Third Edition **February 2007**

Mass·Spec·Focus IMAC Chip Handbook

Mass·Spec·Focus IMAC Chips

For purification of phosphopeptides
for MALDI mass spectrometry analysis

QIAGEN®

Sample & Assay Technologies

Contents

Pack Contents

Mass·Spec·Focus IMAC Chips

	Compatible instrument	Chips per pack	Sample sites per chip	Cat. no.
Type 1	Shimadzu Kratos	6	16	49400
Type 2	Waters Corporation	1	96	49401
Type 3	Applied Biosystems	1	25	49402
Type 4	Applied Biosystems	1	64	49403
Type 5	Thermo Electron	6	16	49404

As part of our commitment to expand the range of instruments compatible with Mass·Spec·Focus IMAC Chips, the contents of your chip package may differ from this table. For an up-to-date list of supported instruments, and pack content and ordering information, please refer to the QIAGEN mass spectrometry web page (www.qiagen.com/maldiprep).

Storage and Stability

Mass·Spec·Focus Chips should be stored in their original packaging in dry conditions at room temperature (15–25°C) and protected from dust and light. After initial opening, the foil pouch should be kept closed and the chips stored under argon or nitrogen to prolong their shelf life.

Mass·Spec·Focus Chips can be stored under these conditions for up to 6 months without showing any decrease in performance.

Note: Unused sites on the chip surface can be affected by the conditions during MALDI analysis. Therefore, it is not advisable to repeatedly use part of a chip, perform analysis, remove the chip, and perform subsequent analyses in unused sites. Where possible, all sites of a chip should be used and analyzed in the same session. However if this is not possible, we recommend analyzing all sites on a chip within three sessions. In addition, the amount of time that chips spend in the instrument should be limited to that required for analysis.

Once a site has had matrix applied, the surface is no longer affected by the MALDI conditions in the instrument and the site can be archived and repeatedly analyzed. Archived chips should be stored at room temperature (15–25°C) in the dark in their original packaging, in dry conditions and protected from dust.

Reagents in the Mass·Spec·Focus Chip Solvent Kit should be stored at room temperature (15–25°C).

Quality Control

In accordance with QIAGEN's ISO-certified Total Quality Management System, each lot of Mass·Spec·Focus IMAC Chips is tested against predetermined specifications to ensure consistent product quality.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany, Tel: +49-6131-19240

Product Use Limitations

Mass·Spec·Focus IMAC Chips are developed, designed, and sold for research purposes only. They are not to be used for human diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this text.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside back cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in life sciences and the use of QIAGEN® products. If you have any questions or experience any difficulties regarding Mass·Spec·Focus IMAC Chips or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see inside back cover).

Introduction

The complexity of the proteome derives not only from the number of individual proteins present in a cell, but also from their post-translational modifications. Additionally, splice variants may be present, adding further to the complexity of the system. One of the most common post-translational modifications of proteins is phosphorylation of serine, threonine, and tyrosine residues. Phosphorylation of proteins plays a vital role in cell signaling, oncogenesis, apoptosis, and immune disorders. Around a third of all eukaryotic gene products can be post-translationally phosphorylated.

Mass·Spec·Focus IMAC chips utilize IMAC (immobilized metal ion affinity chromatography) to enrich the phosphorylated peptides in a digested protein sample. The phosphorylated peptides are detected with high sensitivity by MALDI mass spectrometry.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) was developed in the late 1980s for mass spectral analysis of polypeptides and proteins. The main advantage of MALDI MS over conventional MS is that during the soft-ionization process, little or no fragmentation of analytes takes place, greatly facilitating identification of analyte molecular ions, even within mixtures. The technique has become a mainstay of proteomics research, due in large part to its ability to identify proteins from their characteristic peptide mass "fingerprints".

The MALDI experimental process

Adding a low molecular weight organic matrix to a more massive analyte prevents molecular photodissociation of the sample ions induced by direct laser irradiation. The laser target is formed from a dilute solution of the analyte in a matrix of molecules that efficiently absorb laser light. Such molecules include α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2,5-dihydroxybenzoic acid (DHB). The analyte/matrix mixture is applied to the target stage of a mass spectrometer and allowed to dry. Upon drying, a crystalline deposit is formed, comprising the matrix with evenly dispersed analyte molecules. The matrix is then irradiated by a short-duration pulse of laser light, which causes desorption (vaporization) of the analyte and matrix. In the "plume" of matrix and analyte vapor generated above the target, analytes are protonated by the photo-excited matrix. As the ions are effectively generated instantaneously, they can be separated in time when they are accelerated by a strong electric field under vacuum. After they hit a detector, their time-of-flight (TOF) is calculated. This time is a function of an ion's mass-to-charge (m/z) ratio, enabling an ion's mass to be derived from its TOF.

The sensitivity and speed of MALDI-MS analysis have led to its being used for the majority of protein and oligonucleotide analyses in high-throughput proteomics and genomics projects.

The Mass·Spec·Focus IMAC Chip principle

Mass·Spec·Focus IMAC Chips are disposable devices for purification and MALDI-MS analysis of phosphopeptides in proteomics samples using SPOC (Sample Preparation On a Chip) technology. The chips contain a number of sample sites that allow the processing of biological samples directly on the chip surface (Figure 1).

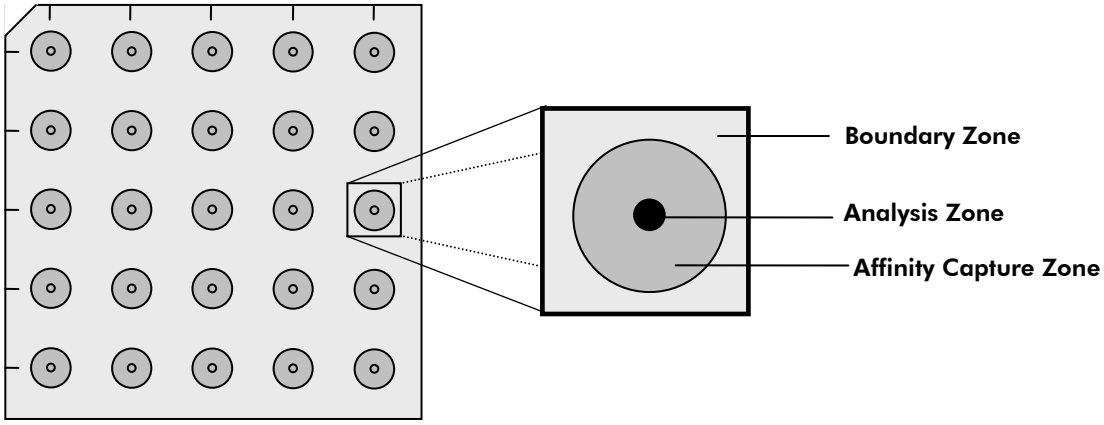


Figure 1 Structure of a Mass·Spec·Focus IMAC Chip sample site (shown here on a 5 x 5 Mass·Spec·Focus Type 3 Chip; see Appendix E for other formats).

