

# Капиллярный электрофорез для фармацевтического анализа

Марина Зайнуллина, Химэксперт

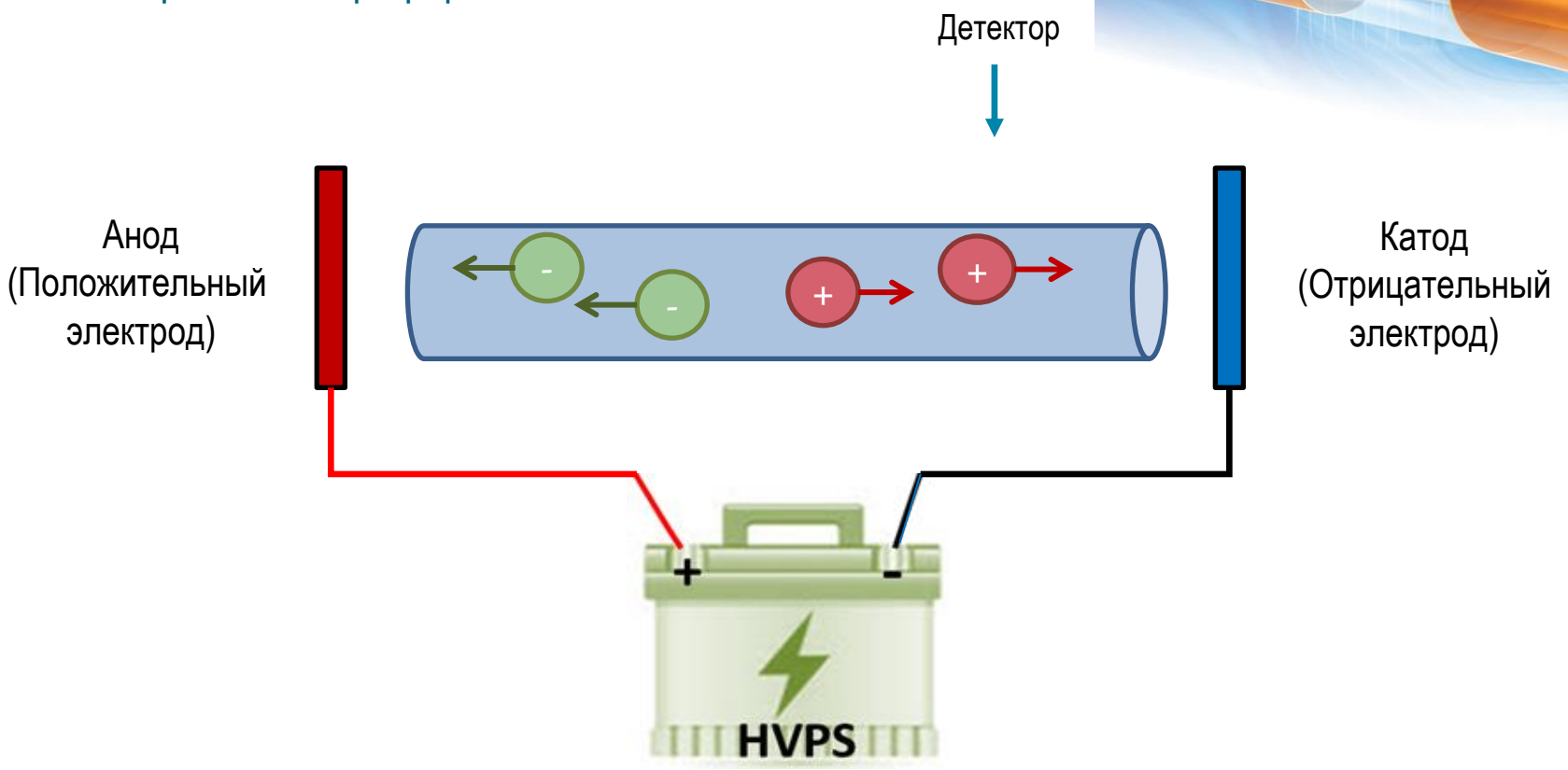
ХИМЭКСПЕРТ

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# Капиллярный электрофорез



Если аналит **заряжен** или имеет **полярность**, то вам нужен Капиллярный электрофорез



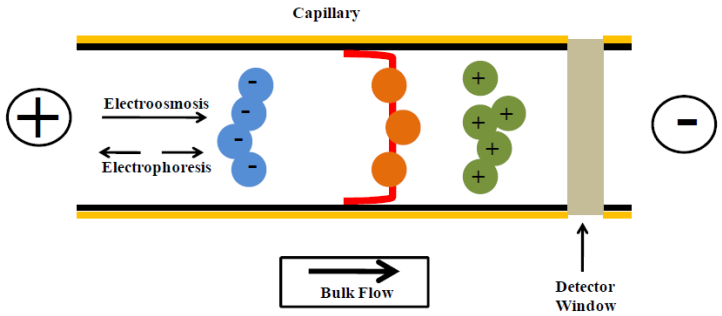

Капиллярный электрофорез – движение заряженных или полярных молекул в капилляре под воздействием однородного электрического поля

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# Капиллярный электрофорез

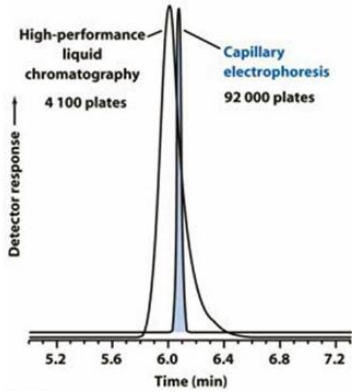
**Разделение**

- Различие в миграции под действием электрического поля
- Дополнение или замена жидкостной хроматографии




**Разрешающая способность**

- Пик эффективности> 1,000,000 теоритически возможных разделений




**Детекторы**

- UV\Vis детектор
- PDA
- LIF
- CE-Mass Spectrometer

**Объем образца**

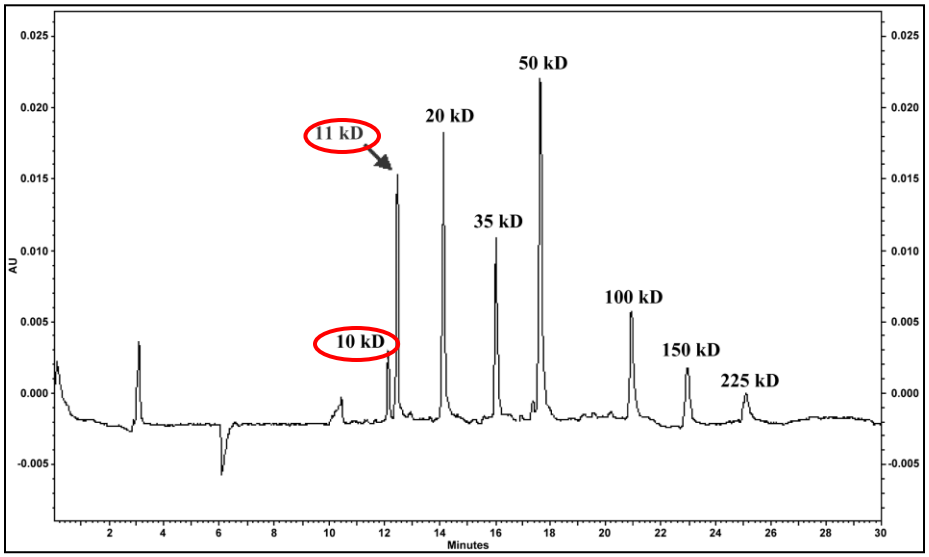
- от 5 мкл с использованием нановиал



**Вариации разделения**

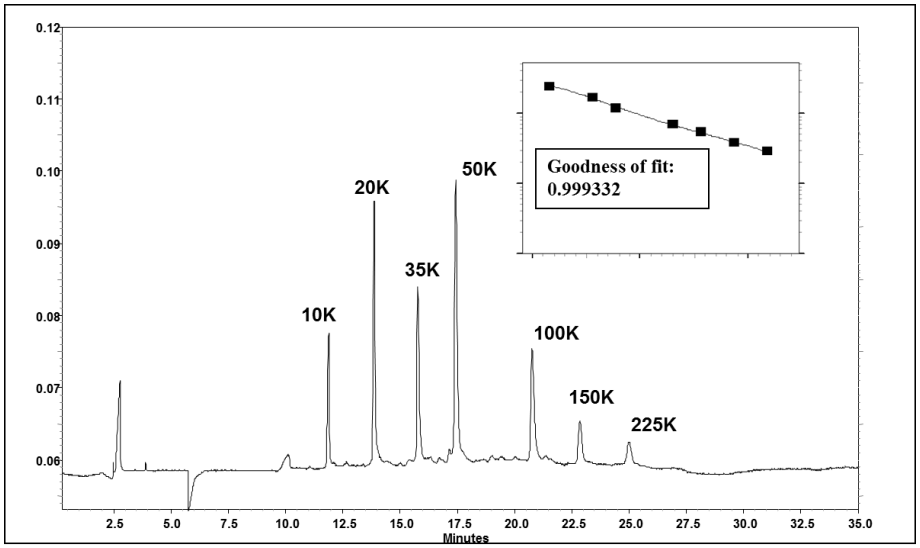
- поверхности, диаметр, длина капилляра
- выбор буфера, методики, типа введения и др.

# SDS-Gel Analysis: Разрешение и Диапазон разделения




## Resolution

Separation of 10KDa and 11KDa spiked molecular weight standards

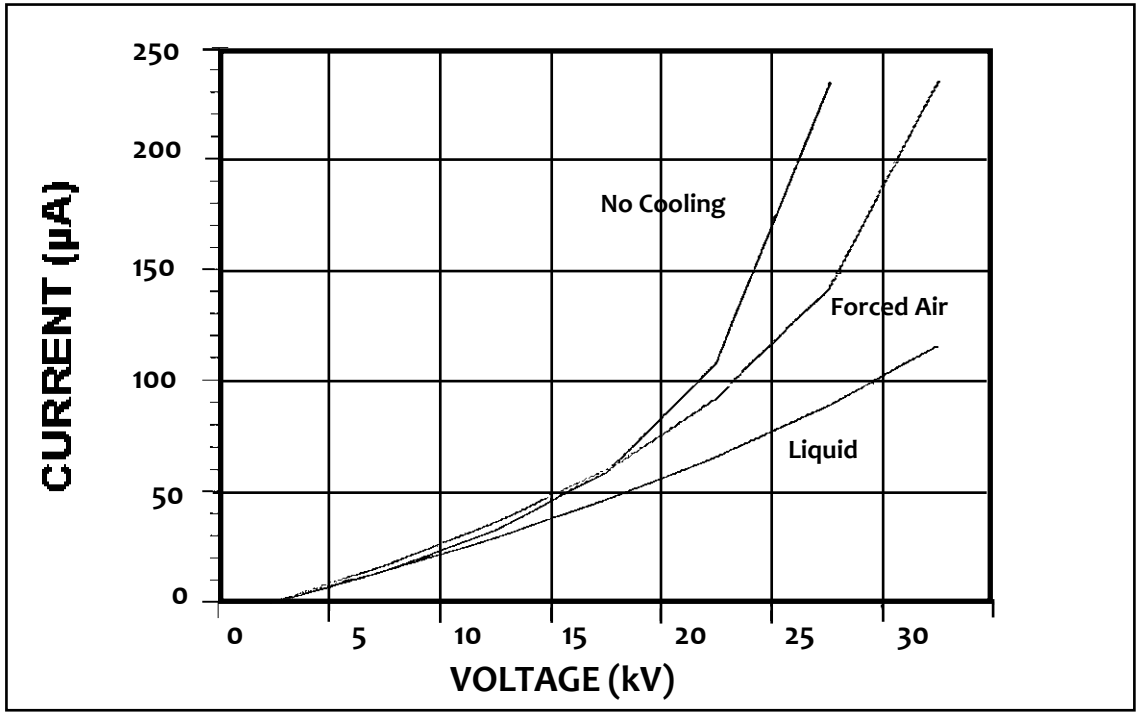


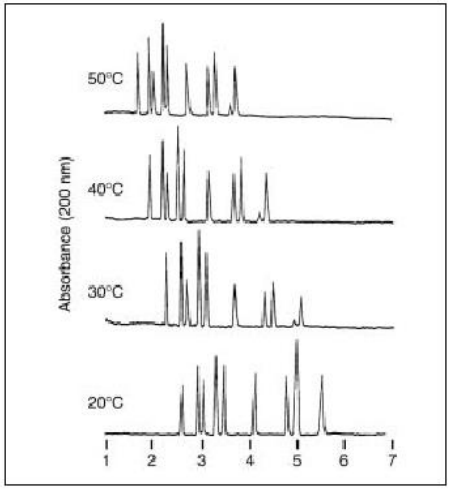

## Broad Range of Separation

Linear from 10KDa to 225KDa


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# **Воспроизводимость –** важнейший элемент в фармацевтической промышленности




**Закон Ома**

**(50 mM Phosphate buffer, pH 4.3 with 75 µm ID capillary at 25°C)**

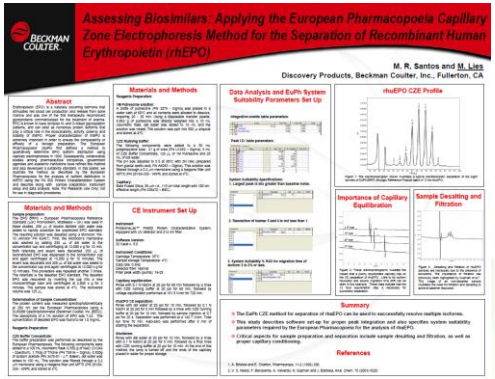
**Контроль нагревания при прохождении тока:**

- Уменьшение внутреннего диаметра капилляра
- Использование буферов с низкой ионной силой и проводимостью
- Ограничение тока
- Термостатирование поверхности капилляра

**Жидкостное охлаждение позволяет:**

- Работать с высоким напряжением
- Использовать буфер с высокой ионной силой
- Применять большой диаметр капилляров

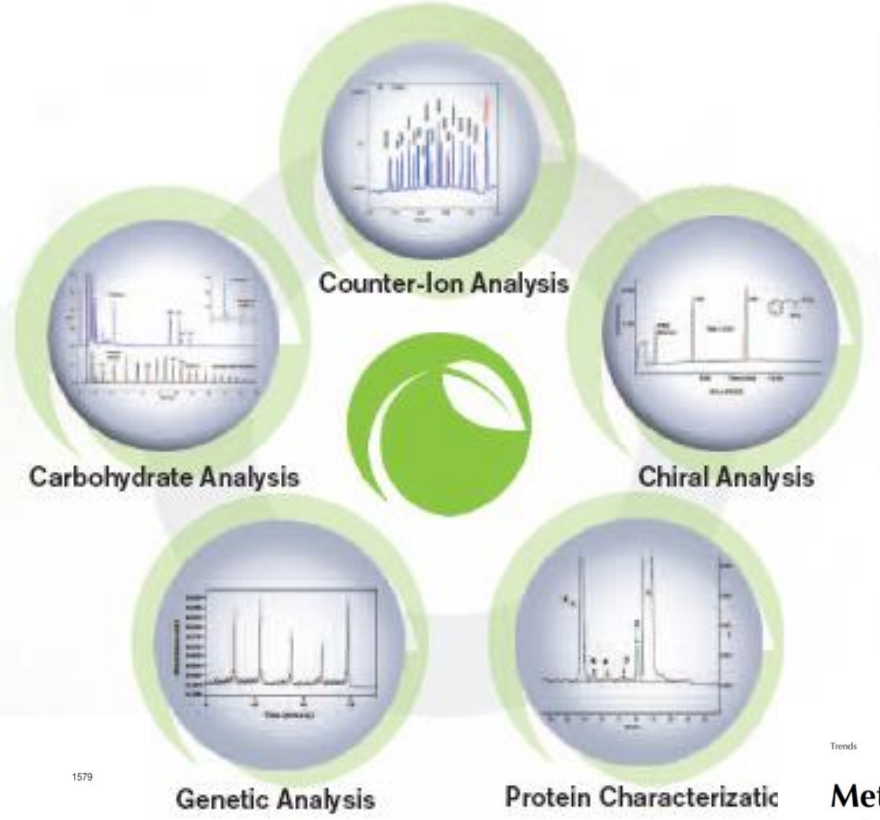
# Применение Капиллярного электрофореза

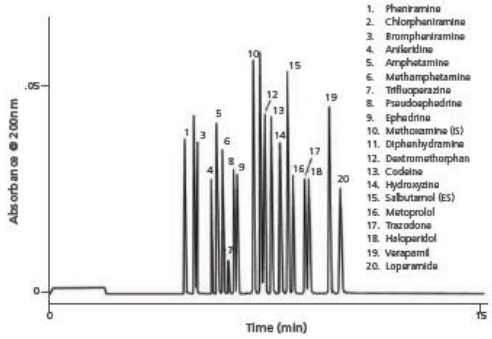


Assessing Biosimilars: Applying the European Pharmacopoeia Capillary Zone Electrophoresis Method for the Separation of Recombinant Human Erythropoietin (rhEPO)

