	Exposure of Chariot-macromolecule complex to cells was insufficient	Leave the Chariot-macromolecule complex on the cells for a minimum of two hours.
	Using a cell line other than those tested with Chariot	Try the transfection in a cell line proven to work with Chariot. Please visit our website for complete in formation on cell lines that have been tested
	A suboptimal amount of macromolecule was used for the transfection	You may need to titrate the amount of macromolecule (protein, peptide, or antibody) used in the transfection.

Problem	Possible Cause	Recommendation
	A suboptimal amount of Chariot was used for the transfection	You may need to titrate the amount of Chariot used in the transfection. Chariot interacts via hydrophobic interactions. Each peptide or protein will have a different hydrophobicity and may require a different amount of Chariot. Depending on the sensitivity required for antibody detection it may be necessary to alter the amount of Chariot used. Detection of fluorescently labeled antibodies in cells requires a large amount of antibody to be delivered. This may mean increasing the amount of Chariot used.
	Diluting Chariot into DMSO may improve antibody delivery	Diluting Chariot into DMSO has improved the delivery of some antibodies. If delivery of your antibody is low, try diluting Chariot into 60-80% DMSO instead of water. After the Chariot dilution is combined with the antibody dilution, the complex mix ture will have a final concentration of 30-40% DMSO. This may need to be lowered for certain cell lines. However, a final concentration of complex mixture below 10-20% DMSO has not been shown to improve antibody delivery.
Protein delivery is localized to a few regions of the cell	Diluting Chariot into DMSO may make protein delivery more uniform	With some proteins that we have tested, delivery of the protein seems to be localized to specific regions in the cell. Diluting Chariot into DMSO instead of water has made the delivery of some of these proteins more uniform. Dilute the Chariot into 40-60% DMSO. After the Chariot dilution is combined with the protein dilution, the complex mixture will have a final concentration of 20-30% DMSO.
Aggregate formation	Improper storage of Chariot	Chariot may form aggregates over time if not stored properly. You may need to sonicate the Chariot dilution just before complex formation. We recommend placing the Chariot dilution in a waterbath and sonicating the waterbath with an ultrasonic processor, such as a Sonics Vibra-cell™, for 1 minute at an amplitude of 30 and a 1-second pulse.
	Excess Chariot used	Decrease the amount of Chariot used

Problem	Possible Cause	Recommendation
Signs of cytotoxicity **Note:** Chariot is non-cytotoxic to cells at the recommended concentrations	Transfected macromolecule may be cytotoxic	Transfect the macromolecule at a lower concentration. Compare cells only, cells with Chariot alone and cells with macromolecule alone. Chariot should not affect cell viability. Transfect the β-galactosidase control protein.
	Culture may be contaminated with mycoplasma	Treat the cells to eliminate mycoplasma.
	Cells may not be healthy	Check the incubator (CO_2 and temperature levels). Check the media
	Too much DMSO present	Reduce the final concentration of DMSO to a final concentration below 40%

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

North America	Toll free:	877 222 9543
	Direct:	760 431 1263
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