Each site is created by the juxtaposition of proprietary surface chemistries on a flat surface in discrete concentric circular zones of wettability. The central, most wettable area is the Analysis Zone, which is surrounded by the less wettable Affinity Capture Zone. The Affinity Capture Zone provides a surface for phosphopeptide binding. Encircling both zones is the non-wettable Boundary Zone.

At first glance, it appears that Mass·Spec·Focus IMAC Chips have no surface features. Depending on the type of chip, registration marks on the chip may define a grid on which the centers of the sample sites are located. For those chips that have marks on their edges, it is recommended that the site closest to a corner is used first, as they are the easiest to locate. Once this site contains liquid, the positions of the other wells — which are based on the Society for Biomolecular Screening's standard 9 mm or 4.5 mm center-to-center spacing — are easy to locate.

"On-chip" purification and concentration of phosphopeptides

Using Mass·Spec·Focus IMAC Chips, purification and concentration of phosphopeptides is accomplished as shown in the flowchart on page 10. Firstly, sample sites are loaded with ferric (Fe^{3+}) ions that will bind the phosphopeptides. After washing, sample is applied and phosphopeptides are bound in the Affinity Capture Zone. Non-phosphorylated peptides are then removed by washing the chip. Addition of an Elution Solution focuses the sample into the Analysis Zone. Addition of matrix resolubilizes the analytes and as the matrix/sample mixture dries, analyte and matrix are co-focused and crystallized in the Analysis Zone. A typical example of an enrichment of phosphopeptides using Mass·Spec·Focus IMAC Chips is shown in Figure 2.

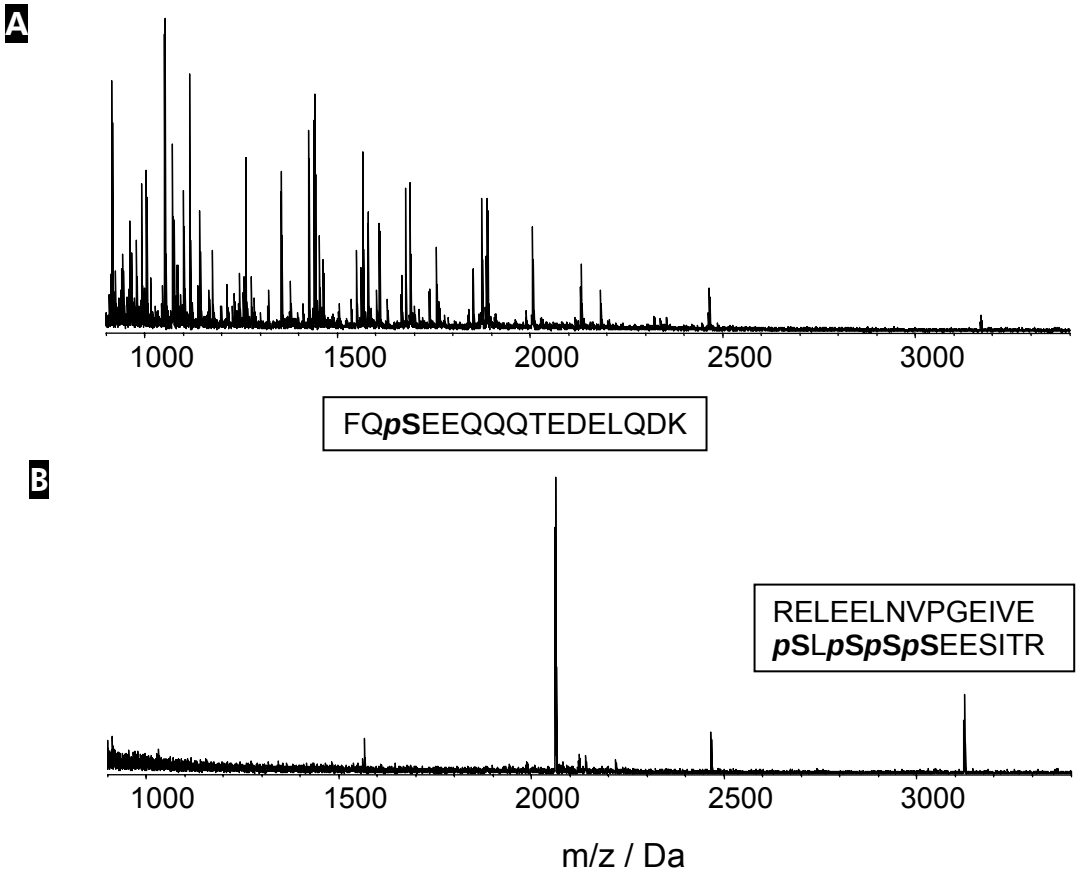
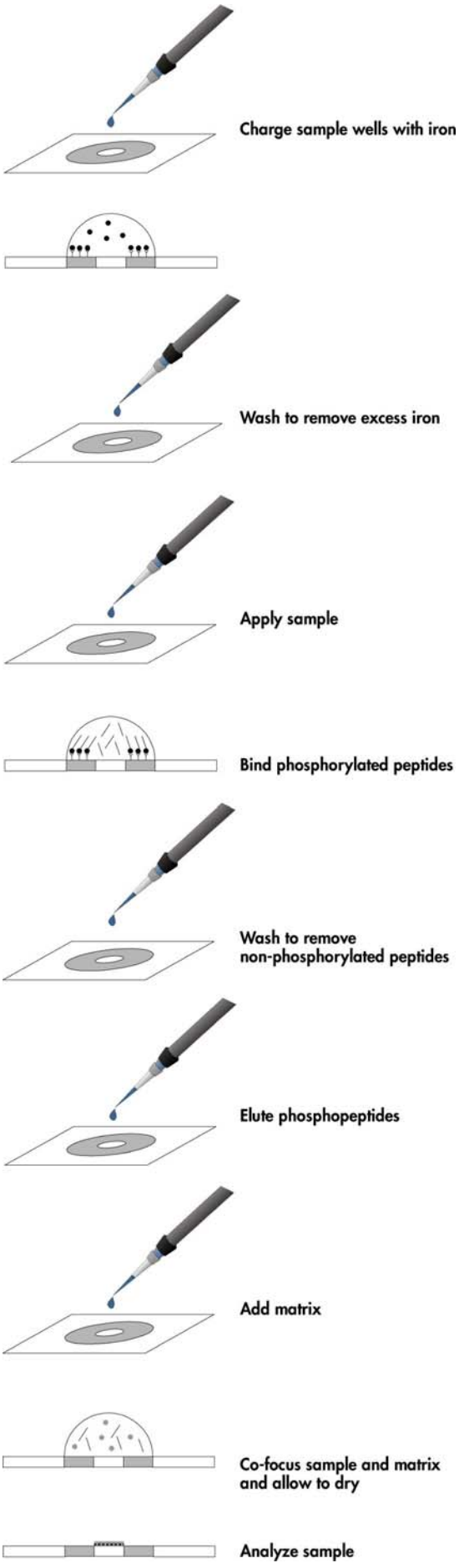