M. R. Santos and M. Lies

Discovery Products, Beckman Coulter, Inc., Fullerton, CA




*Electrophoresis* 2009, *30*, 1579–1586 1579

László Székely
Steffen Kiessig
Maria A. Schwarz
Franka Kálmán

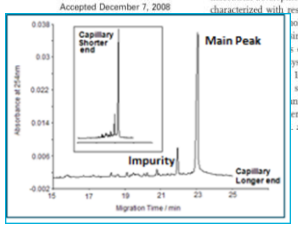
Protein Analytics-Development, Solvias AG, Basel, Switzerland

Received June 17, 2008
Revised November 30, 2008
Accepted December 7, 2008

Research Article

## Capillary gel electrophoresis of therapeutic oligonucleotides – Analysis of single- and double-stranded forms

Recently, several therapeutic double-stranded (ds) oligonucleotides (ODNs) are in pharmaceutical development. During quality control, these therapeutic molecules have to be characterized with respect to their identity, their content and their impurity profile. It olecule as well as its process- and product-related impurities have to ingle strands are considered as process as well as product-related drug substance. Applying well known, conventional, single-base ystems developed for the quality control of single-stranded antisense 1990s, it turned out that the ds ODNs under investigation are splitted peaks between the peaks reaction zones are observed. It ntification of the single strands in the drug substance as well as er product-related impurities, *e.g.* *n*-1; *n*-2 (loss of one and two bases are not possible without adaptation of the test system. The paper




Trends

Trends in Analytical Chemistry, Vol. 25, No. 9, 2006

## Metallodrug research and analysis using capillary electrophoresis

A.R. Timerbaev, C.G. Hartinger, B.K. Keppler

**We review current and emerging capabilities of capillary electrophoresis (CE) as employed for metallodrug development. The application of gentle separation schemes and successful combinations with element-specific and molecule-specific mass-spectrometric detectors have increased the importance of CE as a tool in this area of biochemical speciation analysis.**

**This article highlights recent progress of the method in assessing drug stability, separating metallodrugs and their metabolites, characterizing related metal-bioligand species, describing the kinetics and equilibrium of the corresponding metabolic processes, and analyzing biofluids.**

**Finally, we discuss strategies for developing CE methods in metabolomic and proteomic analysis, centered on metal-based drugs so as ultimately to facilitate their discovery and preclinical investigation.**

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Keywords: Anti-cancer drug; Biological analysis; Biospeciation; Chiral separation;

can be employed to deliver sterilizing radiation to targeted sites (e.g., small tumors) in the body. In addition, some complexes (copper, zinc) find medicinal application due to their bacteriostatic, fungicidal or antiphlogistic activity.

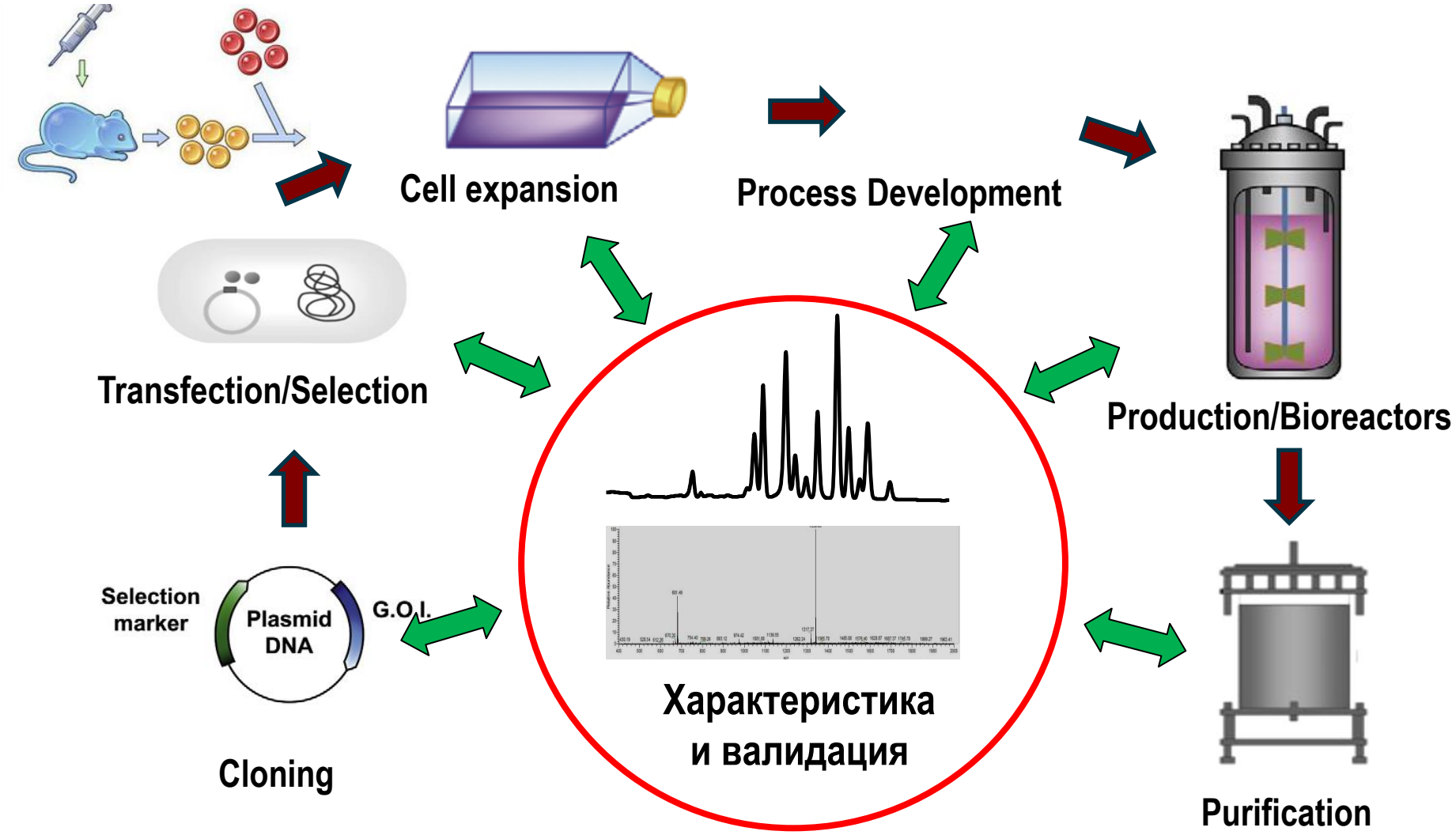
It is widely accepted that the process of creating new medicines, including metallodrugs, is far from efficient. The most commonly cited estimate suggests that to launch one new drug, a drug company spends $800 m and the average pharmaceutical development takes more than a decade (i.e. from the time research begins until approval is received to market the

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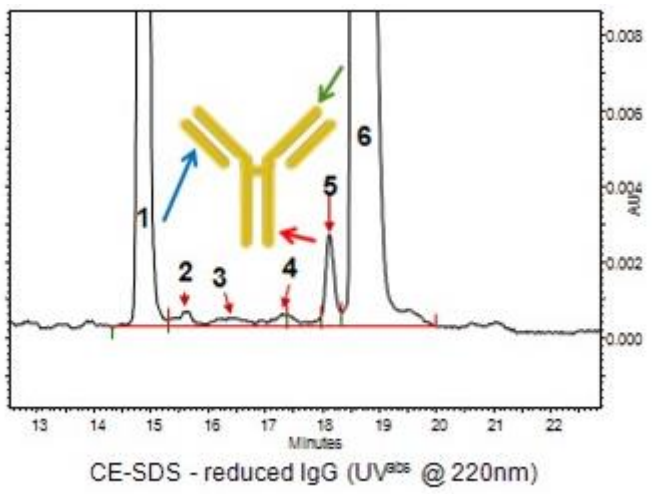
# Top 10 Фармацевтических продуктов в 2016
## Доминирование терапевтических белков

| | Название | Молекула | Компания | Терапевтическая категория |
|---|---|---|---|---|
| 1 | Humira | IgG1 | AbbVie | Ревматоидный артрит (аут. тер) |
| 2 | Sovaldi | small molecule | Gilead | Антивирусные препараты |
| 3 | Remicade | IgG1 химерн | Janssen | Болезнь Крона (аут. тер) |
| 4 | Rituxan | IgG1 химерн | Genentech | Противоопухолевые препараты |
| 5 | Enbrel | IgG1 химерн | Amgen | Ревматоидный артрит (аут. тер) |
| 6 | Lantus | пептид | Sanofi | Диабет |
| 7 | Avastin | IgG1 гуманиз | Genentech | Противоопухолевые препараты |
| 8 | Herceptin | IgG1 | Genentech | Противоопухолевые препараты |
| 9 | Advair | small molecule | GSK | ХОБЛ |
| 10 | Crestor | small molecule | Shionogi/AZ | Снижение холестерина |

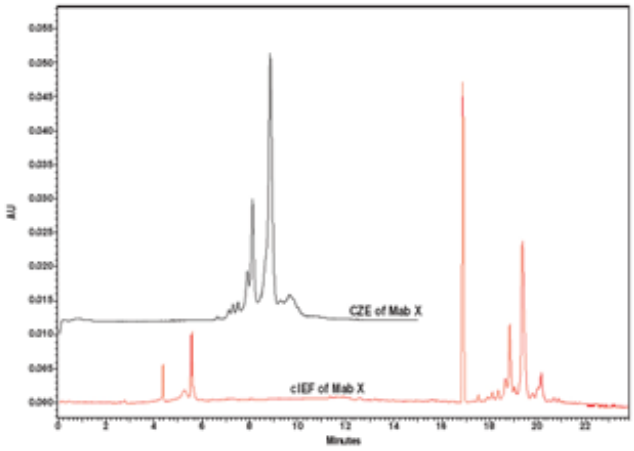
# Производство рекомбинантных белков или терапевтических mAb



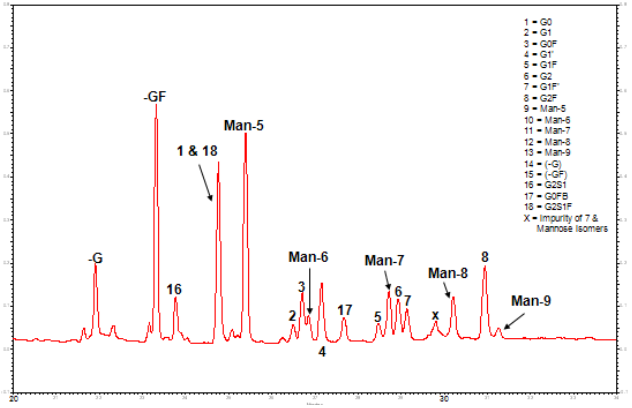
# Характеристика терапевтических mAb




1. CE-SDS для анализа чистоты

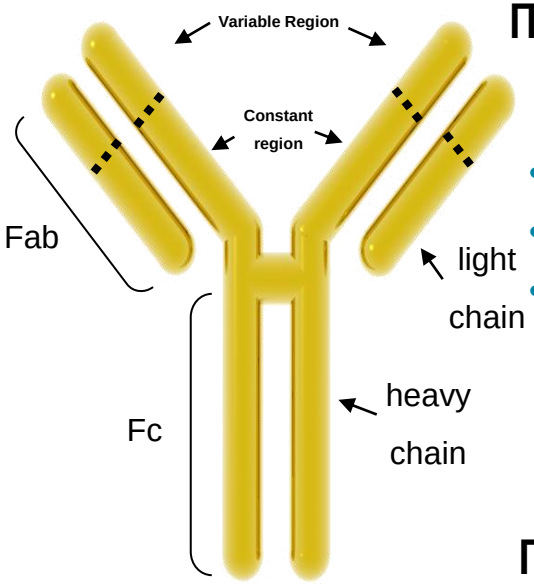



2. cIEF & CZE для анализа гетерогенности заряда




3. Определение углеводного профиля

# 1. CE-SDS для анализа чистоты: загрязнения mAb подвергают опасности здоровье пациента и эффективность препарата в целом

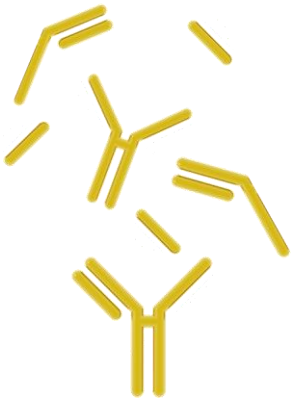



## Побочные продукты на различных стадиях процесса производства:

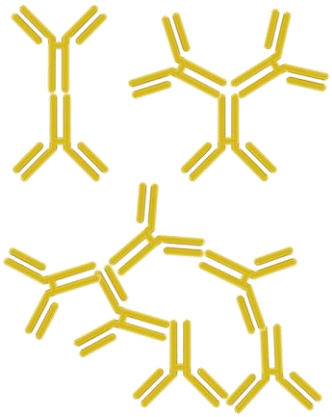
- Клеточные: вирусы, белки клеток хозяина или нуклеиновые кислоты
- Культуральная среда: индукторы, антибиотики, компоненты среды
- Компоненты отчистки: растворители, ферменты, соли

## Побочные продукты самого процесса продуцирования:

Фрагменты

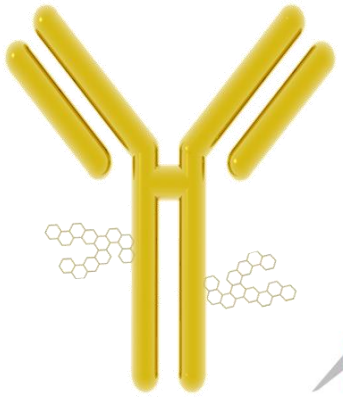


Агрегаты



Модифицированные формы:

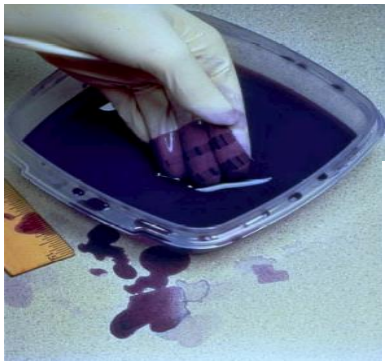
гликозилирование, изомеризация



# Переход от SDS-PAGE к Капиллярному электрофорезу

SDS-PAGE

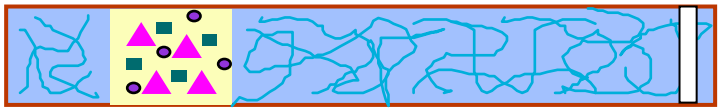
- ручной & маркий
- токсичные реагенты
- низкая точность
- полуколичественный
- низкое разрешение






Капиллярный электрофорез

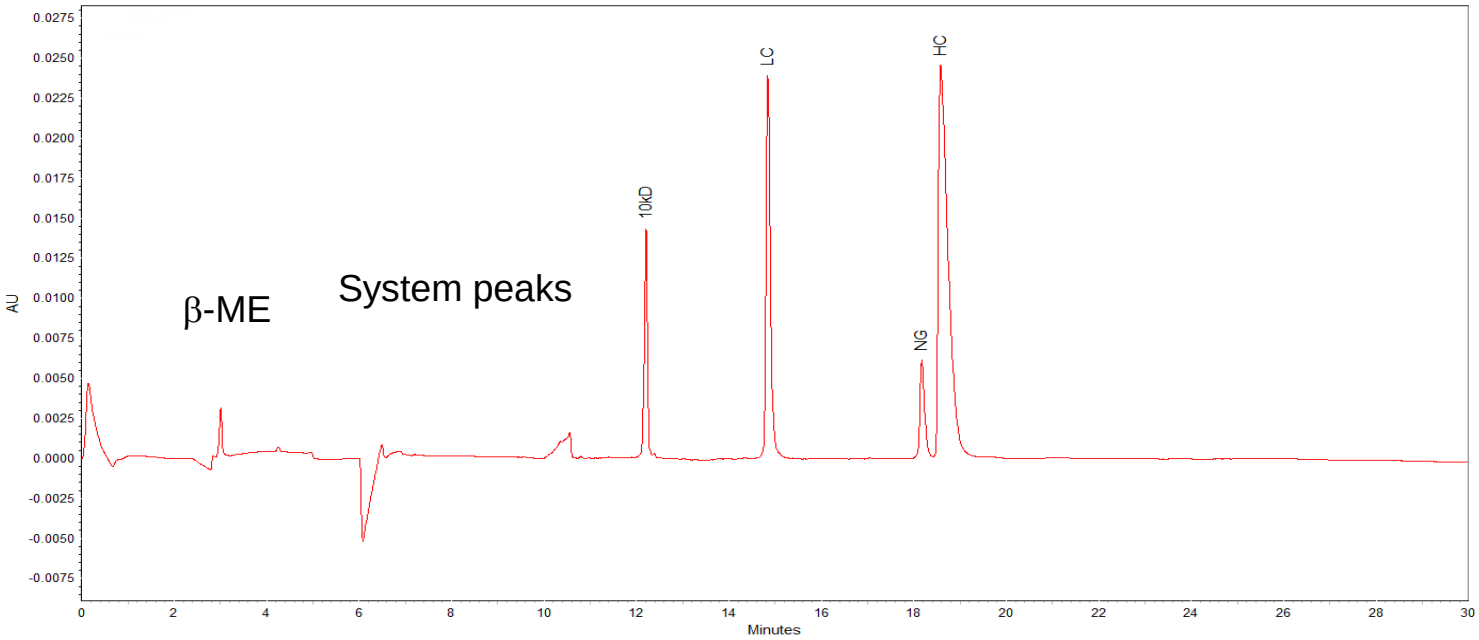
- сменный гель в капиллярах
- электрофоретическое разделение

