Multiplex PCR to detect respiratory viral infections

M. Ieven

ESCMID Venice

25.10. 2010

Vaccine & Infectious Disease Institute

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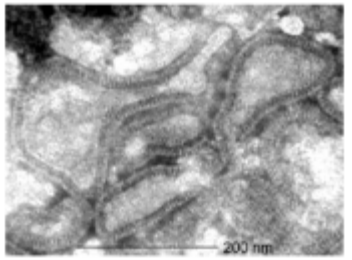
Molecular methods for diagnosis of respiratory tract infections

- Virus discovery ⟹ new molecular diagnostic tests
- Will revolutionize infectious disease diagnosis and patient care
- Early detection of infection impacts outbreak management
- Impact on epidemiological knowledge
  - Role Distribution of respiratory viruses in LRTI
    - In +/- 75% of outbreaks of RTI in the community never have an etiological agent identified
- Medical impact on individual patient care
  - Stop inappropriate AB use: reduces overall costs, resistance:
    - Although viruses account for 80% of all human respiratory tract infections, 60% of patients will receive antibiotics
  - Decrease unnecessary diagnostic studies

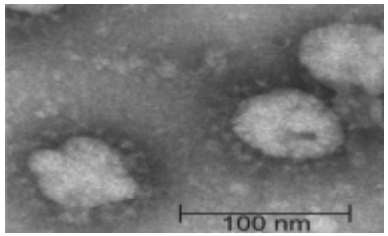
A newly discovered human pneumovirus isolated from young children with respiratory tract disease

2001: hMPV

Bernadette G. van den Hoogen[1], Jan C. de Jong[1], Jan Groen[1], Thijs Kuiken[1], Ronald Groot[2], Ron A.M. Fouchier[1] & Albert D.M.E. Osterhaus[1]



The NEW ENGLAND JOURNAL of MEDICINE

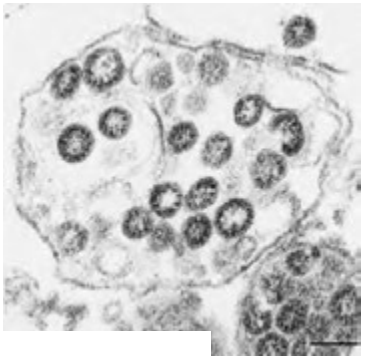


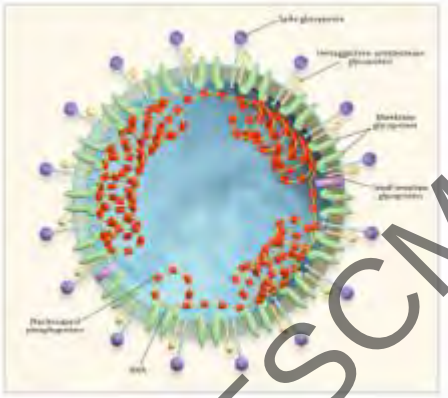
ORIGINAL ARTICLE

2003: HCoV SARS

Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome

Christian Drosten, M.D., Stephan Günther, M.D., Wolfgang Preiser, M.D., Sylvie van der Werf, Ph.D., Hans-Reinhard Brodt, M.D., Stephan Becker, Ph.D., Holger Rabenau, Ph.D., Marcus Panning, M.D., Larissa Kolesnikova, Ph.D., Ron A.M. Fouchier, Ph.D., Annemarie Berger, Ph.D., Ana-Maria Burguière, Ph.D., Jindrich Cinatl, Ph.D., Markus Eickmann, Ph.D., Nicolas Escriou, Ph.D., Klaus Grywna, M.Sc., Stefanie Kramme, M.D., Jean-Claude Manuguerra, Ph.D., Stefanie Müller, M.Sc., Volker Rickerts, M.D., Martin Stürmer, Ph.D., Simon Vieth





nature medicine

2005: HCoV NL 63

Identification of a new human coronavirus

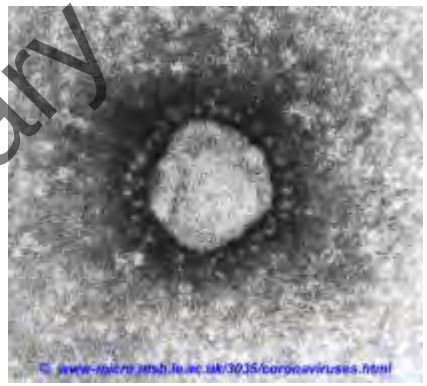
Lia van der Hoek[1], Krzysztof Pyrc[1], Maarten F Jebbink[1], Wilma Vermeulen-Oost[2], Ron J M Berkhout[2], Katja C Wolthers[1], Pauline M E Wertheim-van Dillen[3], Jos Kaandorp[4], Joke Spaargaren[2] & Ben Berkhout[1]

JOURNAL OF VIROLOGY, Jan. 2005, p. 884–895
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.2.884–895.2005

Vol. 79,

Characterization and Complete Genome Sequence of a Novel Coronavirus, Coronavirus HKU1, from Patients with Pneumonia

Patrick C. Y. Woo,[1,2]† Susanna K. P. Lau,[1,2]† Chung-ming Chu,[3] Kwok-hung Chan,[1] Hoi-wah Tsoi,[1] Yi Huang,[1] Beatrice H. L. Wong,[1] Rosana W. S. Poon,[1] James J. Cai,[1] Wei-kwang Luk,[4] Leo L. M. Poon,[1,2] Samson S. Y. Wong,[1,2] Yi Guan,[1,2] J. S. Malik Peiris,[1,2] and Kwok-yung Yuen[1,2]*