Figure 2 A mixture of 10 fmol β-casein tryptic digest (two phosphopeptides) and 100 fmol phosphorylase B digest (no phosphopeptides) was processed and analyzed on **A** a Mass·Spec·Focus Chip and **B** a Mass·Spec·Focus IMAC Chip. The two specifically enriched peptides isolated using the IMAC chip correspond to Arg48–Lys63 with a phosphorylated serine residue at position 50 (m/z = 2062) and Arg16–Arg40 with phosphorylated serine residues at positions 30, 32, 33, and 34 (m/z = 3123).


Charge sample wells with iron
Wash to remove excess iron
Apply sample
Bind phosphorylated peptides
Wash to remove
non-phosphorylated peptides
Elute phosphopeptides
Add matrix
Co-focus sample and matrix
and allow to dry
Analyze sample

Alternative protocols and applications for Mass·Spec·Focus Chips

The protocols in this handbook describe the basic application of Mass·Spec·Focus IMAC chips for phosphopeptide enrichment with iron cations. However, Mass·Spec·Focus IMAC chips can be used with other metal ions and for other applications in proteomics. For additional protocols for Mass·Spec·Focus IMAC chips, please, check the QIAGEN mass spectrometry web page (www.qiagen.com/maldiprep).

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques or alternative protocols for Mass·Spec·Focus IMAC chips. To contact us send an email to MassSpec@qiagen.com.

Materials and reagents compatible with Mass·Spec·Focus IMAC Chips

The extreme sensitivity of MALDI-MS analysis requires that reagents used to prepare samples are of the highest quality. The components of the Mass·Spec·Focus Chip Solvent Kit (cat. no. 49200) have been proven to deliver excellent results with Mass·Spec·Focus IMAC Chips. We strongly recommend using the materials in this kit to obtain optimal performance.

Solvents

Because of the concentrating effect of Mass·Spec·Focus IMAC Chips, it is critical that all solvents and reagents are of the highest purity. Lower-grade solvents interfere with both focusing and spectrum acquisition.

Plasticware

Many plastics are incompatible with Mass·Spec·Focus chips because they may release plasticizers, polymers, or other stabilizing agents from the manufacturing process. This often results in the presence of strong polymer signals in MS spectra or poor-to-incomplete focusing and crystallization. For this reason, tubes (and any other plasticware such as pipet tips) labeled as “Low-Retention” or “Siliconized” should be strictly avoided.

Table 1. Materials and Reagents Certified for Use with QIAGEN Mass·Spec·Focus chips

Item	Recommended supplier
Thermometer/Hygrometer	Fisher Scientific, cat no. 11.661.14VWR, cat. no. 35519-049 Control Company, product number 4093
15 ml and 50 ml sterile (DNase and RNase free) PP-tubes	Greiner Bio-One GmbH, Frickenhausen, Germany; cat. nos. 188261 & 227270
Rainin LTS pipet tips (10, 250, and 1000 µl)	Rainin Instruments, LLC., Woburn, MA, USA; cat. no., various types e.g., cat. nos. GPS-L10, GPS-L250, & GPS-L1000
Rainin traditional pipet tips (10, 250, and 1000 µl)	Rainin Instruments, various types, e.g., cat. nos. RT-10, RT-250, & RT-1000
epT.I.P.S pipet tips	Eppendorf AG, Hamburg, Germany or Brinkmann Instruments, Westbury, NY, USA, various types
Safe-Lock microcentrifuge tubes (0.5 ml and 1.5 ml)	Eppendorf AG, cat . nos. 0030 121.023 & 0030 120.086 or Brinkmann Instruments, cat. nos. 22363611 & 22363204 (distributed by Fisher Scientific)
Iron (III) Chloride Hexahydrate	Sigma-Aldrich, St. Louis, MO, USA, cat. no. 236489 or Fluka cat. no. 44944
Urea (Sequanal Grade)	Pierce, Rockford, IL, USA, cat. no. 29700
85% Phosphoric acid (100 ml)	Sigma-Aldrich, cat. no. 345245
Acetonitrile (30 ml)	QIAGEN, Mass·Spec·Focus Chip Solvent Kit, cat. no. 49200 or Riedel del Häen (Sigma-Aldrich, cat. no. 34967)
Water, ACS reagent	Sigma-Aldrich, cat. no. 320072
Octyl β-D-glucopyranoside (OBG), SigmaUltra (500 mg)	Sigma-Aldrich, cat. no. O9882
Acetic acid p.a.	Merck (VWR , cat.no. 1.00063.1000)
Phosphopeptide standard mixture	Invitrogen/Molecular Probes; cat. no. P33357
Rubis K35a Plastic Tweezers	Electron Microscopy Sciences, Hatfield, PA, USA, cat. No. 78193-01

Measuring humidity

Crystallization of sample and matrix on Mass·Spec·Focus Chips is sensitive to humidity changes. Controlling humidity is important to the ultimate reproducibility of this process in the Analysis Zone. To check temperature and relative humidity (%RH) we strongly recommend using one of the combination thermometer/hygrometers from the Certified Materials list or an equivalent instrument. It will also help the user diagnose any problems outlined in the Troubleshooting Guide (see page 19).

Focusing matrix solutions in a humidity chamber

The process of matrix focusing is dependent on relative humidity. Both CHCA and DHB will focus well if the relative humidity is between 50% and 70%. However, if the relative humidity is outside of this range then either a controlled humidity chamber or a refocusing step may be needed. The QIAGEN Mass·Spec·Focus Humidity Chamber (cat. no. 49903) has been proven to deliver excellent results with Mass·Spec·Focus Chips. A simple controlled humidity chamber can also be constructed by placing a water-saturated sponge in a box with sides at least 5 cm higher than the top of the sponge. The chip is placed on top of the sponge for matrix application.