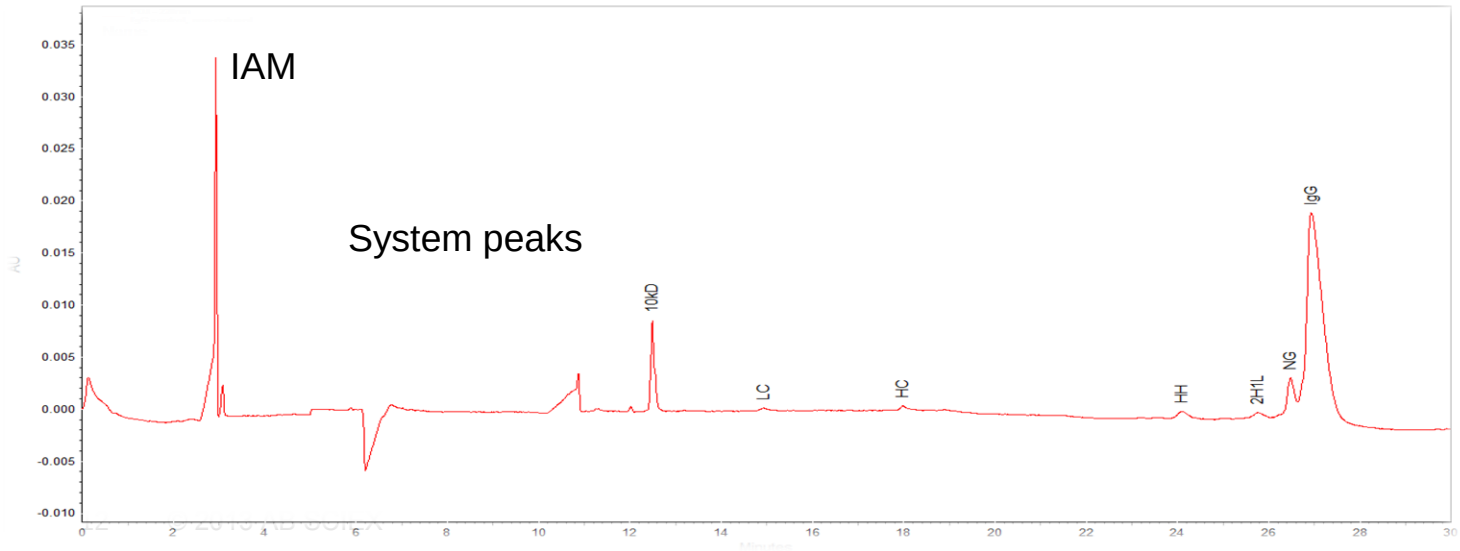



Разделение и детектирование

- формирование комплекса SDS-белок
- одинаковое соотношение заряд размер
- прямое детектирование UV поглощения

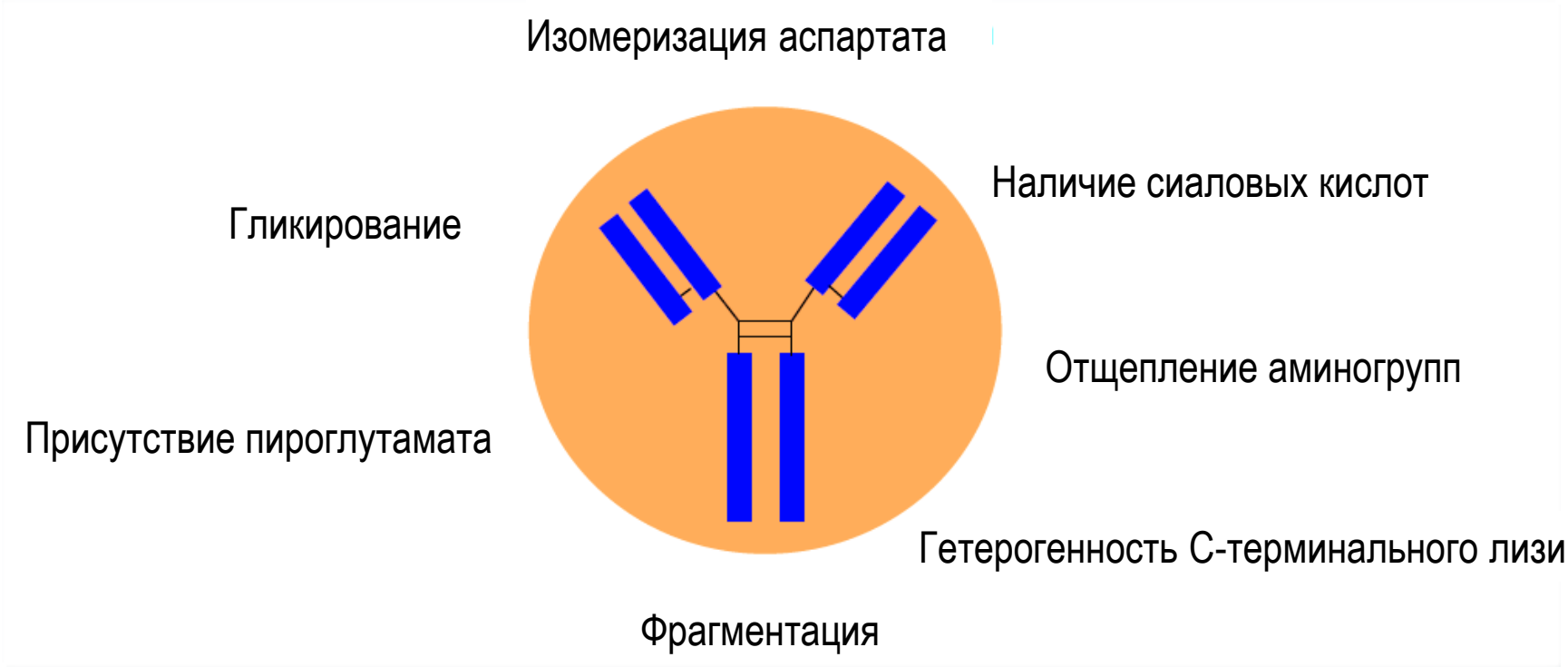
# Анализ чистоты препаратов: Разделение редуцированных и интактных IgG в CE-SDS



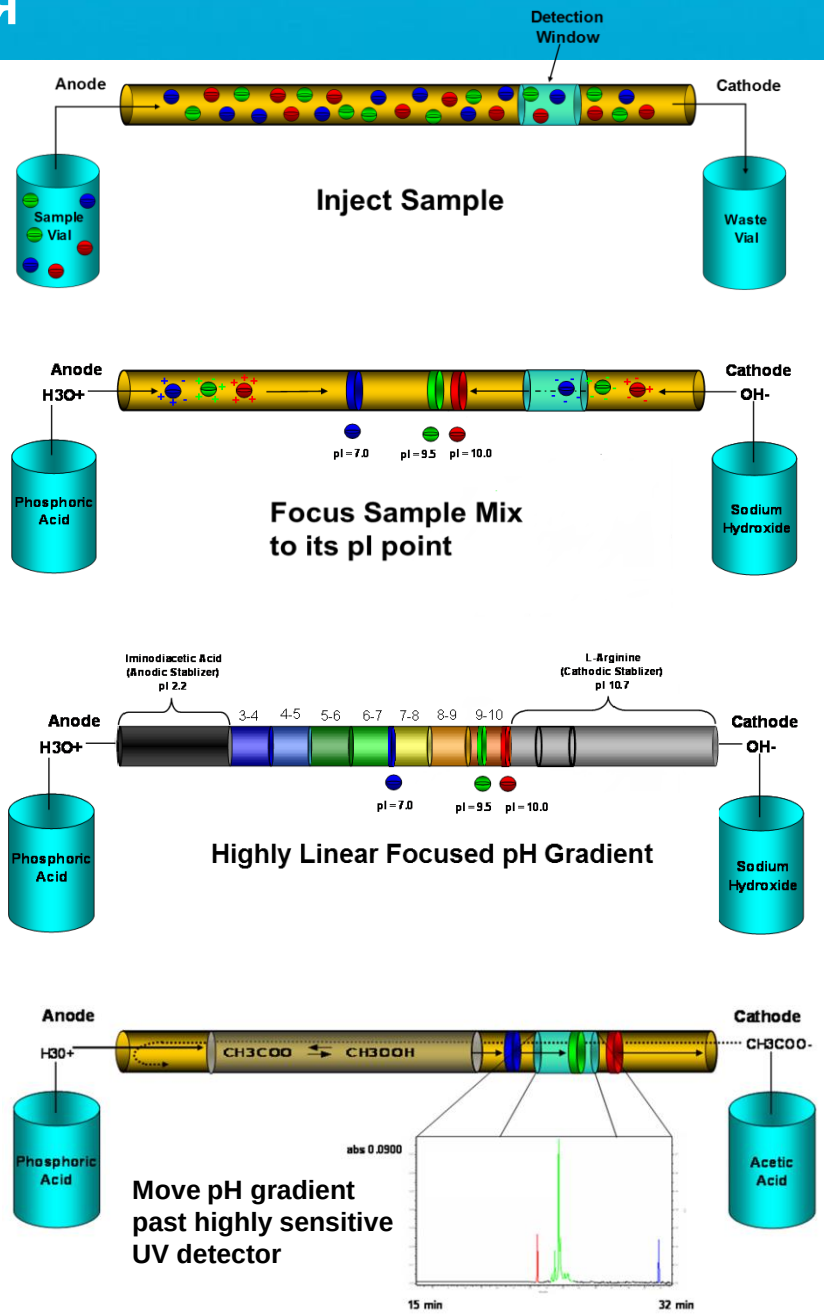

Анализ редуцированного и нередуцированного IgG стандарта методом проверки чистоты IgG. Пик 1: Внутренний стандарт (10 kDa); 2: Легкая цепь (L); 3: NG тяжелая цепь ; 4: Тяжелая цепь (H); 6: Тяжелая-тяжелая цепь (2H); 7: 2 тяжелая 1 легкая цепь (2H1L).

# 2. Гетерогенность заряда биопрепаратов



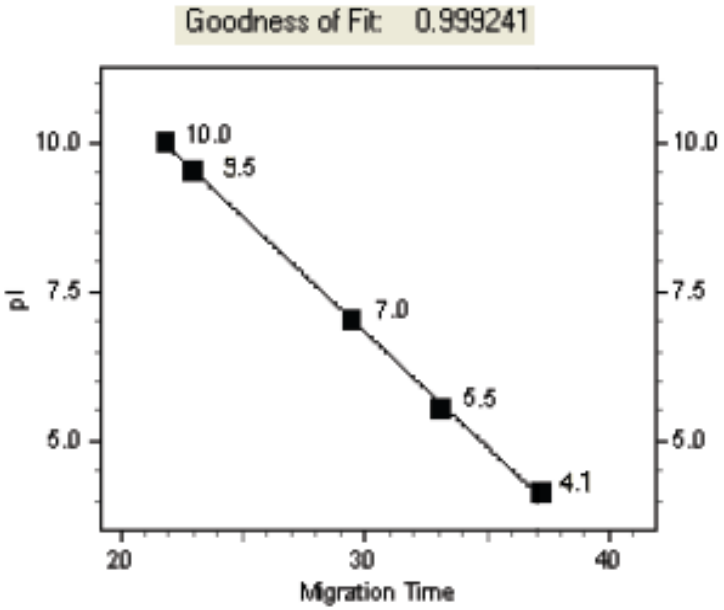

**Модификации, влияющие на качество продукта**

# Анализ гетерогенности заряда методом cIEF: Изоэлектрического фокусирования

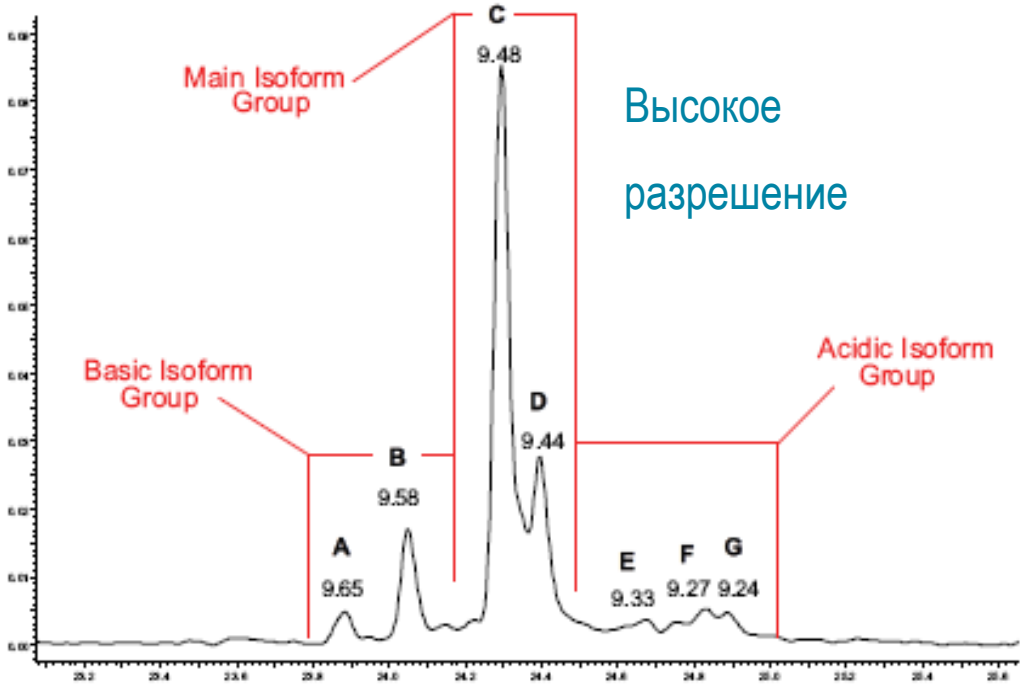


# cIEF Изоэлектрическое фокусирование

Линейная зависимость времени миграции и значений точек изофокусирования




Воспроизводимость




Высокое разрешение

mAb

n = 25

**Isoform Group Percent Composition**

| Group | Average | Std Dev | CV |
|---|---|---|---|
| Basic | 13.94% | 0.42% | 3.04% |
| Main | 71.97% | 0.46% | 0.64% |
| Acidic | 14.09% | 0.34% | 2.38% |

**Calculated pI**

| Peaks | Average | Std Dev | CV |
|---|---|---|---|
| A | 9.65 | 0.01 | 0.05% |
| B | 9.58 | 0.01 | 0.06% |
| C | 9.48 | 0.01 | 0.07% |
| D | 9.44 | 0.01 | 0.09% |
| E | 9.33 | 0.01 | 0.08% |
| F | 9.27 | 0.01 | 0.07% |
| G | 9.24 | 0.01 | 0.08% |