Protocol: On-Chip Capture of Phosphorylated Peptides from Digests

Important points before starting

- The reagents, tubes, and mixing vials in the Mass·Spec·Focus Chip Solvent Kit (cat. no. 49200) have been proven to deliver excellent results with Mass·Spec·Focus IMAC Chips. We strongly recommend using the materials in this kit to obtain optimal performance.
- To achieve optimum performance, follow all protocol steps and recommendations carefully.
- Wear the appropriate personal protective equipment during all procedures.
- When handling Mass·Spec·Focus Chips use plastic tweezers and only grip the edges of the boundary zone. Avoid touching the chip surfaces (especially the analysis and liquid retention zones) with gloves and tweezers.
- Dust or other foreign material on the chip surface — in particular on the liquid retention or analysis zones — may interfere with the focusing of either analyte or matrix solutions. The use of a dust shield is highly recommended to prevent any foreign material from contaminating the chip surface.
- It is vital that the iron (III) chloride hexahydrate solution (Iron Charging Solution) is freshly prepared before use in clean glassware and applied within one hour. The solution should be clear. If the solution becomes yellow-orange in color, discard it and prepare a fresh solution.
- Typical working volumes for sample are 5–20 µl.
- Use only ultra-high purity (18 MΩ) water and the highest purity matrices (included in the Mass·Spec·Focus Chip Solvent Kit, cat. no. 49200) to ensure optimal performance.
- Unused sites on the chip surface can be affected by the conditions during MALDI analysis. Therefore, it is not advisable to repeatedly use part of a chip, perform analysis, remove the chip, and perform subsequent analyses in unused sites. Where possible, all sites of a chip should be used and analyzed in the same session. However if this is not possible, we recommend analyzing all sites on a chip within three sessions. In addition, the amount of time that chips spend in the instrument should be limited to that required for analysis.

Reagents and equipment to be supplied by user*

- **Sample Diluent** — 1 M acetic acid, 800 mM urea, 0.1% octyl-beta-glucoside (OBG)
- **Wash Solution 1** — 100 mM acetic acid
- **Wash Solution 2** — 100 mM acetic acid, 800 mM urea, 0.1% OBG
- **Elution Solution** — 90% (v/v) acetonitrile:10% (v/v) 0.1% phosphoric acid, i.e., 9 volumes acetonitrile mixed with 1 volume 0.1% phosphoric acid
- **Iron Charging Solution** — 0.1 mM iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) in 1 mM acetic acid. Prepare this solution immediately before use in clean glassware and apply it to the chip sample sites within one hour.
- **DHB Matrix Working Solution** — 1.0 mg/ml
- **CHCA Matrix Working Solution** — 0.1 mg/ml

For a detailed description of the solution compositions see Appendix C (matrix solutions) and Appendix D (other solutions).

Procedure

1. **Adjust pH of samples to <3 with Sample Diluent or Wash Solution 2.**
   For samples from in-gel digestions in 50 mM ammonium bicarbonate, add 5 µl Sample Diluent per 10 µl sample. For neutral or acidic samples (e.g., peptide standards) adjust the pH using Wash Solution 2. To obtain the highest specificity of phosphopeptide binding a pH below 3 is crucial to suppress non-specific binding of acidic peptides.
2. **Charge the sample sites with iron by pipetting 10 µl freshly prepared Iron Charging Solution (0.1 mM $FeCl_3 \cdot 6H_2O$ in 1 mM acetic acid) onto each site. Incubate for 15 min at room temperature (15–25°C). After 15 min, remove any remaining solution with a pipet.**
3. **Pipet 10 µl Wash Solution 1 onto each site and wash by gently pipetting solution up and down 5 times. Leave Wash Solution 1 to incubate on the site for 2 min and remove with a pipet. Repeat.**
4. **Pipet 10 µl Wash Solution 2 onto each site and wash by gently pipetting solution up and down 5 times. Leave Wash Solution 2 to incubate on the site for 2 min and remove with a pipet. Repeat.**
5. **Apply sample to chip and incubate for 20 min at room temperature, ideally in a humidity chamber (see page 13). After incubation, remove sample with a pipet.**

*When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

6. **Pipet 10 µl Wash Solution 2 onto each site and wash by gently pipetting solution up and down 5 times. Leave Wash Solution 2 to incubate on the site for 2 min and remove with a pipet. Repeat.**
7. **Pipet 10 µl Wash Solution 1 onto each site and wash by gently pipetting solution up and down 5 times. Leave Wash Solution 1 to incubate on the site for 2 min and remove with a pipet. Repeat.**
8. **Allow chip to air-dry.**
9. **Pipet 2 µl Elution Solution onto each well and focus to the Analysis Zone, ideally in a humidity chamber. After focusing, dry under ambient conditions.**
10. **Proceed with the protocol "Co-Focusing Sample and Matrix on Mass·Spec·Focus Chips" on page 14.**

Protocol: Co-Focusing Sample and Matrix on Mass·Spec·Focus Chips

Use this protocol to co-focus analytes and matrix after analyte deposition. Either 2,5-Dihydroxybenzoic acid (DHB) or α-Cyano-4-hydroxycinnamic acid (CHCA) matrices can be used with Mass·Spec·Focus chips. The choice of matrix should be determined by the user's instrument and the nature of the analyte(s). CHCA and DHB and the solvents required to make a working solution are supplied in the Mass·Spec·Focus Chip Solvent Kit, cat. no. 49200.

Important points before starting

- The reagents, tubes, and mixing vials in the Mass·Spec·Focus Chip Solvent Kit (cat. no. 49200) have been proven to deliver excellent results with Mass·Spec·Focus Chips. We strongly recommend using the materials in this kit to obtain optimal performance.
- To achieve optimum performance, follow all protocol steps and recommendations carefully.
- Wear the appropriate personal protective equipment during all procedures.
- Matrix solutions contain low boiling point organic solvents and TFA. These solutions should be used in well ventilated areas.
- When handling Mass·Spec·Focus Chips use plastic tweezers and only grip the edges of the chip. Avoid touching the chip surface (especially the analysis and liquid retention zones) with gloves and tweezers.
- Dust or other foreign material on the chip surface — in particular on the Affinity Capture or Analysis Zones — may interfere with the focusing of either analyte or matrix solutions. The use of a dust shield is highly recommended to prevent any foreign material from contaminating the chip surface.
- It is strongly recommended that one or more sites on the chip are used for calibration standards.
- Use only ultra-high purity (18 MΩ) water and the highest purity matrices (included in the Mass·Spec·Focus Chip Solvent Kit, cat. no. 49200) to ensure optimal performance.