# Сравнение cIEF: изоэлектрофокусирования и CZE: капиллярного зонного электрофореза

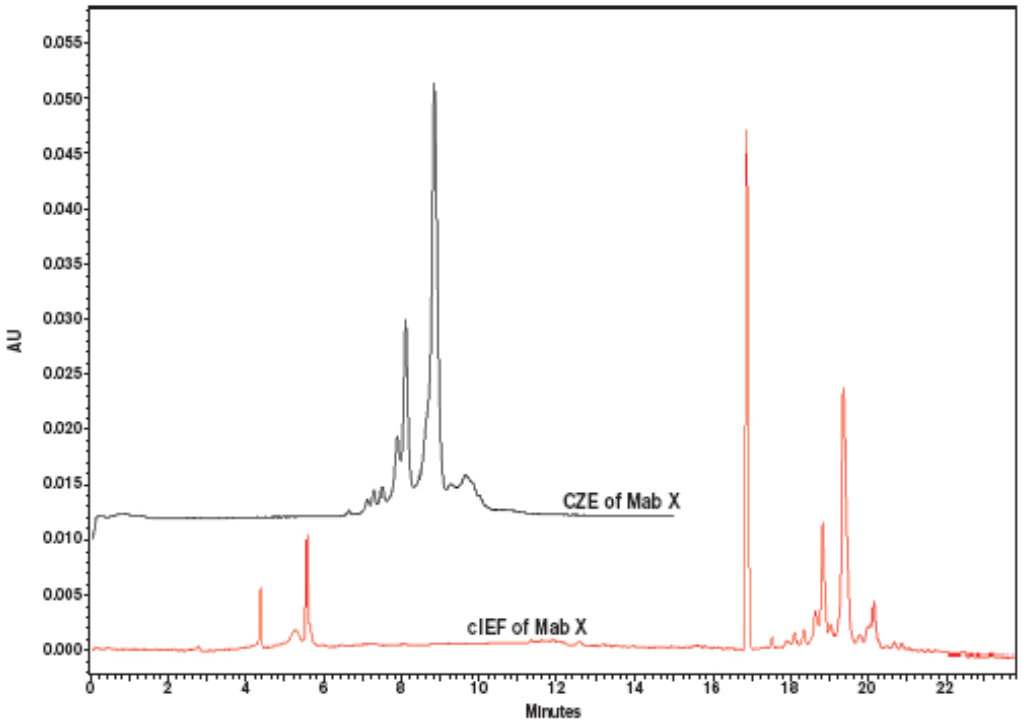



**Figure 3.** Comparison between cIEF and CZE of MAb X.

| | MT (main) |
|---|---|
| | min |
| 30 cm | 7.7 |
| 50 cm | 23 |

**CZE = технология разделения на основе мобильности**

Заряд

Гидродинамический размер

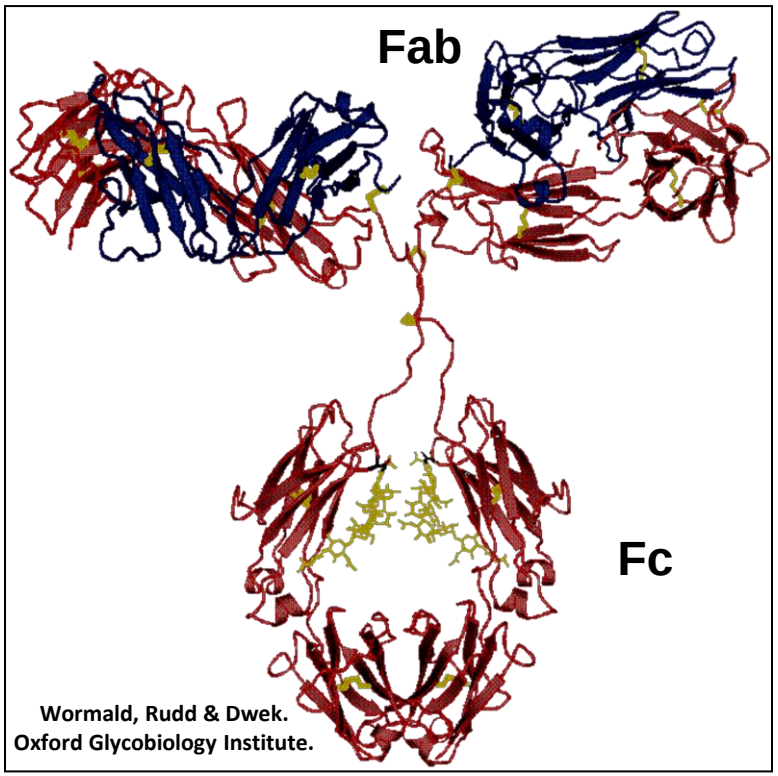
**cIEF = технология на базе заряда**

**Значительное сокращение времени разделения без серьезной потери разрешения!**

**→ CZE для высокой пропускной способности**

SCIEX

# 3. Определение углеводного профиля - анализ гликанов



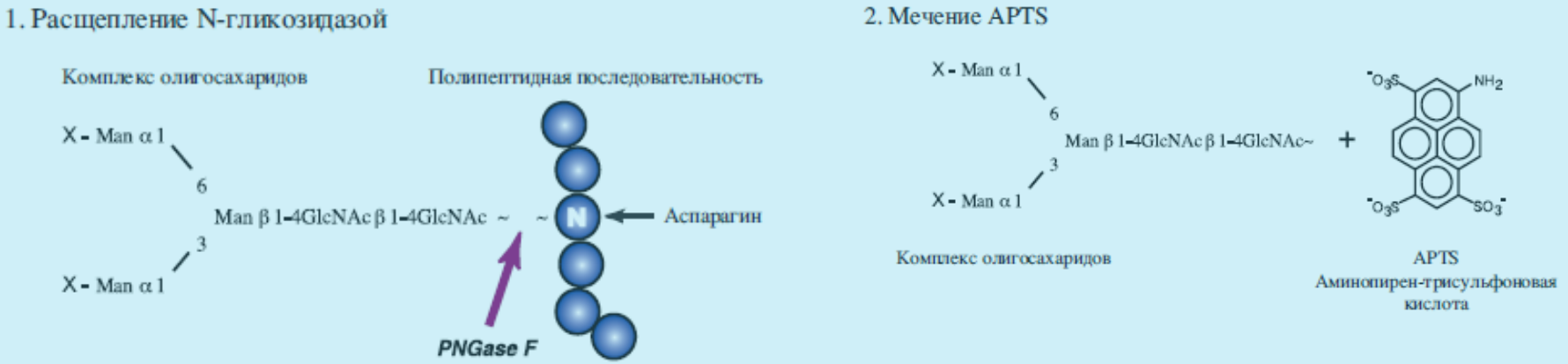

- Присоединение одного олигосахарида к NH(2)-группе боковой цепи остатка аспарагина Asn 297.
- Вариабельность процесса зависит от клеточной линии и условиях экспрессии.
- Структурное разнообразие: Fuc, Gal, Neu5AC, GlcNAc.
- Гликозилирование определяет:
  - биологическую активность,
  - физико-химические свойства,
  - ADCC и CDC функции.
- Ключевое в анализе гликозилирования IgG:
  - выбор клона,
  - характеристика самого продукта-терапевтического mAb,
  - выпуск однородной партии.

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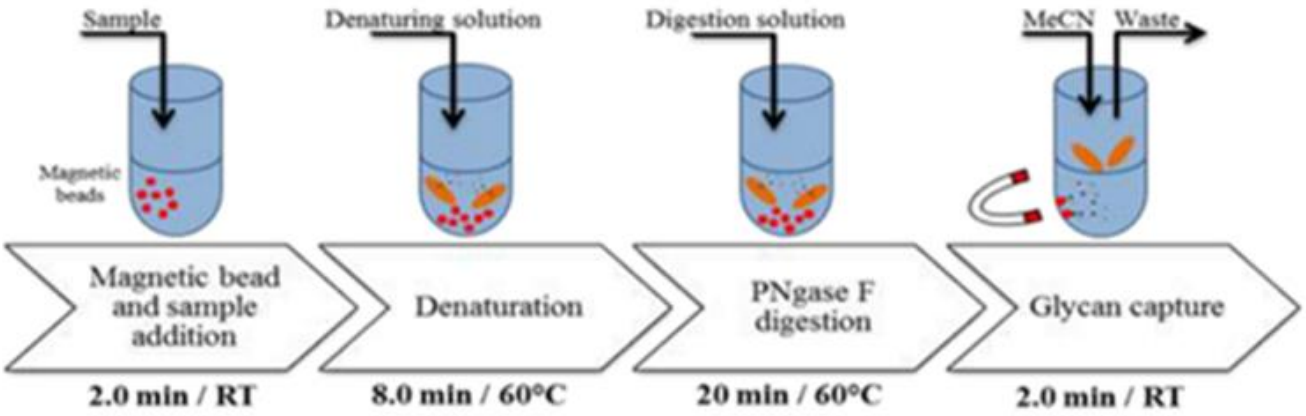
# Идентификация с высокой разрешающей способностью и количественная оценка гликанов

- Количественное определение N-связанных олигосахаридов
- Методы разделения высокого разрешения, с помощью которых можно дифференцировать позиционные изомеры
- Утвержденная методология, разработанная для рутинного использования
- Немедленная идентификация гликанов на базе глюкозных единиц (GU)

## Анализ меченых олигосахаридов с использованием LIF детектора :



# Новое решение SCIEX - Fast glycan labeling and analysis kit



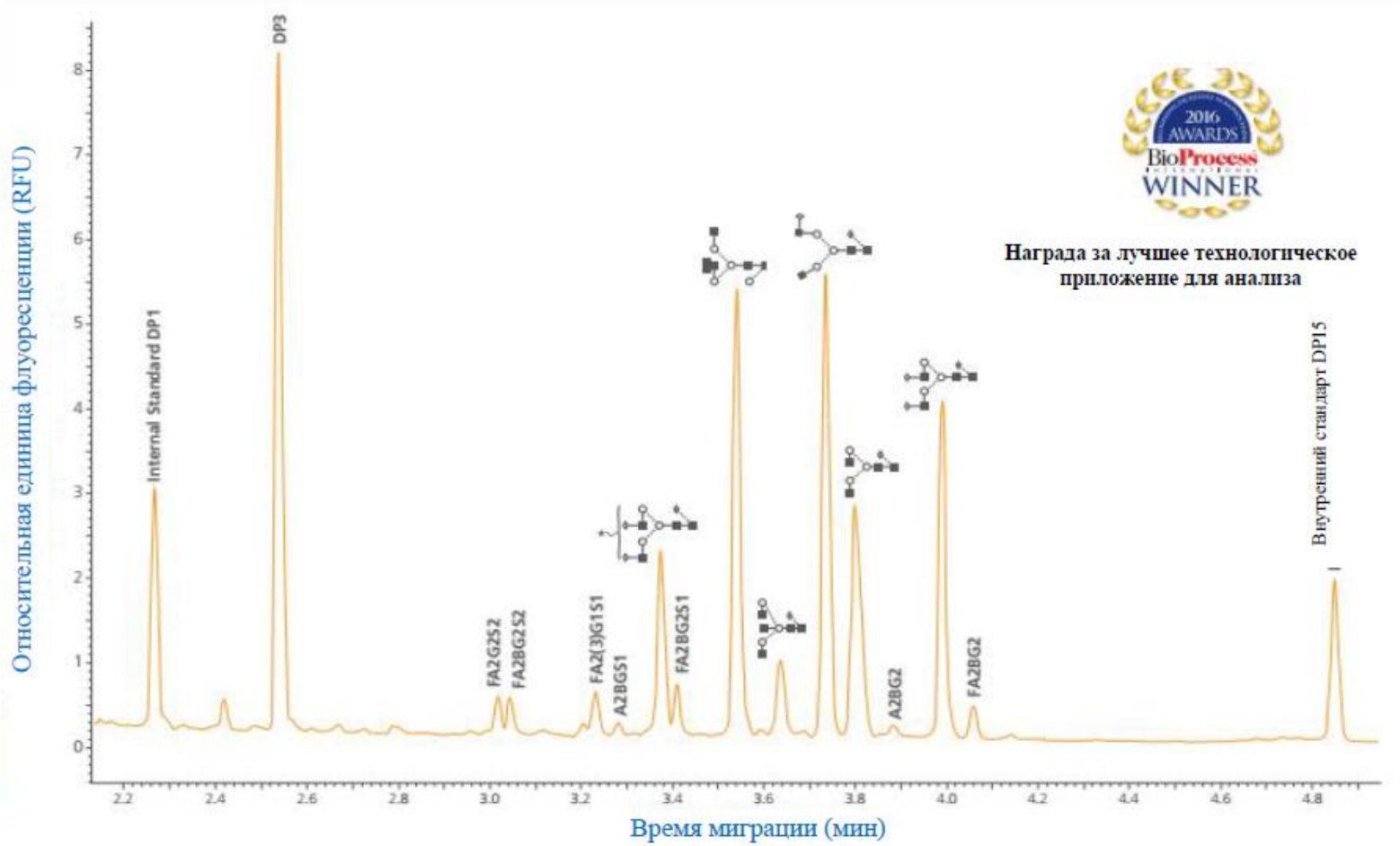






# Новое решение SCIEX - Fast glycan labeling and analysis kit

Отчет **Быстрого анализа гликанов** на платформе **PA 800 Plus** включает в себя электрофореграмму с высоким разрешением и информацию о значении GU вместе с данными о структуре гликана для идентификации.



# Биофармацевтические компании с PA 800 Plus

Biocard™

Р-ФАРМ
Инновационные технологии здоровья

ФАРМА ПАРК

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Alcon

Johnson&Johnson

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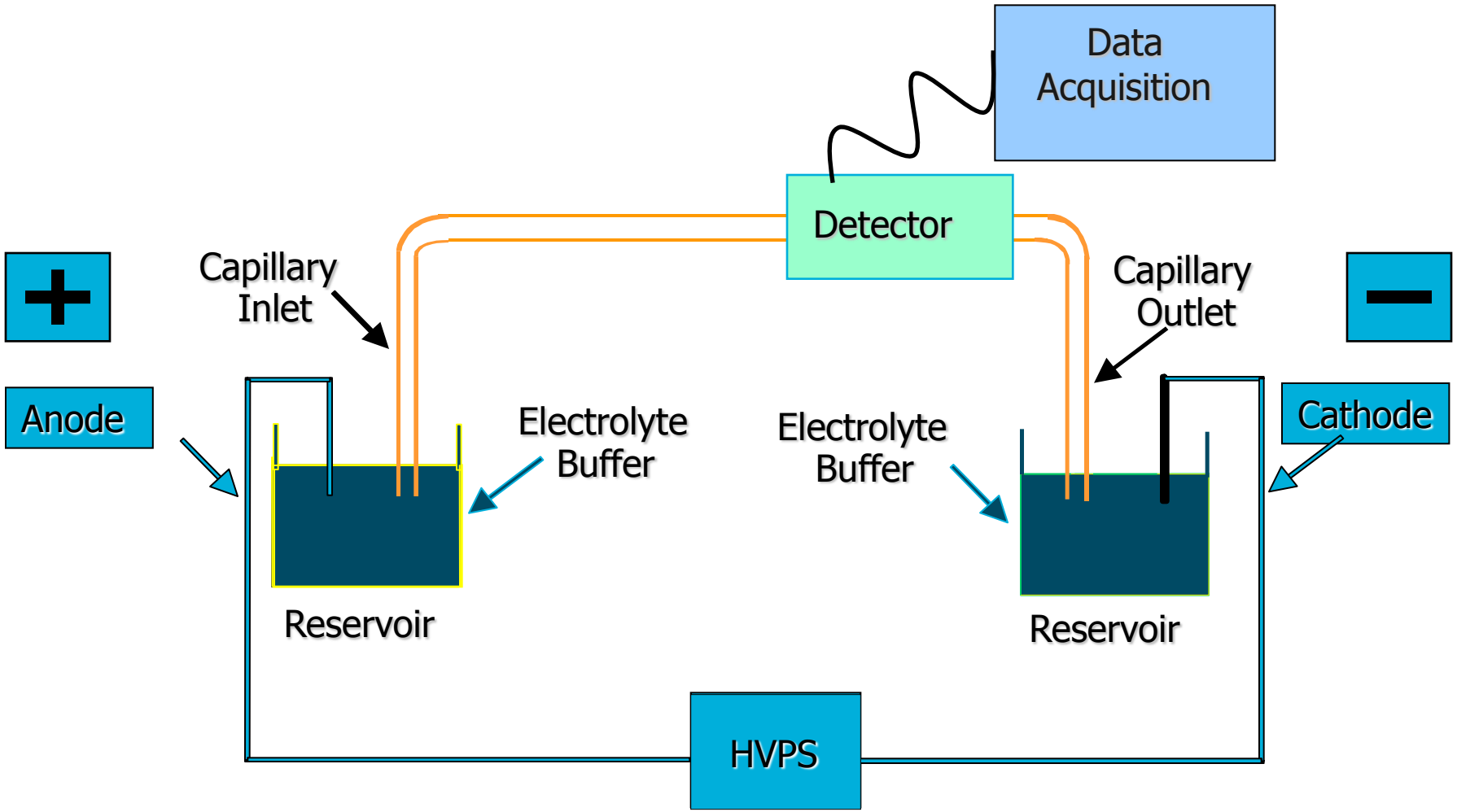
Pfizer

GlaxoSmithKline

SCIEX

# Метод капиллярного электрофореза с масс-спектрометрическим детектированием - **CESI-MS** расширение возможностей

# Традиционная схема системы капиллярного электрофореза





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# Сочетание CE & ESI-Mass Spectrometry

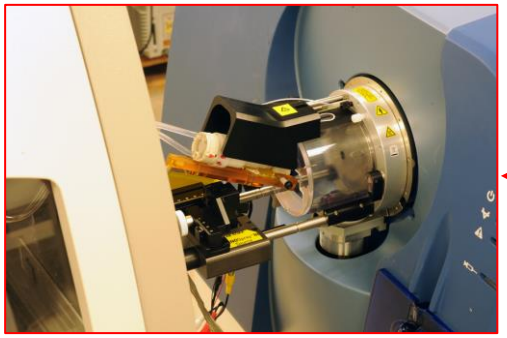
## CESI 8000 High Performance Separation-ESI Module