Reagents and equipment to be supplied by user

- Matrix working solution (see Appendix C, page 25). All components are supplied in the Mass·Spec·Focus Chip Solvent Kit (cat. no. 49200).
- Optional: Thermometer/hygrometer and humidity chamber providing a relative humidity of 50–70%.

Procedure

1. **Apply 2 µl of matrix working solution to each sample site containing analyte.**

   To avoid polymer contamination, rinse the pipet tip by pipetting matrix solution up and down several times before applying matrix solution to the chip.

2. **Allow the matrix solution to focus and dry under ambient conditions and collect data.**

   If the matrix focuses properly, an almost perfectly circular matrix layer of 500–600 µm diameter is formed in the central Analysis Zone. If the matrix spot diameter is much broader and/or the spot shows a pronounced frayed rim, the focusing has not worked properly, probably due to an unsuitable relative humidity. Proceed using steps 3a – 4a or 3b – 4b for re-focusing. Consider using a humidity chamber (see page 13) to avoid the re-focusing step in future preparations.

3a. If the ambient relative humidity is less than 50%, place the chip in a humidity chamber (see page 13) and apply 1 µl of 90:10 ACN:0.1% TFA refocusing solution to each site containing analyte.

To avoid polymer contamination, rinse the pipet tip by pipetting solution up and down several times before applying refocusing solution to the chip. Note that the refocusing solution does not contain ammonium citrate or matrix.

4a. Allow the matrix solution to refocus and dry in the humidity chamber and collect data.

OR

3b. If the ambient relative humidity is greater than 70%, apply 1 µl of 98:2 ACN:0.1% TFA refocusing solution to each well containing analyte.

To avoid polymer contamination, rinse the pipet tip by pipetting solution up and down several times before applying refocusing solution to the chip. Note that the refocusing solution does not contain ammonium citrate or matrix.

4b. Allow the matrix solution to refocus and dry at ambient conditions and collect data.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see back page for contact information).

	Comments and suggestions
Contamination of spectrum by a 44 or 58 Da ladder	
a) Plasticware was contaminated	Use recommended tubes (see page 10) and/or change pipet tip vendor.
b) Glove touched chip surface	Do not touch the surface of Mass·Spec·Focus IMAC Chips with gloves.
c) Sample is contaminated with a non-ionic detergent	Remove detergent from sample or prepare sample without using non-ionic detergent.
Matrix does not focus (see also page 17)	
a) Dust contamination on Chip surface	Repeat analysis using a new chip and a dust-shield.
b) Wrong matrix solvent was used	Repeat analysis using the correct matrix solvent.
c) Extreme humidity conditions in laboratory	Measure relative humidity with a hygrometer. In the case of low relative humidity (<50%) refocus matrix using 1 µl 90:10 (ACN:0.1% TFA). In the case of high relative humidity (>70%) refocus matrix using 1 µl 98:2 (ACN:0.1% TFA). Consider using a humidity chamber (see page 13) to avoid the re-focusing step in future preparations.
Poor matrix crystallization	
a) Polymer contamination	Use only specified plasticware. Do not use plasticware that has been sterilized, siliconized, or molded using releasing agents.
b) Wrong matrix solvent was used	Repeat analysis using the correct matrix solvent.

	Comments and suggestions
c) Extreme humidity conditions in laboratory	Measure relative humidity with a hygrometer. In the case of low relative humidity (<50%) refocus matrix using 1 µl 90:10 (ACN:0.1% TFA). In the case of high relative humidity (>70%) refocus matrix using 1 µl 98:2 (ACN:0.1% TFA). Consider using a humidity chamber (see page 13) to avoid the re-focusing step in future preparations.
Poor phosphopeptide specificity	
Sample pH is too high	Tryptic digests have an alkaline pH. Check sample pH. If >3 mix sample with higher volume of sample diluent containing 1 M acetic acid.

Appendix A: Troubleshooting Decision Tree

Troubleshooting outline for poor focus and/or crystallization:

1. **Is the relative humidity within the 50–70% range?**

   Yes – Go to step 3.

   No – Try the recommended refocusing step.

2. **Does refocusing step work?**

   Yes – Problem solved. Proceed with experiment, using refocusing step or humidity chamber for future preparations.

   No – Go to step 3 (using controlled % relative humidity for all subsequent steps if possible).

3. **Does matrix working solution focus and crystallize on chip without sample?**

   Yes – Go to step 7.

   No – Test matrix solvent stock solution on chip to see if it will focus. Go to step 4.

4. **Does the solvent stock solution focus on chip?**

   Yes – Make fresh matrix working solution, carefully following directions. Return to step 3.

   No – Re-make solvent stock solution, adding components in the specified order. Go to step 5.

5. **Does the remade solvent stock solution focus on chip?**

   Yes – Make fresh matrix solution with remade solvent solution. Return to step 3.

   No – Go to step 6.

6. **Do the individual solvents ACN, 0.1% TFA (without ammonium citrate!) focus on the chip, leaving no visible residue in the Analysis Capture Zone?**

   Yes – Re-make solvent stock solution, carefully adding components in the specified order. Return to step 5.

   No – Failing solvent is contaminated. Replace with higher purity material (if using recommended materials, take a fresh aliquot into recommended container). Repeat step 6.

7. **Does sample solution focus on chip?**

   Yes – Go to step 10.

   No – Sample solution is contaminated. Dilute sample, go to step 8.

8. **Does diluted sample solution focus on chip?**

   Yes – Go to step 11.

   No – Test sample diluent. Go to step 9.

9. **Does sample diluent focus on chip?**

   Yes – Sample is contaminated. Prepare fresh sample.

   No – Remake sample diluent with fresh aliquots of highest grade ACN and TFA, then make a fresh sample in the remade diluent. Return to step 8 (or step 7, if contaminated sample diluent was used to prepare initial sample).

10. **Does matrix focus and crystallize on chip after sample has been applied and focused?**

    Yes – Problem solved. Proceed with the experiment.

    No – Sample is either too concentrated or contaminated. Dilute sample, and go to step 11.

11. **Does matrix focus and crystallize on chip after diluted sample has been applied?**

    Yes – Problem solved. Proceed with the experiment using diluted sample.

    No – Sample is contaminated. Prepare fresh sample.

Appendix B: In-Gel Tryptic Digestion

This protocol can be used to prepare tryptic digests of proteins in gel spots or bands. Proteins are separated by electrophoresis. Following electrophoresis, the gel is stained using GelCode® Blue Stain Reagent from Pierce Biotechnology, Inc. (Cat. no. 24590). The stained spot or band is excised, destained, and digested with trypsin in situ. The resulting digests are analyzed using Mass·Spec·Focus IMAC Chips.

Equipment and reagents to be supplied by user*

- GelCode Blue Stain Reagent
- Ultrapure water
- Shaker or rocker table
- Staining/destaining bath
- Reducing solution – 100 mM ammonium bicarbonate (NH_4HCO_3), 10 mM DTT
- Alkylation solution – 100 mM ammonium bicarbonate (NH_4HCO_3), 55 mM iodoacetamide
- 100 mM ammonium bicarbonate (NH_4HCO_3)
- 50 mM ammonium bicarbonate (NH_4HCO_3)
- Acetonitrile (100%)
- Destain solution – 50% acetonitrile in 100 mM ammonium bicarbonate (NH_4HCO_3)
- Trypsin (15 ng/μl in 50 mM NH_4HCO_3)

Procedure

Staining and destaining

1. **Separate proteins by electrophoresis and remove gel from electrophoresis assembly.**
2. **Place gel into a container suitable for gel-staining. Add water until the gel is completely covered. Rinse gel on a rocker or shaker platform for 15 min, changing the water every 5 min.**
3. **Stain gel with GelCode Blue for 1 h.**
4. **Destain gel background for 1 hour using several changes of water (Water Wash Enhancement™ step).**

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

5. **Excise protein bands/spots and cut each one into small pieces (approx. 1 mm x 1 mm) using a sterile scalpel. Place the gel pieces from each band/spot into a separate microcentrifuge tube.**
6. **Add 200 µl of acetonitrile to each tube, close the tube, and incubate at room temperature for 15 min. After incubation, remove liquid using a pipet and allow gel slice to air-dry or dry under vacuum.**

   Because protein alkylation is normally performed during 2D-PAGE analysis, steps 7–12 can be omitted if spots from 2D gels are being processed.

Protein reduction

7. **Add 200 µl of 100 mM NH_4HCO_3, 10 mM DTT to each tube and incubate for 30 min at 56°C. After incubation remove any excess solution using a pipet.**
8. **Repeat step 6 to dry the gel slices again.**

Protein alkylation

9. **Add 200 µl of 100 mM NH_4HCO_3, 55 mM iodoacetamide to each tube and incubate for 20 min at room temperature (15–25°C) in the dark. After incubation remove any excess solution using a pipet.**
10. **Wash gel pieces with 150 µl of 100 mM NH_4HCO_3 for 15 min at room temperature (15–25°C). After incubation remove any excess solution using a pipet.**
11. **Repeat step 6 to dry the gel slices again.**
12. **If gel pieces still contains stain, add 500 µl 50% acetonitrile in 50mM NH_4HCO_3 and incubate for 30 min at 37°C. After incubation remove any excess solution using a pipet. Repeat if necessary. Once the gel pieces are completely destained, dry them with acetonitrile as in step 6.**

Trypsin digestion

13. **To each tube, add sufficient trypsin (15 ng/µl) in 50 mM NH_4HCO_3 to cover the gel pieces. Incubate at 4°C for 1 hour. Remove any excess enzyme solution.**
14. **Add sufficient 50 mM NH_4HCO_3 to each tube to cover the gel pieces and incubate overnight at 37°C.**

    If the entire sample is to be loaded onto a single sample site, it is important that the volume of solution is adapted appropriately.

15. **Remove the supernatant and transfer the solution to a clean microcentrifuge tube.**

    This is the digested protein sample that will be applied to the Mass·Spec·Focus Desalting Chip.

16. **Add acetonitrile to 25% of the total volume.**

    For example, if the digested protein sample has a volume of 15 µl, add 5 µl acetonitrile.

Appendix C: Matrix Working Solution Compositions

For best results, solutions should be used on the day they are prepared and prepared as described below using the reagents, tubes, and mixing vials supplied with the the Mass·Spec·Focus Chip Solvent Kit (cat. no. 49200).

DHB Matrix Working Solution (1.0 mg/ml in 84:13:3 acetonitrile : ethanol : 5 mM ammonium citrate in 0.1% TFA)

1. **Prepare a 50 mM (= 11.3 mg/ml) ammonium citrate (dibasic) solution in 0.1 % TFA in a 1.5 ml microcentrifuge tube. Mix by vortexing.**
2. **Dilute the solution prepared in step 1 by a factor of ten. Pipet 100 µl of 50 mM ammonium citrate into 900 µl 0.1% TFA and mix by vortexing.**
3. **Make a Solvent Stock Solution of 84% (v/v) acetonitrile: 13% (v/v) ethanol: 3% (v/v) 5 mM ammonium citrate in 0.1% TFA. Pipet 1000 µl acetonitrile, 155 µl ethanol, and 36 µl 5 mM ammonium citrate in 0.1% TFA (prepared in step 2) into a 1.5 ml microcentrifuge tube and mix by vortexing.**
4. **Make a DHB Matrix Stock Solution by weighing out 3–8 mg DHB into an empty Matrix Mixing Vial and dissolve in the amount of Solvent Stock Solution (prepared in step 3) required to obtain a concentration of 10 mg/ml.**

   For example, if you weigh out 4.2 mg DHB, dissolve in 420 µl solvent stock solution.
5. **Make a DHB Matrix Working Solution by diluting the Matrix Stock Solution by a factor of ten. Pipet 35 µl of the Matrix Stock Solution prepared in step 4 into a clean microcentrifuge tube containing 315 µl of Solvent Stock Solution (prepared in step 3). Mix by vortexing.**

CHCA Matrix Working Solution (0.1 mg/ml in 84:13:3 acetonitrile : ethanol : 5 mM ammonium citrate in 0.1% TFA)

1. **Prepare a 50 mM (= 11.3 mg/ml) ammonium citrate (dibasic) solution in 0.1 % TFA in a 1.5 ml microcentrifuge tube. Mix by vortexing.**
2. **Dilute the solution prepared in step 1 by a factor of ten. Pipet 100 µl of 50 mM ammonium citrate into 900 µl 0.1% TFA and mix by vortexing.**

3. **Make a Solvent Stock Solution of 84% (v/v) acetonitrile: 13% (v/v) ethanol: 3% (v/v) 5 mM ammonium citrate in 0.1% TFA. Pipet 1000 µl acetonitrile, 155 µl ethanol, and 36 µl 5 mM ammonium citrate in 0.1% TFA (prepared in step 2) into a 1.5 ml microcentrifuge tube and mix by vortexing.**
4. **Make a CHCA Matrix Stock Solution by weighing out 3–8 mg CHCA into an empty Matrix Mixing Vial and dissolve in the amount of Solvent Stock Solution (prepared in step 3) required to obtain a concentration of 10 mg/ml.**