High resolution, ultra-low flow rate CE separations coupled with high resolution, high sensitivity MS

**Ultra-low flow rates of < 30nL/min**

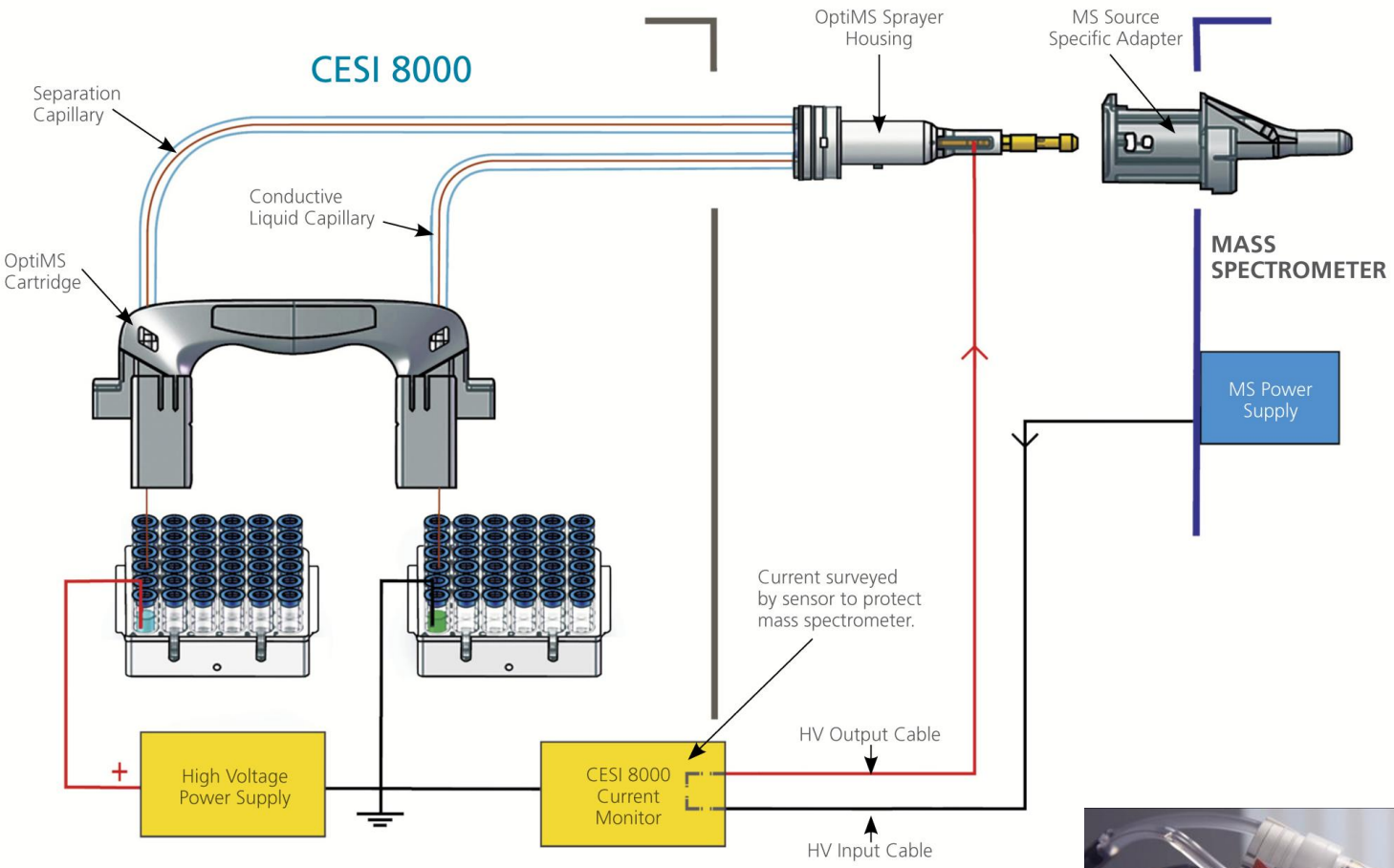


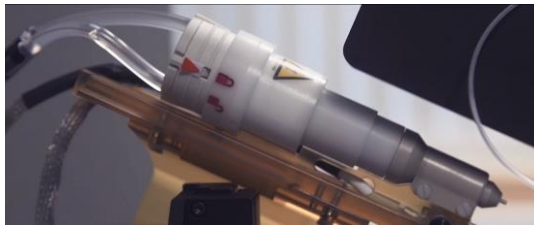
**TripleTOF® 5600+ and 6600 Systems**

**CESI - “CESI 8000 Интеграция капиллярного электрофореза (CE) с ионизацией электроспреем (ESI) в единый динамический процесс в одном устройстве”**


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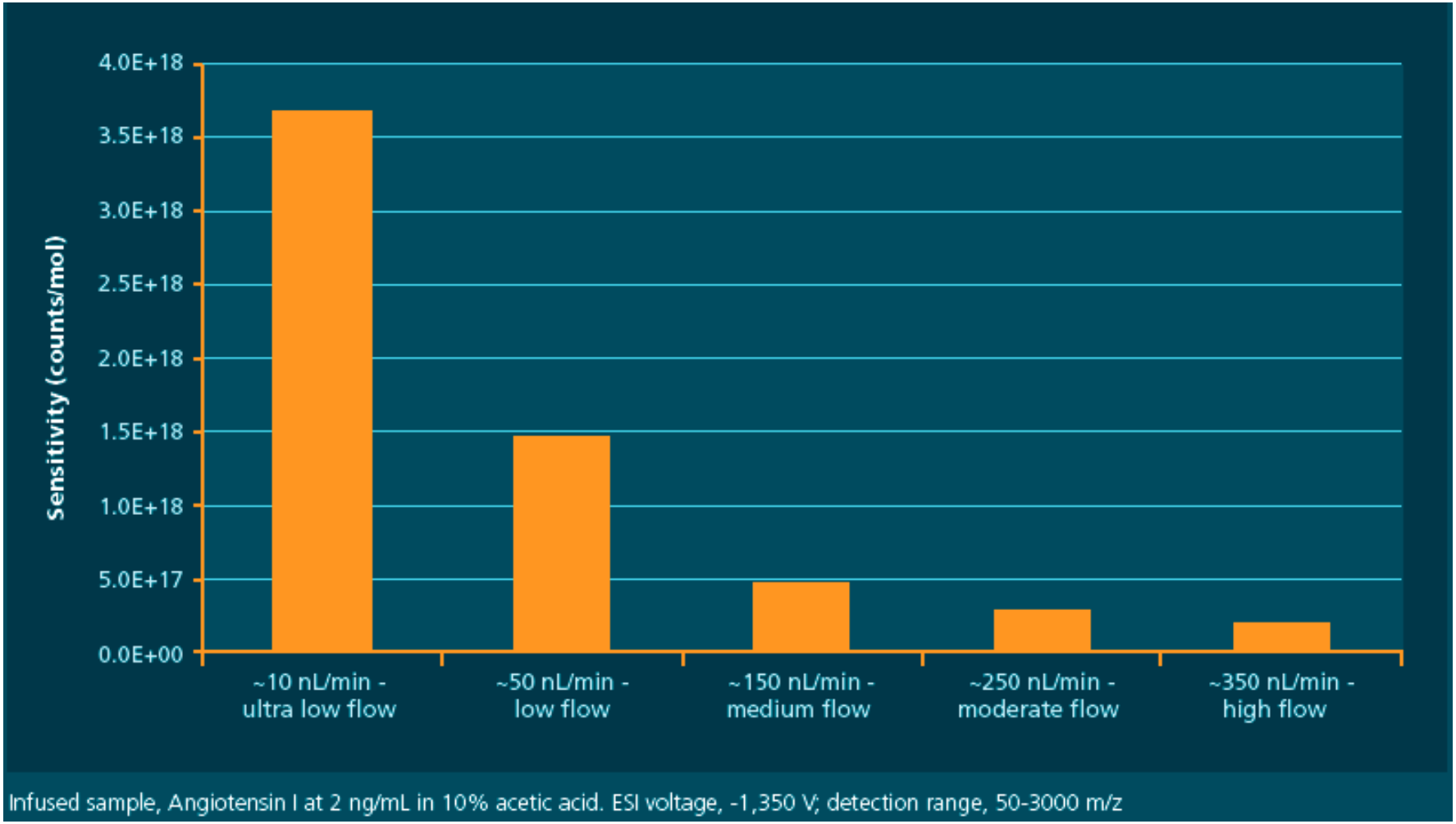
# Подключение модуля CESI к MS




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# Зависимость чувствительности от потока




*Evolution of the peak intensity of Angiotensin I (a) and detection sensitivity as a function of the flow rate (b)* Experimental conditions: capillary electrophoresis; bare fused silica capillary with a porous tip, total length 88.5 cm × 30 *μ*mi.d. × 150 *μ*mo.d.; Infused sample, Angiotensin I at 2 ng/mL in 10% acetic acid. Mass spectrometry; capillary voltage,-1350 V; detection range, 50-3000*m/z*,


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# Новый метод для биопрепаратов нового поколения

- Recent reviews on mAb characterization

FOCUS ON THERAPEUTIC ANTIBODIES

PERSPECTIVES

TIMELINE

## Strategies and challenges for the next generation of therapeutic antibodies

*Alain Beck, Thierry Wurch, Christian Bailly and Nathalie Corvaia*

Abstract | Antibodies and related products are the fastest growing class of therapeutic agents. By analysing the regulatory approvals of IgG-based biotherapeutic agents in the past 10 years, we can gain insights into the successful strategies used by pharmaceutical companies so far to bring innovative drugs to the market. Many challenges will have to be faced in the next decade to bring more efficient and affordable antibody-based drugs to the clinic. Here, we discuss strategies to select the best therapeutic antigen targets, to optimize the structure of IgG antibodies and to design related or new structures with additional functions.

The research and development of monoclonal antibodies is a rapidly progressing field[1,2]. In the past 25 years, more than 30 immunoglobulins (IgGs) and their derivatives have been approved for use in various indications[3,4] (TIMELINE). The currently marketed antibody-based drugs have been approved not only to treat diseases affecting large numbers of patients (such as cancer and inflammatory diseases) but also for more specialized indications owing to special regulatory procedures for rare medical conditions (orphan diseases), such as paroxysmal nocturnal haemoglobinuria (for which, eculizumab (Soliris; Alexion pharmaceuticals) therapy was approved in 2007). Interestingly, 9 out of the 26 antibodies currently in Phase III clinical trials (35%) have 'orphan drug' designation[4].

Since the first generation of mouse, chimeric and humanized IgG1 antibodies reached the market in the late 1990s, the variety of antibody structures has been considerably extended. Humanized and human antibodies of other IgG isotypes (IgG2 and IgG4)[5] have been developed, as well as a large number of IgG-related products[6]. By analysing the successful regulatory approvals of IgG-based biotherapeutic agents in the past 10 years (TIMELINE), we can gain insights into the strategies developed by biopharmaceutical companies. Here, we discuss strategies to select therapeutic antigen targets based on previous clinical or experimental validation or on functional approaches; strategies to optimize the antibody structure and to design related or new structures with additional functions; as well as challenges to bring more affordable treatments to the most appropriate patient populations screened for validated biomarkers.

> Since the first generation of... IgG1 antibodies reached the market in the late 1990s, the variety of antibody structures has been considerably extended.

**Strategies to select the best targets**

Antigen target selection can be classified in broad terms into two main approaches. The first approach involves the development of antibodies directed against so-called 'validated targets', either because prior antibodies have clearly shown proof of activity in humans (first-generation approved antibodies on the market for clinically validated targets) or because a vast literature exists on the importance of these targets for the disease mechanism in both *in vitro* and *in vivo* pharmacological models (experimental validation; although this does not necessarily equate to clinical validation). Basically, the strategy consists of developing new generations of antibodies specific for the same antigens but targeting other epitopes and/or triggering different mechanisms of action (second- or third-generation antibodies, as discussed below) or even specific for the same epitopes but with only one improved property ('me better' antibodies). This validated approach has a high probability of success, but there are many groups working on this class of target proteins and freedom to operate is decreased. By contrast, one can identify new or less well studied target proteins that confer particular functions to cells that might be involved in pathogenic disorders. This second 'functional approach' — in which antibodies are selected based on a functional screen, and the targets to which they bind are then identified using proteomic or cell-based approaches (reverse pharmacology), for example — is associated with greater potential for innovation and intellectual property rights but increased risk of development failure.

***Clinically validated targets.*** 'Blockbuster' antibodies such as rituximab (Rituxan/Mabthera; Genentech/Roche/Biogen Idec), infliximab (Remicade; Centocor/Merck), trastuzumab (Herceptin; Genentech/Roche) and cetuximab (Erbitux; ImClone Systems), directed against now highly clinically validated targets such as CD20, tumour necrosis factor (TNF), human epidermal growth factor receptor 2 (HER2; also known as ERBB2) and epidermal growth factor receptor (EGFR), respectively, are tremendous success stories[1]. Second-generation antibodies directed against these same antigens have alterations such as improved variable domains to decrease immunogenicity and/or to



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2010

## CESI 8000 Plus launched in 2014

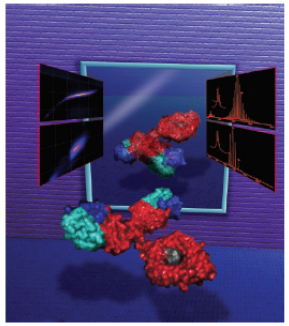
analytical chemistry — Feature — pubs.acs.org/ac

### Biosimilar, Biobetter, and Next Generation Antibody Characterization by Mass Spectrometry

**This Feature will introduce the strategies of therapeutic antibodies (mAbs) in-depth characterization by mass spectrometry (MS) and discuss analytical comparison of biosimilar to originator mAbs, with the cases of trastuzumab and cetuximab. In addition, the structural and functional insights gained both by state-of-the art and emerging MS methods used for biobetters and next generation antibodies design and optimization will also be highlighted.**

Alain Beck,*[†] Sarah Sanglier-Cianférani,[‡] and Alain Van Dorsselaer[‡]

[†]Centre d'Immunologie Pierre Fabre (CIPF), 5 Av. Napoléon III, BP 60497, 74164 Saint-Julien-en-Genevois, France
[‡]Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Université de Strasbourg, IPHC, 25 rue Becquerel 67087 Strasbourg, France and CNRS, UMR7178, 67037 Strasbourg, France



Robert Gates

Monoclonal antibodies (mAbs) and related products are the fastest growing class of therapeutic agents. Biosimilar antibodies are copy versions of the original ones that will be out of patent in the next decade.[1] In contrast to generic versions of small molecules (150−600 Da), it is not possible to produce exact molecular copies of such large and complex glycoproteins (150 000 Da). Biosimilars are produced from different cell clones and different manufacturing processes. As a consequence, differences in glycosylation and other microvariations can be observed and can impact (or not) quality, safety, and potency.[2] Only very small differences between biosimilar and reference mAbs, with reassurance that these are not of clinical relevance, may be accepted by health authorities. Copy versions of the original biopharmaceuticals are already available in several countries but no consistent worldwide requirements for their registration are established so far.[3] Copies of rituximab (Reditux; Dr. Reddy's laboratories) and abciximab (Clotinab; Abu Abxis) have, for example, been approved in India and in South Korea, respectively.[4] The initial versions were originally discovered and developed by Roche/Biogen Idec (Rituxan/Mabthera) and Johnson & Johnson (Reopro), respectively. Further major biosimilar candidates currently in development include copies of infliximab (Remicade; Johnson & Johnson/Merck), trastuzumab (Herceptin; Roche), cetuximab (Erbitux; Lilly/Merck-Serono), bevacizumab (Avastin; Roche), and also etanercept, a Fc-fusion protein (Enbrel; Amgen/Pfizer), to name a few.[5] The European Medicine Agency (EMA) has recently released a draft guideline on similar medicinal products containing mAbs.[6] This guidance discusses relevant animal model, nonclinical, and clinical studies that are recommended to establish the similarity and the safety of a biosimilar compared to an originator reference mAb approved in the European Union (EU) and should enter in application in 2012. From a regulatory standpoint, antibodies that are not highly similar must undergo greater preclinical and clinical evaluation. Conversely, abbreviated clinical programs should be suitable for biosimilar mAbs showing molecular profiles and characteristics within the range of originator versions. Manufacturing process changes are tightly regulated by the International Committee of Harmonization (ICH) guidelines. Changes of quality attributes are only acceptable if they do not alter safety and efficacy.[7] The biosimilar specifications for batch release should be as tight as the current originator specifications. In the leading generic companies, a high degree of similarity is achieved based on extensive characterization and on iterative process development. In such laboratories, only when a biosimilar antibody is shown to be "highly similar" at the analytical level, preclinical and clinical studies are engaged as illustrated below.[8]