   For example, if you weigh out 4.2 mg CHCA, dissolve in 420 µl Solvent Stock Solution.
5. **Make a CHCA Matrix Working Solution by diluting the Matrix Stock Solution 1 in 100 to a concentration of 0.1 mg/ml. Pipet 5 µl of the Matrix Stock Solution prepared in step 4 into a clean microcentrifuge tube containing 495 µl of Solvent Stock Solution (prepared in step 3). Mix by vortexing.**

Matrix Solution Reagent Summary

Solvent stock solution	1000 µl acetonotrile, 155 µl ethanol, and 36 µl 0.1% TFA
DHB matrix stock solution (10 mg/ml)	10 mg/ml DHB in solvent stock solution
DHB matrix working solution (1 mg/ml)	35 µl DHB matrix stock solution; 315 µl solvent stock solution
CHCA matrix stock solution (10 mg/ml)	10 mg/ml CHCA in solvent stock solution
CHCA matrix working solution (0.1 mg/ml)	35 µl CHCA matrix stock solution diluted in 315 µl solvent stock solution. 35 µl of the resulting solution diluted in 315 µl solvent stock solution.

Appendix D: MALDI-MS Solution Formulations

For recommendations for plasticware, pipet tips, etc., please see page 12.

Sample Diluent — 0.1% OBG, 800 mM urea, 1 M acetic acid

For 10 ml: Weigh 10 mg OBG and 480 mg urea into a 15 ml tube and fill to 10 ml with 1M acetic acid (0.572 ml glacial acetic acid + 9.43 ml water).

Wash Solution 1 — 100 mM acetic acid

For 10 ml: Add 1 ml 1M acetic acid to 9 ml water.

Wash Solution 2 — 0.1% OBG, 800 mM urea, 100 mM acetic acid

For 10 ml: Weigh 10 mg OBG and 480 mg urea into a 15 ml tube and fill to 10 ml with 100 mM acetic acid (1 ml 1M glacial acetic acid + 9 ml water).

Elution Solution — 90% (v/v) Acetonitrile: 10% (v/v) 0.1% Phosphoric Acid
IMPORTANT! Use glass vials to prepare all components of the Elution Solution.

For 1ml: Add 100 µl 0.1% phosphoric acid (12 µl 8.5% phosphoric acid* + 988 µl water) to 900 µl acetonitrile.

* Make 8.5% phosphoric acid by diluting 85% phosphoric 1 in 10, i.e., add 100 µl 85% phosphoric acid to 900 µl water.

Iron Charging Solution — 0.1 mM $FeCl_3$, 1 mM acetic acid
IMPORTANT! Use glass vials to prepare all components of the Iron Charging Solution.

For 1ml: Prepare 1 mM $FeCl_3 \cdot 6H_2O$ by dissolving 2.7 mg $FeCl_3 \cdot 6H_2O$ in 10 ml 1 mM acetic acid. Prepare 1 mM acetic acid by adding 300 µl 100 mM acetic acid to 29.7 ml water. Prepare Iron Charging working solution by adding 100 µl 1 mM $FeCl_3$ (in 1 mM acetic acid) to 900 µl 1 mM acetic acid.

Phosphopeptide Standard Working Solution — 1 in 15 dilution of Phosphopeptide Standard Mixture

Mix 3 µl Phosphopeptide Standard Mixture with 27 µl 100 mM acetic acid = Wash Solution (see above) and 15 µl Sample Diluent (see above).

DHB Matrix Working Solution — See Appendix C.

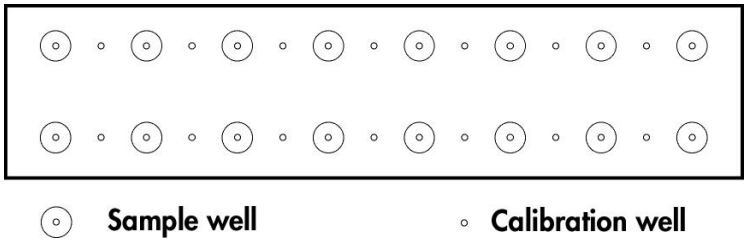
CHCA Matrix Working Solution — See Appendix C

Appendix E: Mass·Spec·Focus Chip Geometries*

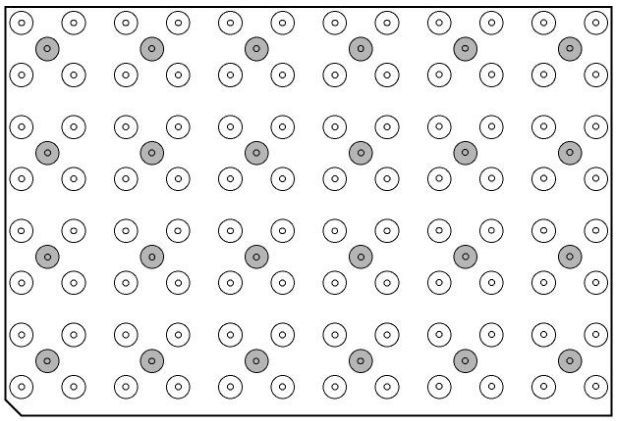
The laminated pipetting guide supplied with Chip Types 1 and 2 helps to locate sample sites. Chip Types 3 and 4 have etched guidelines. Type 5 Chips have etched guide spots between the sample wells to aid pipetting (see below).†

In addition to sample sites, Mass·Spec·Focus Chip types 1, 2, and 5 contain calibration sites. On type 1, 5, and 6 chips, the calibration sites contain only an Analysis Zone. Type 2 chip calibration sites have the same functionality (i.e., desalting or purification) as sample wells. Type 3 and 4 Mass·Spec·Focus Chips do not have designated calibration sites. Depending on the experimental approach, the user is free to choose one or more sample sites for "nearest neighbour" external calibration.

Type 1 Chips for Shimadzu Kratos MALDI-MS instruments (cat. nos. 49201, 49300, and 49400) 2 x 8 sample sites + 14 calibration sites



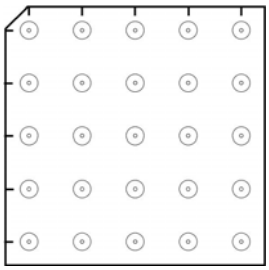
Type 2 Chips for Waters MALDI-MS instruments (cat. nos. 49202, 49301, and 49401) 12 x 8 sample sites + 24 calibration sites (grey)



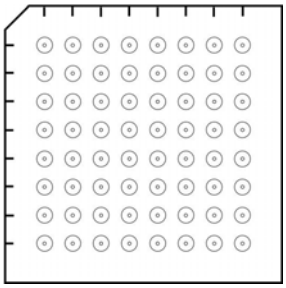
* Chips are not drawn to scale

† The laminated pipetting guide or the guide spots may be replaced for your actual chip version by a more precise direct labeling for each sample and calibrant position.