### ■ MONOCLONAL ANTIBODIES USED AS THERAPEUTICS

**Therapeutic Antibody Structural Features.** mAbs are highly selective antigen binders forming immune complexes

Published: April 17, 2012



2012

## CESI-MS referenced

Special feature: tutorial — Journal of MASS SPECTROMETRY

Received: 28 July 2014 Revised: 18 November 2014 Accepted: 24 November 2014 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/jms.3554

### Cutting-edge mass spectrometry characterization of originator, biosimilar and biobetter antibodies

Alain Beck,[a]* François Debaene,[b] Hélène Diemer,[b] Elsa Wagner-Rousset,[a] Olivier Colas,[a] Alain Van Dorsselaer[b] and Sarah Cianférani[b]*

**The approval process for antibody biosimilars relies primarily on comprehensive analytical data to establish comparability and high similarity with the originator. Mass spectrometry (MS) in combination with liquid chromatography (LC) and electrophoretic methods are the corner stone for comparability and biosimilarity evaluation. In this special feature we report head-to-head comparison of trastuzumab and cetuximab with corresponding biosimilar and biobetter candidates based on cutting-edge mass spectrometry techniques such as native MS and ion-mobility MS at different levels (top, middle and bottom). In addition, we discuss the advantages and the limitations of sample preparation and enzymatic digestion, middle-up and -down strategies and the use of hydrogen/deuterium exchange followed by MS (HDX-MS). Last but not least, emerging separation methods combined to MS such as capillary zone electrophoresis-tandem MS (CESI-MS/MS), electron transfer dissociation (ETD), top down-sequencing (TDS) and high-resolution MS (HR-MS) that complete the panel of state-of-the-art MS-based options for comparability and biosimilarity evaluation are presented. Copyright © 2015 John Wiley & Sons, Ltd.**

*Additional supporting information may be found in the online version of this article at the publisher's web-site.*

**Keywords:** antibody; biosimilar; biobetter; trastuzumab; cetuximab; glycosylation; mass spectrometry; critical quality attribute

#### Introduction

Biosimilar antibodies are copy versions of already approved biologics that are or will be out of patent soon.[1] In contrast to generic versions of small molecules (150 to 600 Da range), it is not possible to produce exact molecular copies of such large and complex tetrameric glycoproteins (150 000 Da). By nature, biosimilars are produced from different cell clones and different manufacturing processes as the originators. As a consequence differences in glycosylation and other microvariations are observed and may impact (or not) on quality, safety and potency of the drug.[1,2] Only very small differences between biosimilar and reference mAbs, with reassurance that these are not of clinical relevance, may be accepted by health authorities. In 2007, copies of rituximab (Dr Reddy's Reditux®) and abciximab (Isu Abxis', Clotinab®) were first approved in India and in South Korea, respectively, paving the ways for regulatory guidelines for biosimilar antibodies in other parts of the world including highly regulated regions such as Europe, the USA and Japan.[3]

The European Medicine Agency (EMA) has pioneered the regulatory framework for approval of biosimilar products since 2005,[4] resulting to date in marketing authorizations for 16 recombinant drugs including somatropins, erythropoetins, filgastrims and more recently the first two mAbs in the European Union (EU).[4,5] Both mAbs were biosimilar versions of infliximab (Celltrion's Remsima® and Hospira's Inflectra®) that should be followed by biosimilar versions of other blockbuster mAbs such as rituximab, trastuzumab, cetuximab, adalimumab, bevacizumab as well as etanercept, an Fc-fusion protein. Following several workshops starting in 2009, the EMA has released a guideline for 'similar biological medicinal products'. The document discusses relevant animal-model, nonclinical and clinical studies that are recommended to establish the similarity and the safety of a biosimilar compared with a reference mAb or Fc-fusion product approved in the EU.[4,6] Importantly, a revised version was recently released and will come into effect in December 2014 (www.ema.europa.eu).

The approval process for biosimilars relies primarily on comprehensive analytical data to establish comparability and high similarity with the originator biologic. On a practical side and from a regulatory standpoint, antibodies that are not highly similar must undergo greater preclinical and clinical evaluation. Conversely, abbreviated clinical programs may be suitable for biosimilars mAbs showing molecular profiles and characteristics within the range of originator versions (quality target product profile, QTPP). Manufacturing process changes are tightly regulated by the International Committee of Harmonization (ICH) guidelines. Changes of quality attributes are only acceptable if they do not alter safety and efficacy (Critical Quality Attributes). The biosimilar release specifications should be close to the current originator specifications. In the leading generic companies, high degree of

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* *Correspondence to: Sarah Cianférani, Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Université de Strasbourg, IPHC, 25 rue Becquerel 67087 Strasbourg, France. CNRS, UMR7178, 67087, Strasbourg, France. E-mail: sarah.cianferani@unistra.fr*
a *Centre d'Immunologie Pierre Fabre (CIPF), 5 Av. Napoléon III, BP 60497, 74164, Saint-Julien-en-Genevois, France*
b *Laboratoire de Spectrométrie de Masse BioOrganique (LSMBO), Université de Strasbourg, IPHC, 25 rue Becquerel 67087 Strasbourg, France. CNRS, UMR7178, 67087, Strasbourg, France*



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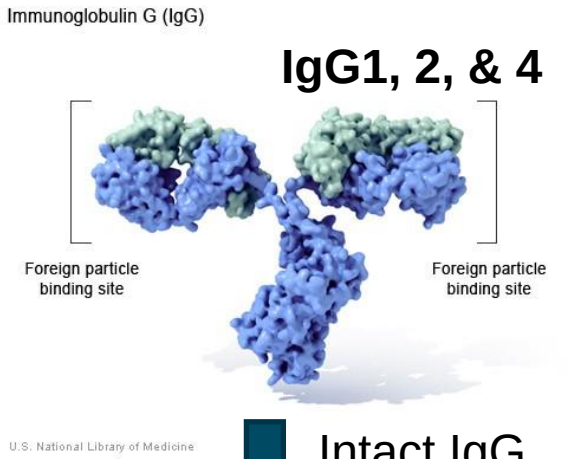
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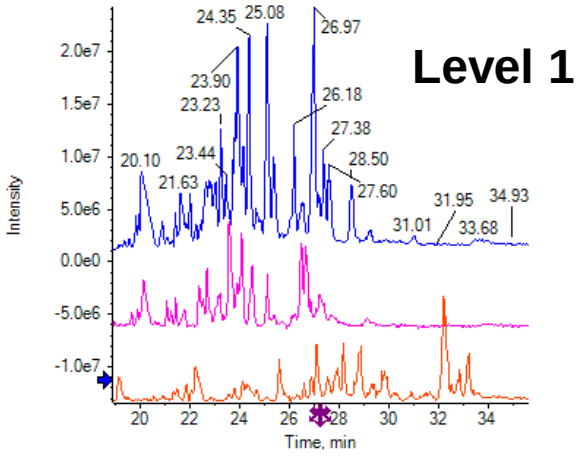
# Многоуровневая характеристика моноклональных антител CESI-MS

Evaluation of CESI-MS for the comprehensive characterization of mAb forms



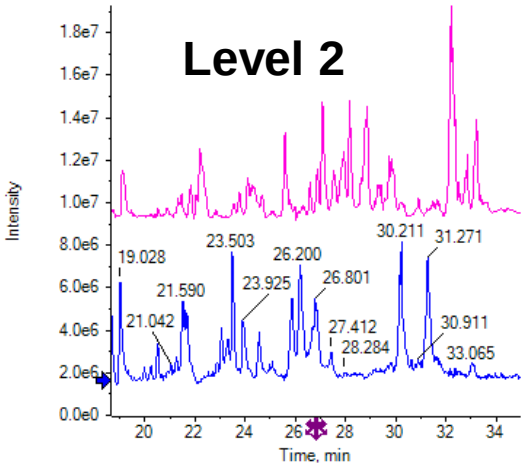

Peptide mapping

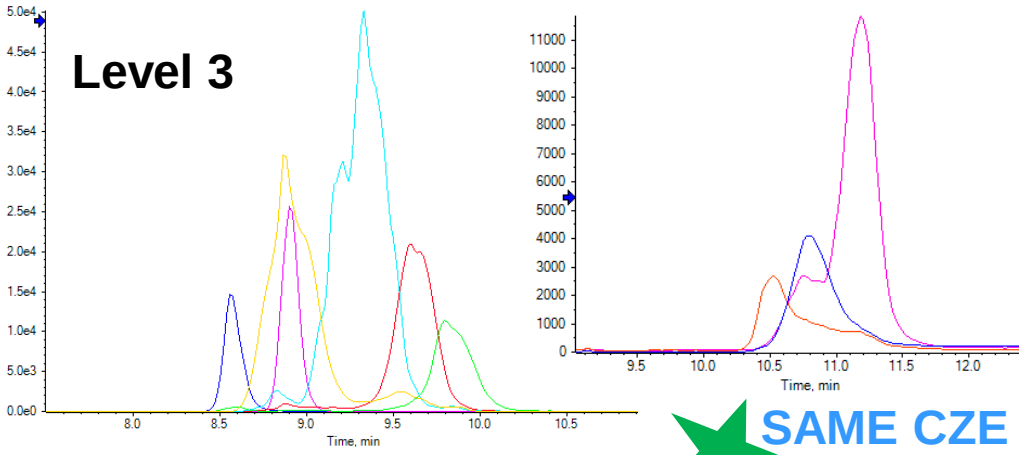
Reduce, alkylate, & digest




Disulfide mapping
Alkylate & digest

Intact IgG
Charge Heterogeneity
& Reduced Analysis



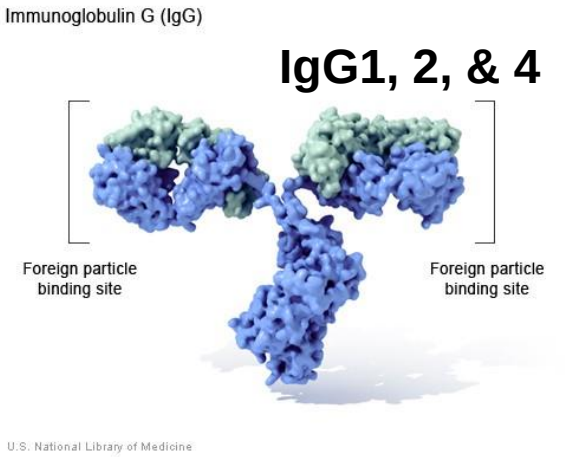




**SAME CZE METHOD & REAGENTS USED FOR ALL ANALYSES**


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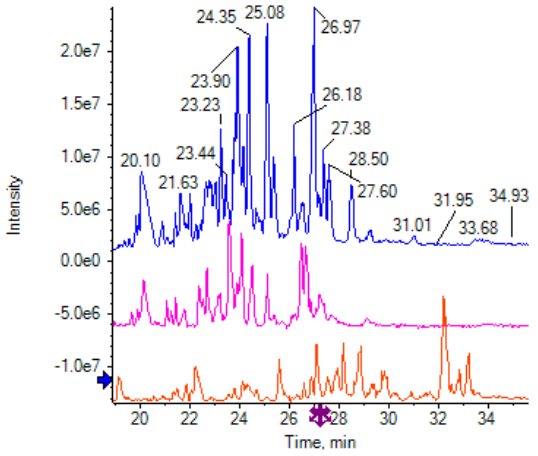
# Подход "снизу-вверх" (bottom-up)

Evaluation of CESI-MS for the comprehensive characterization of mAb forms




Peptide mapping

Reduce, alkylate, & digest

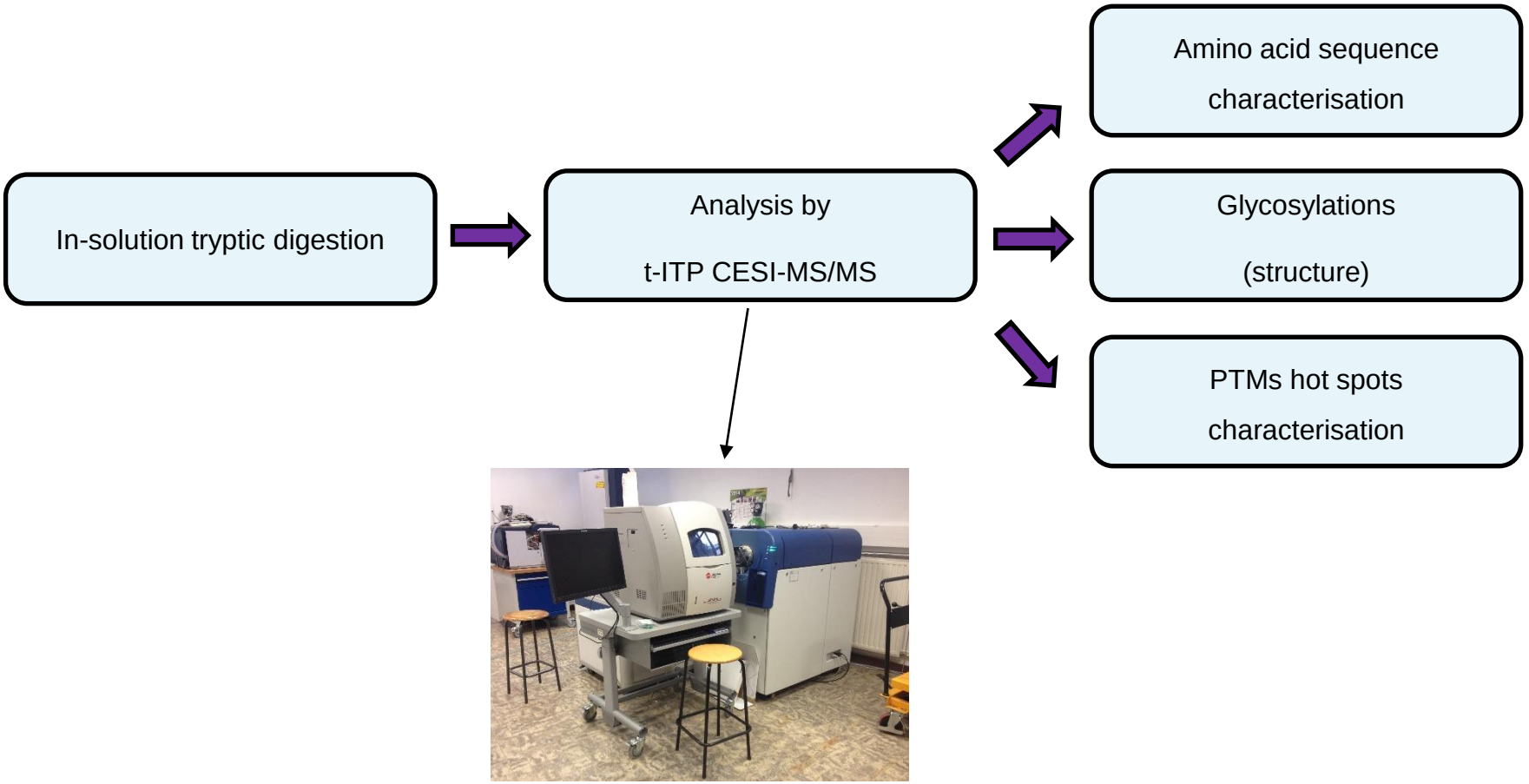




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# mAb Peptide Mapping

- Характеристика первичной структуры основана на стратегии «снизу-вверх»



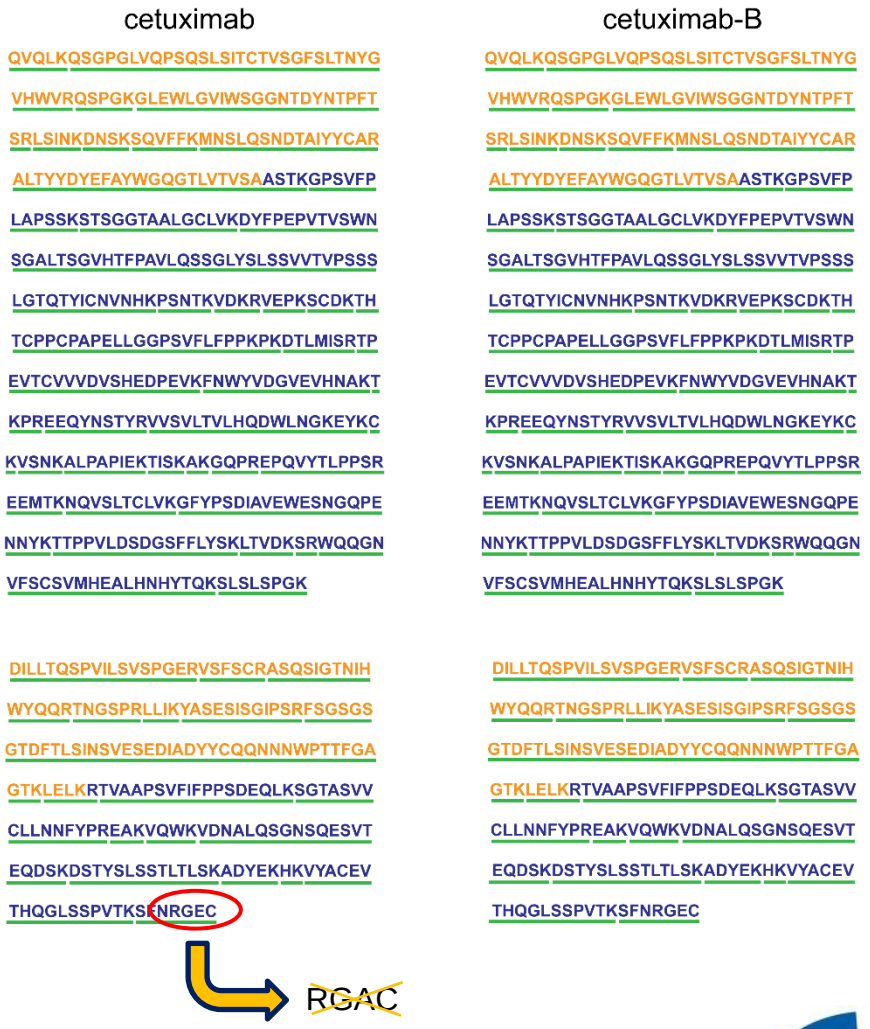

*CESI8000 coupled to 5600 TripleTOF MS*

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Slide courtesy of Dr Yannis Francois, University of Strasbourg