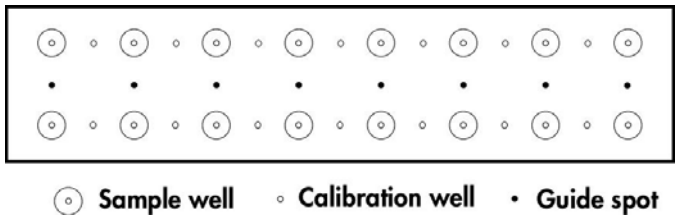
Type 3 Chips for Applied Biosystems MALDI-MS instruments (cat. nos. 49203, 49302, and 49402) 5 x 5 sample sites



Type 4 Chips for Applied Biosystems MALDI-MS instruments (cat. nos. 49204, 49303, and 49403) 8 x 8 sample sites



Type 5 Chips for Thermo Electron MALDI-MS instruments (cat. nos. 49205, 49304, and 49404) 2 x 8 sample sites + 14 calibration sites



Appendix F: Loading and Running Mass·Spec·Focus Chips on MALDI Mass Spectrometers

Mass·Spec·Focus IMAC Chips are available for a wide range of different MALDI mass spectrometers. To run the chips on a particular mass spectrometer the chip has to be placed in a special adapter or customized holder. These holders are available either from the original instrument manufacturer or directly from QIAGEN (depending on the instrument). You will find a comprehensive overview on the latest instrument compatibility including ordering information for the adapter, interface, or holder that will match your MALDI instrument on the QIAGEN mass spectrometry web page (www.qiagen.com/maldiprep).

For Mass·Spec·Focus Chips types 2, 3, and 4, only one chip can be placed in the corresponding holder. For Mass·Spec·Focus Chips types 1 and 5, up to six chips can be placed in a sample holder. If the user wishes to use less than the maximum 6 chips in the holder, then the remaining positions must be filled with blanks loaded in the slots instead of chips. The blanks are reusable and should be stored after the experiment for future use.

Additionally, the actual plate layout, which can differ for different MS instruments, has to be correctly set in the instrument software. This is typically done either by choosing a predefined plate layout in the software or by importing the corresponding plate or geometry file. You can find detailed instructions on how to set the correct plate layout in your MALDI instrument software on the QIAGEN mass spectrometry web page (www.qiagen.com/maldiprep).

Ordering Information

Product	Contents	Cat. no.
Mass·Spec·Focus IMAC Chip Type 1	For Shimadzu Kratos MALDI-MS instruments: 6 chips with 16 sites for on-chip purification and concentration of phosphopeptides from MALDI samples	49400
Mass·Spec·Focus IMAC Chip Type 2	For Waters MALDI-MS instruments: 1 chip with 96 sites for on-chip purification and concentration of phosphopeptides from MALDI samples	49401
Mass·Spec·Focus IMAC Chip Type 3	For Applied Biosystems MALDI-MS instruments: 1 chip with 25 sites for on-chip purification and concentration of phosphopeptides from MALDI samples	49402
Mass·Spec·Focus IMAC Chip Type 4	For Applied Biosystems MALDI-MS instruments: 1 chip with 64 sites for on-chip purification and concentration of phosphopeptides from MALDI samples	49403
Mass·Spec·Focus IMAC Chip Type 5	For Thermo Electron MALDI-MS instruments: 6 chips with 16 sites for on-chip purification and concentration of phosphopeptides from MALDI samples	49404
Mass·Spec·Focus Chip Solvent Kit	For preparing MALDI samples: acetonitrile, ethanol, 0.1% TFA, CHCA, DHB, ammonium citrate, peptide standard, tubes	49200
Mass·Spec·Focus Humidity Chamber	Controlled humidity chamber for Mass·Spec·Focus Chips: chamber, lid, support tray, and sponge	49903

As part of our commitment to expand the range of instruments compatible with Mass·Spec·Focus IMAC Chips, the contents of your chip package may differ from this table. For an up-to-date list of supported instruments, and pack content and ordering information, please refer to the QIAGEN mass spectrometry web page (www.qiagen.com/maldiprep).

Notes

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QIAGEN Companies

Please see the back cover for contact information for your local QIAGEN office.

QIAGEN Distributors and Importers

For a complete, up-to-date list of QIAGEN distributors and importers and contact information, visit www.qiagen.com/contact/qiagenworldwide.aspx

www.qiagen.com

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Austria ■ Orders 0800/28-10-10 ■ Fax 0800/28-10-19 ■ Technical 0800/28-10-11

Belgium ■ Orders 0800-79612 ■ Fax 0800-79611 ■ Technical 0800-79556

Canada ■ Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

China ■ Orders 021-51345678 ■ Fax 021-51342500 ■ Technical 021-51345678

Denmark ■ Orders 80-885945 ■ Fax 80-885944 ■ Technical 80-885942

Finland ■ Orders 0800-914416 ■ Fax 0800-914415 ■ Technical 0800-914413

France ■ Orders 01-60-920-926 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930 ■ Offers 01-60-920-928

Germany ■ Orders 02103-29-12000 ■ Fax 02103-29-22000 ■ Technical 02103-29-12400

Ireland ■ Orders 1800-555-049 ■ Fax 1800-555-048 ■ Technical 1800-555-061

Italy ■ Orders 02-33430411 ■ Fax 02-33430426 ■ Technical 800-787980

Japan ■ Telephone 03-5547-0811 ■ Fax 03-5547-0818 ■ Technical 03-5547-0811

Luxembourg ■ Orders 8002-2076 ■ Fax 8002-2073 ■ Technical 8002-2067

The Netherlands ■ Orders 0800-0229592 ■ Fax 0800-0229593 ■ Technical 0800-0229602

Norway ■ Orders 800-18859 ■ Fax 800-18817 ■ Technical 800-18712

Sweden ■ Orders 020-790282 ■ Fax 020-790582 ■ Technical 020-798328

Switzerland ■ Orders 055-254-22-11 ■ Fax 055-254-22-13 ■ Technical 055-254-22-12

UK ■ Orders 01293-422-911 ■ Fax 01293-422-922 ■ Technical 01293-422-999

USA ■ Orders 800-426-8157 ■ Fax 800-718-2056 ■ Technical 800-DNA-PREP (800-362-7737)

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