# Исследование биоподобия: Cetuximab

- Один анализ каждой выборки достаточен для заключения о полном сходстве в отношении аминокислотной последовательности
- Получение полной последовательности не дает информации о пост-трансляционных модификациях
- CESI-MS / MS позволяет увидеть ошибку, недавно опубликованную в литературе



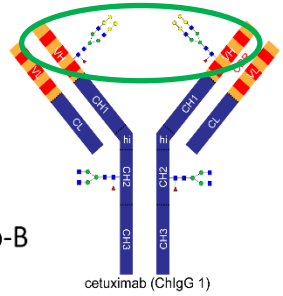

*D. Ayoub et al., mAbs **2013**, 5, 699-710*

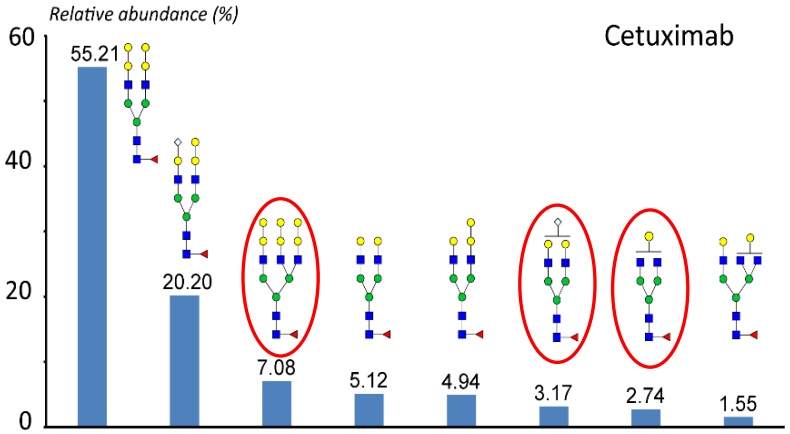
*Gahoual R. et al., mAbs, **2014** (6), 1464-1473*

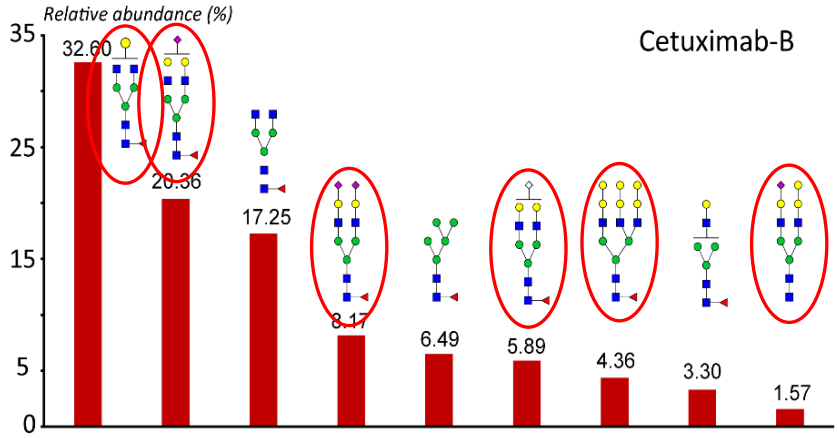

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# Характеристика гликозилирования как оценка биоподобия терапевтических препаратов: Цетуксимаб




Гликоформы претендентов на кандидаты биосимиляра значительно отличаются от Цетуксимаба

- *30 % гликанов содержат N-ацетил нейраминовую кислоту* → Отклонение в качестве биосимиляра

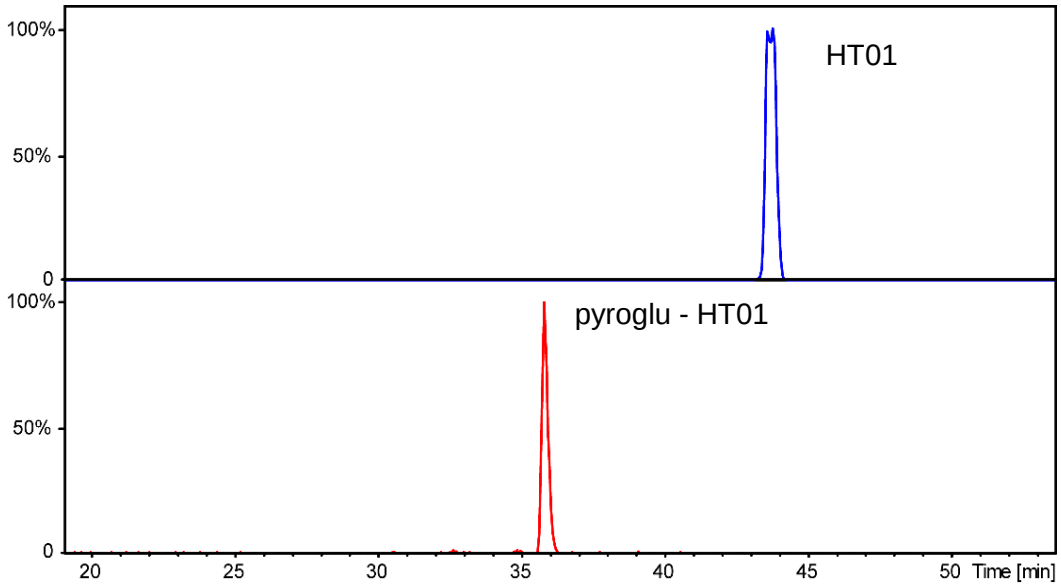
*Gahoual R. et al., mAbs, **2014** (6), 1464-1473*


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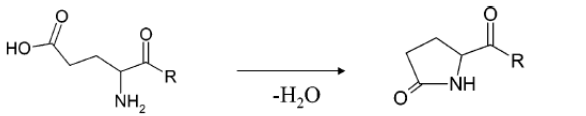
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# Анализ важных пост-трансляционных модификаций- PTM Hot Spot

N-концевая циклизация глутаминовой кислоты




*Extracted ion electropherograms of peptides HT01 and modified HT01*




Результат говорит о наличие частичной модификации образца

- CE позволяет разделить пептид с циклизацией N-терминальной циклизацией глутаминовой кислоты от немодифицированного пептида

*Gahoual R., et al., Anal. Chem.*, ***2014*** (86), 9074-9081


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# Monoclonal antibodies primary structure and biosimilarity assessment in a single analysis using transient isotachophoresis sheathless capillary electrophoresis-tandem mass spectrometry

*Rabah Gahoual[1], Jean-Marc Busnel[2], Johana Chicher[3], Lauriane Kuhn[3], Phillipe Hammann[3], Alain Beck[4], Yannis Nicolas François[1], Emmanuelle Leize-Wagner[1]*

*[1]Laboratoire de Spectrométrie de Masse des Interactions et des Systèmes (LSMIS), CNRS – UMR7140, University of Strasbourg, Strasbourg, France; [2]Beckman Coulter Inc., Marseille, France; [3]Institut de Biologie Moléculaire et Cellulaire (IBMC), University of Strasbourg; [4]Centre d'Immunologie Pierre Fabre, Saint-Julien-en-Genevois, France*

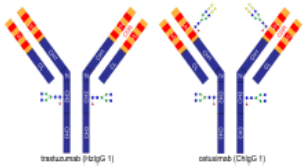
## Overview

Monoclonal antibodies (mAbs) represent currently the therapeutic agent category experiencing the most important progression due to their therapeutic potency and specificity. Some patents regarding mAbs are going to end in the near future, giving the opportunity to alternative actors to produce and market the same protein which is defined in biopharmaceuticals as biosimilars. Here, capillary zone electrophoresis coupled to tandem mass spectrometry by the intermediate of an ultra-low flow interface (CESI-MS/MS) was used to characterize marketed mAbs and their respective candidate biosimilar simultaneously over different facets of their primary structure **using a sole sample injection of 200 fmol of digested peptides**. CESI-MS/MS data enabled to obtain simultaneously **100% sequence coverage**, structures of **15 glycoforms** and the characterization of all PTMs hot-spots present on the studied mAbs samples. CESI-MS/MS allowed to conclude regarding the biosimilarity study between approved mAbs and biosimilar candidate. Characterization results allowed to specifically point out the facets of the candidate which were not complying to be considered as a biosimilar.

## Introduction

Monoclonal antibodies (mAbs) have taken a major market share in the pharmaceutical industry and their development is constantly increasing. Several patents pending upon early approved mAbs are going to end in the next few months, giving the opportunity to different companies to produce "copies" of the concerned antibody, those copies are referred as **biosimilars** or **follow-on biologics**. Since a few years, regulatory agencies have been working toward establishing guidelines designed to determine critical criteria that must be common between an innovator mAb and its biosimilar. mAbs are highly complex proteins that display a wide range of micro heterogeneities that requires multiple analytical methods for full structure assessment and quality control [1]. As a consequence, the characterization of mAbs on different level is particularly product and time-consuming.

In this work, we have developed a transient isotachophoresis CE-ESI-MS/MS methodology, by mean of the CESI-MS system, in order to obtain in the first place the characterization of several mAbs. This characterization, over different level of the protein, was performed in a single analysis of each sample. In a second time, each mAb was compared to a candidate biosimilar in order to establish if the methodology developed could be used to assess the biosimilarity between two samples.




## Methods

**Studied samples.** Trastuzumab, cetuximab in their final formulation and respective candidate biosimilar trastuzumab-B and cetuximab-B were characterized by CESI-MS/MS and the obtained data confronted for biosimilarity assessment

**Samples treatment.** Tryptic digestion in-solution of mAbs. Digestion buffer: 50mM bicarbonate ammonium, reducing reagent: dithiothreitol 100mM, alkylation reagent: Iodoacetamide 100mM. Sample peptide final concentration : 0.3 µg/µL

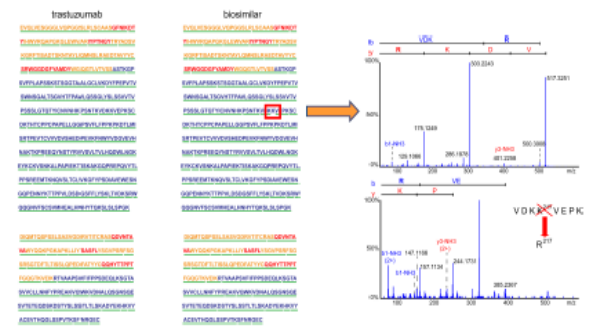
**CE conditions.** Instrument: Sciex separation CESI-MS prototype (Brea, CA, US) equipped with a bare-fused silica capillary cartridge (90 cm, 30 µm I.d.), Injection volume: 100 nL (220 fmol of digest), BGE: 10% acetic acid, separation voltage: +20 kV, duration: 50 min. CESI-MS interface for MS coupling

**Mass spectrometry.** MS/MS was performed using an ESI-Qq-TOF 5600 tripleTOF® (AB Sciex, San Francisco, CA) MS/MS method: IDA (top15 precursor ions), dynamic exclusion (0.15 min after 2 spectra), mass range 100-2000 m/z, duty cycle 1.75 sec, capillary voltage -1.75 kV, curtain gain 5, Source temperature 150°C.

[1] Beck A, Sanglier-Cianférani S, Van Dorsselaer A. , " Biosimilar, biobetter, and next generation antibody characterization by mass spectrometry." Anal Chem 2012, 84:4637-4646
[2] Gahoual R., Biacchi M., Chicher J., Kuhn L., Hammann P., Beck A., François Y-N., Leize-Wagner E., " Monoclonal antibodies biosimilarity assessment using transient isotachophoresis preconcentration-capillary zone electrophoresis-tandem mass spectrometry " 2014, submitted.

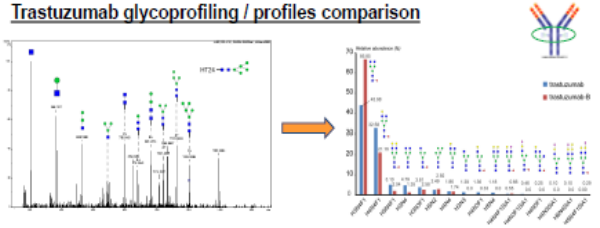
## Results

### Amino acids sequence characterization / comparison




100 % sequence coverage (no miscleavages / PTMs) → 1 AA difference characterized for trastuzumab biosimilar

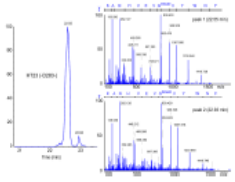
### Trastuzumab glycoprofiling / profiles comparison



Structures deduced from MS/MS spectra — Glycoforms relative abundances established

15 glycosylations structures characterized → Glycoforms distribution comparison for biosimilarity

### PTMs hot-spots characterization / abundance comparison

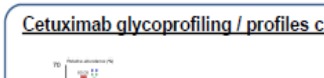


CESI allowed separation of peptides with PTMs

↓

Relative quantitation of each PTMs hot-spots obtained

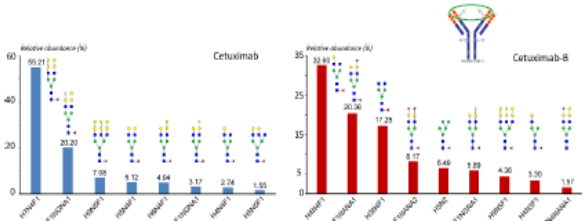
### Cetuximab glycoprofiling / profiles comparison



Glycosylation site dependant characterization

↓

Minor differences exposed between original mAb and biosimilar




Different glycoforms characterized between cetuximab and biosimilar (glycans containing murine sialic acids)

## Conclusions

| | Trastuzumab | Trastuzumab-B | Cetuximab | Cetuximab-B |
|---|---|---|---|---|
| sequence coverage | 100% | 100% | 100% | 100% |
| identified glycosylations | 15 | 10 | 10 | 16 |
| *other PTMs hotspots* | | | | |
| glutamic acid cyclization | 1 / 1 | 1 / 1 | 1 / 1 | 1 / 1 |
| methionine oxidation | 2 / 2 | 2 / 2 | 0 / 0 | 0 / 0 |
| asparagine deamidation | 4 / 4 | 2 / 2 | 4 / 4 | 4 / 4 |
| aspartic acid isomerization | 6 / 6 | 3 / 3 | 2 / 2 | 2 / 2 |

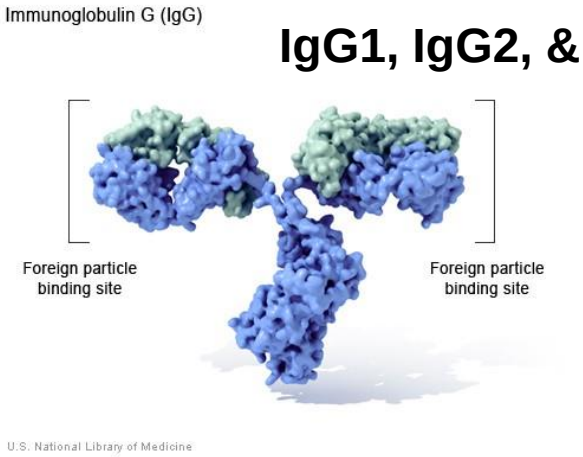
Data obtained from solely one injection of each sample tryptic digest allowed to characterize each mAbs simultaneously over several levels defining their primary structure. **Biosimilarity study results indicated that in both cases, candidate biosimilars exhibited minor differences compared to the originator mAb.** In the case of trastuzumab-B, one AA difference compared to the original mAb leaded to this conclusion while in the case of cetuximab-B uncontrolled expression of murine glycoforms toxic to human metabolism ended up in the rejection candidate biosimilar.

## Acknowledgements

Authors would like to thank AB Sciex for the loan of a prototype CESI system and a 5600 TripleTOF® mass spec. The authors would like to express their gratitude to Dr. M. Anselme (Sciex, Les Ulis, France) for his support and Dr. Joseph Vlasak (Merck, West Point, PA) for critical discussions around antibody structural characterization.

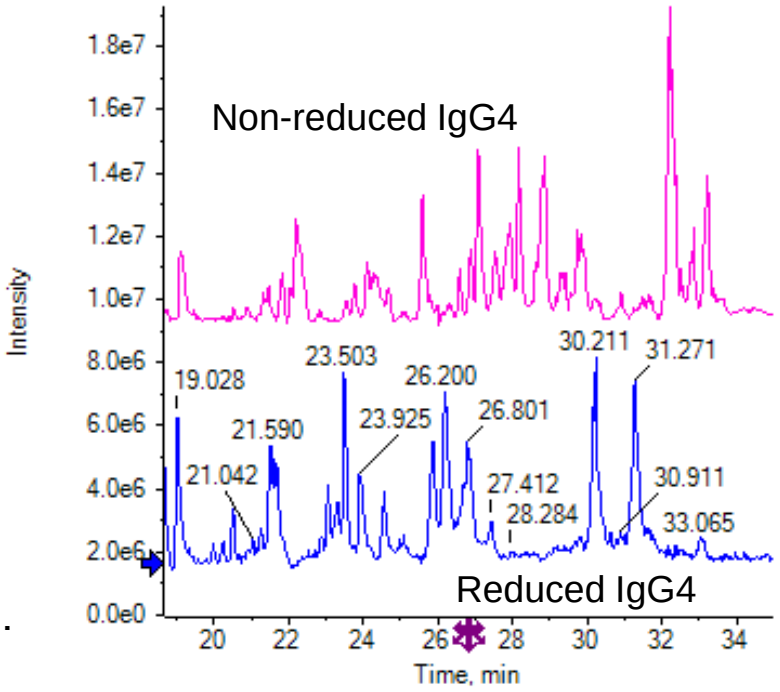

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# Анализ дисульфидных мостиков –подход «с середины вниз» (**middle**-down)




**IgG1, IgG2, & IgG4**

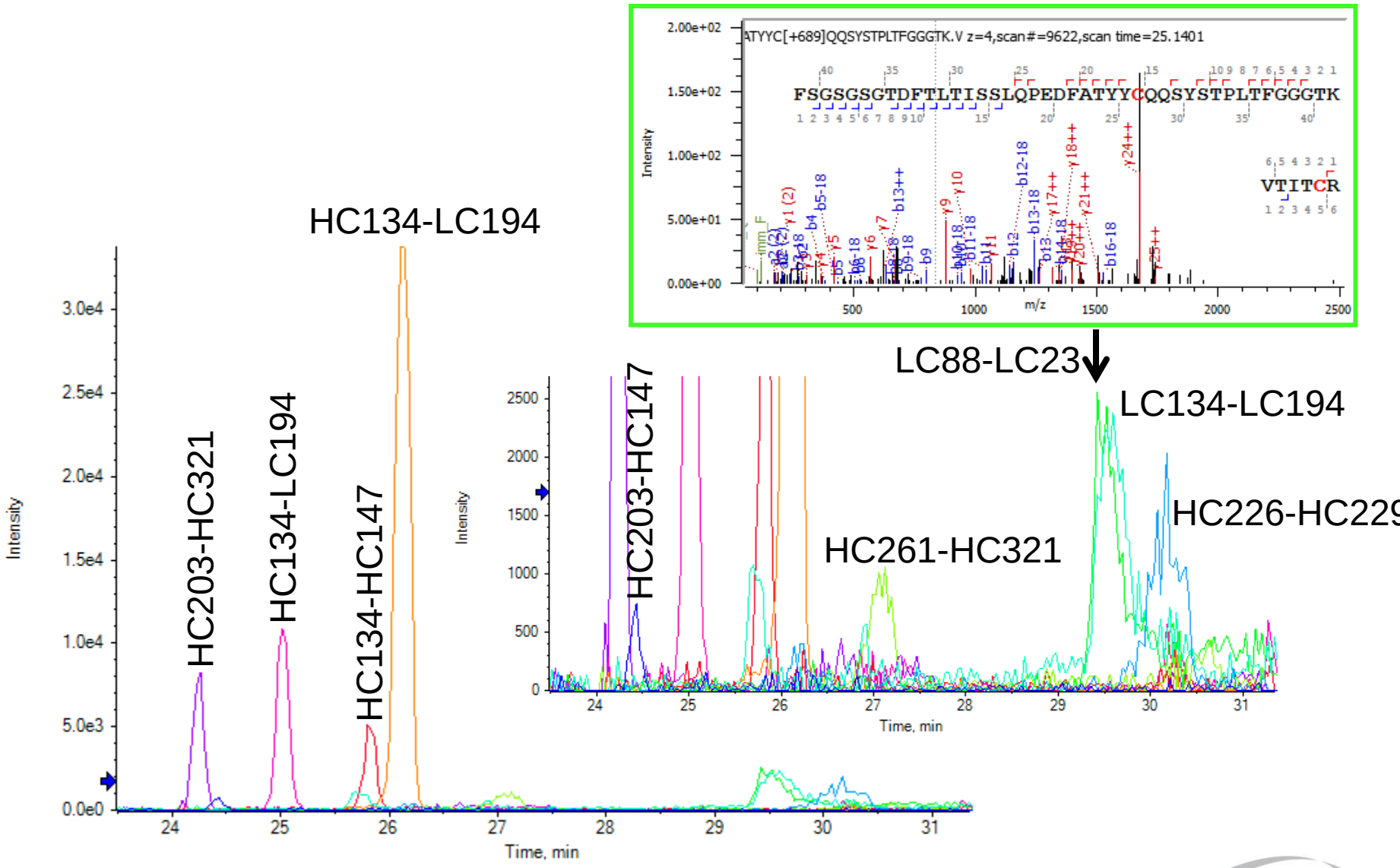
**Disulfide mapping**
**Alkylate & digest**
**~25 ng of digest analyzed**




*Перестановка дисульфидных связей между HC и LC может приводить к функциональным различиям.


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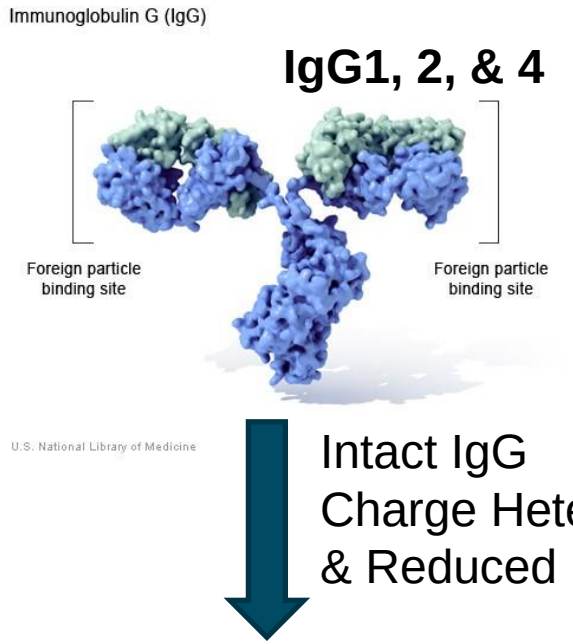
# Идентификация дисульфидных связей IgG4



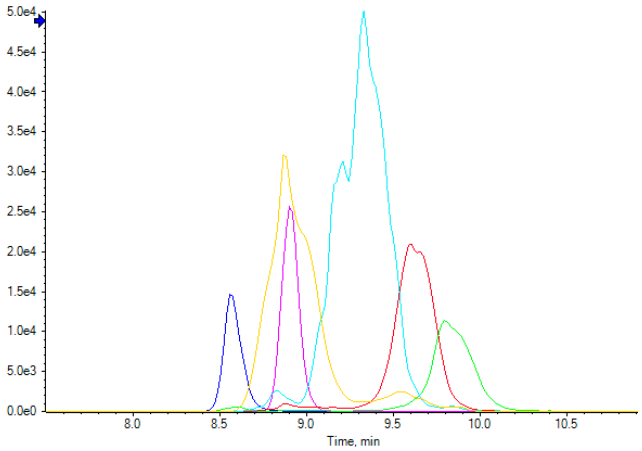


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# Многоуровневая характеристика моноклональных антител CESI-MS – Анализ интактного белка - подход «сверху-вниз» (top-down)




Intact IgG
Charge Heterogeneity
& Reduced Analysis

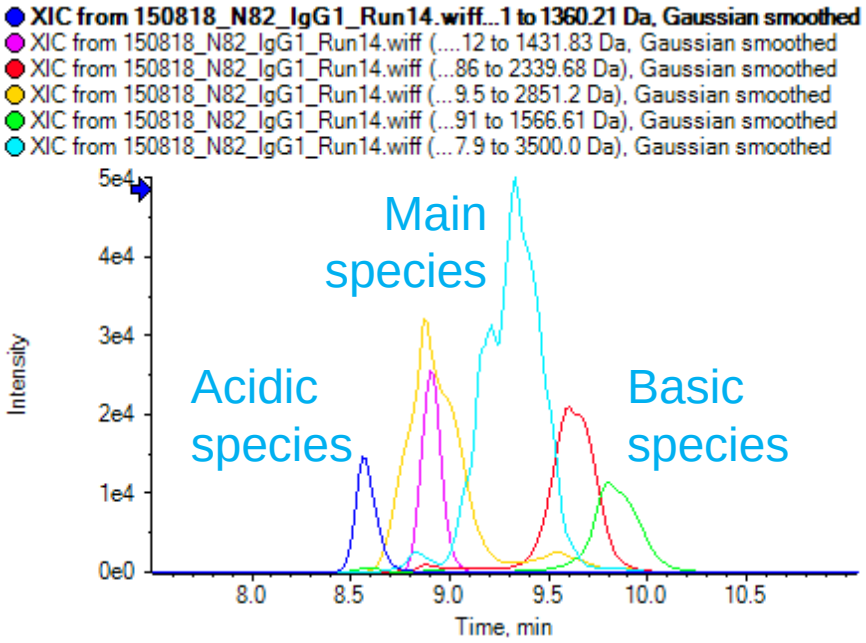


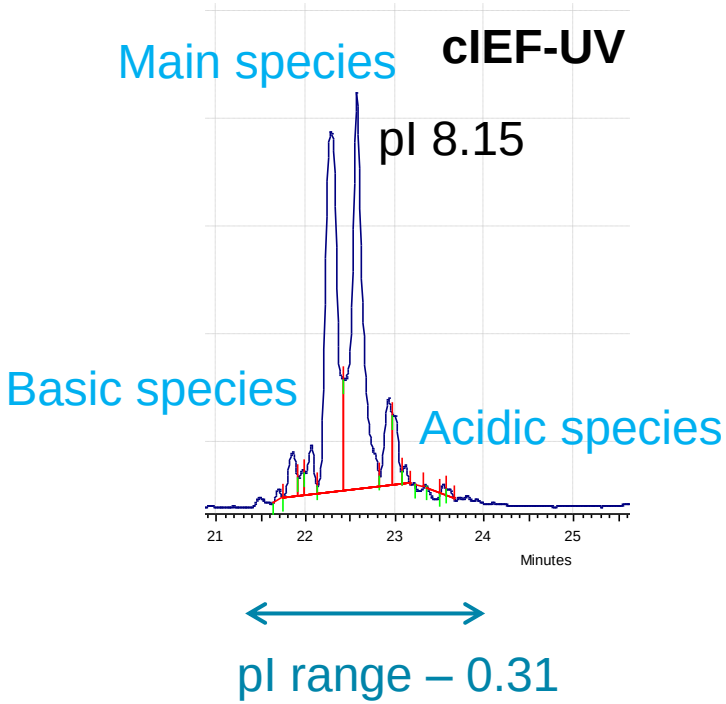


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# Анализ гетерогенности заряда IgG1 с CESI-MS

## CESI-TripleTOF® 6600 MS




~ 10 mg/mL sample desalted into 50 mM ammonium acetate, pH 4

BGE – 3% acetic acid (tITP-CZE mode)

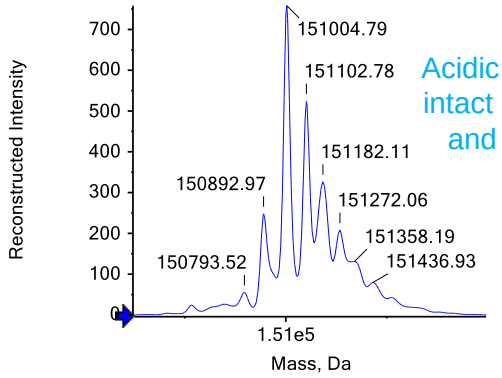
**~3.5 nL injection → ~ 35 ng injected**

***Reverse migration order**

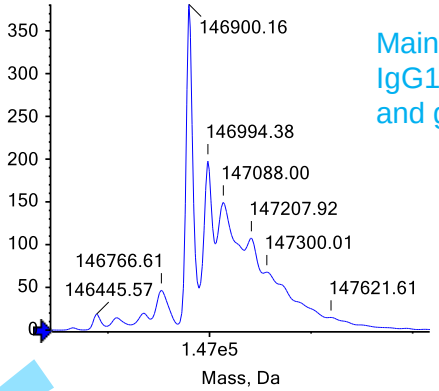

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# Анализ гетерогенности IgG1 с CESI-MS

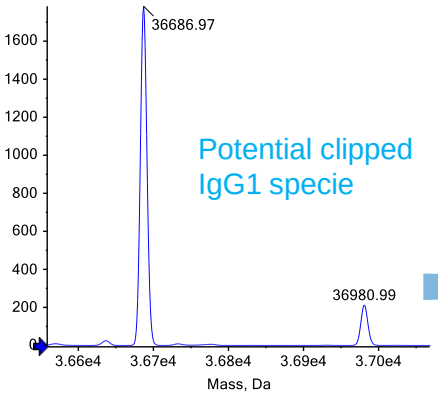
Single analysis at the intact level unifies multiple inferred analyses



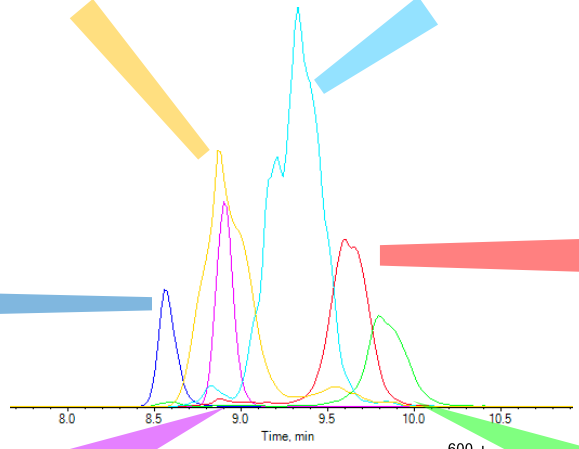

Acidic, higher MW intact IgG1 specie and glycoform

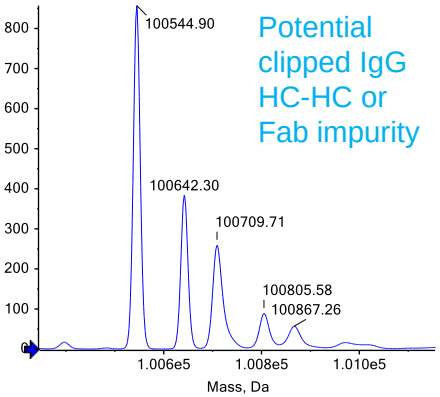



Main intact IgG1 specie and glycoform

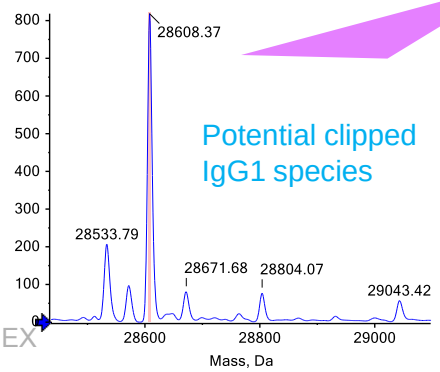



Potential clipped IgG1 specie

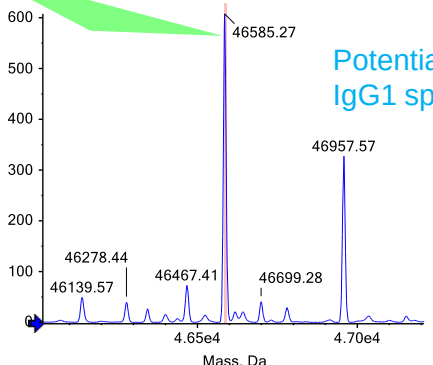



Potential clipped IgG HC-HC or Fab impurity




Potential clipped IgG1 species




Potential clipped IgG1 species


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