2005: HCoV HKU1

PNAS

Cloning of a human parvovirus by molecular screening of respiratory tract samples

Tobias Allander*†‡, Martti T. Tammi§¶, Margareta Eriksson‖, Annelie Bjerkner*, Annika Tiveljung-Lindell*, and Björn Andersson§

2005: Boca virus

Journal of Clinical Virology
Volume 38, Issue 3 , March 2007, Pages 227-237

doi:10.1016/j.jcv.2006.12.016 Cite or Link Using DOI

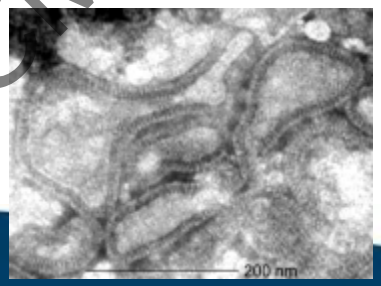
2007: rhinovirus C

Molecular characterization of a variant rhinovirus from an outbreak associated with uncommonly high mortality

David Kiang[a], , , Shigeo Yagi[a], Katherine A. Kantardjieff[b], Euna J. Kim[b], Janice K. Louie[a] and David P. Schnurr[a]

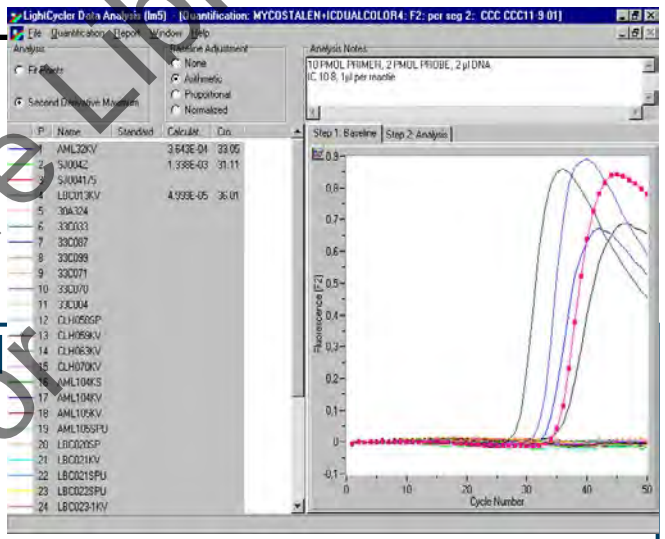
The main respiratory targets in molecular diagnostic tests

- Those in our routine panel:
  - Influenza A (IFVA)
  - Influenza B (IFVB)
  - Parainfluenza (PIV) 1-4
  - Respiratory syncytial virus (RSV)
  - Adenoviruses (ADV)
  - Metapneumovirus (hMPV)



- Those extra assays that many would consider important:
  - Rhinoviruses
  - Enteroviruses
  - Coronaviruses (OC43, 229E, NL63 and HKU1)
  - IFVA sub-typing
  - Bocavirus
  - Atypicals: *M.pn*., *C. pn*., *Leg. pn*., *Bordetella pertussis*

Conventional and Real-Time Mono- and Multiplex NAAT



Author	targets	Species detected
Fan, J et al. 1998	2	RSVA, RSVB
Scheltinga et al. 2005	2	hMPN, RHI
McDonough et al. 2005	4	*M. pn.*, *C. pn.*, *L. pn.*, *B. pertussis*
Gunson et al. 2005	12	IFL A and B, PFL 1,2,3 RHI, hMPN RSVA and B, COR E229, OC 43, NL63 in 4 triplex reactions
Loens et al. 2007	3	*M. pn.*, *C. pn.*, *L. pn*
Choi et al. 2006	12	in 4 multiplex and one monoreaction
Tiveljung et al. 2009	16	in 13 reactions: IFL A and B, RSV A+B, PFL 1+3, PFL 2+ hCoV-229E, ADE, hMPV, RHI, ENT, HCoV-OC43, HCoV-NL63 and HKU, HBoV

Ieven M, J Clin Virol 2007; 40: 259-76

Commercially available Mono- and Multiplex tests

	targets	Species detected
Xpert FluA, Cepheid	2	Influenza A and subtyping
RSV,ASR, Cepheid	2	RSVA, RSVB
ProPneumo-1, Prodesse	2	*M. pneumoniae*, C. *pneumoniae*
RespiFinder plus, Pathofinder	18	IFL A/B, PFL 1-4, RHI, hMPN, RSV A/B, AV, 3 coronaviruses, *M. pn., C.pn., L.pn., Bordetella pertussis*
SeeplexRV, Seegene	19	*S. pneumoniae*, *H. influenzae*, *M. pn., C.pn., L.pn.,* IFL A and B, RSV A/B, PFL 1-3, RHI, 3 coronaviruses, AV, HBoV, EV
xTAG RVP, Luminex	19	IFL A (H1, H3, H5, non-specific) and B, PFL 1-4, RSV A/B, ADE, hMPV, RHI/ENT,SARS-COR, HCoV OC43, HCoV 229E, HCoV NL63 and HKU1

Technological Advances

- Rapid, real-time PCR/TMA/NASBA/LAMP
- Fast cyclers
- New innovative detection systems
- Multiplex capabilities expanding
- Automation for NA extraction
- Automated amplification and detection platforms
- Full integration of MDx steps
- Molecular “Point of Care” testing
- Next gen sequencing

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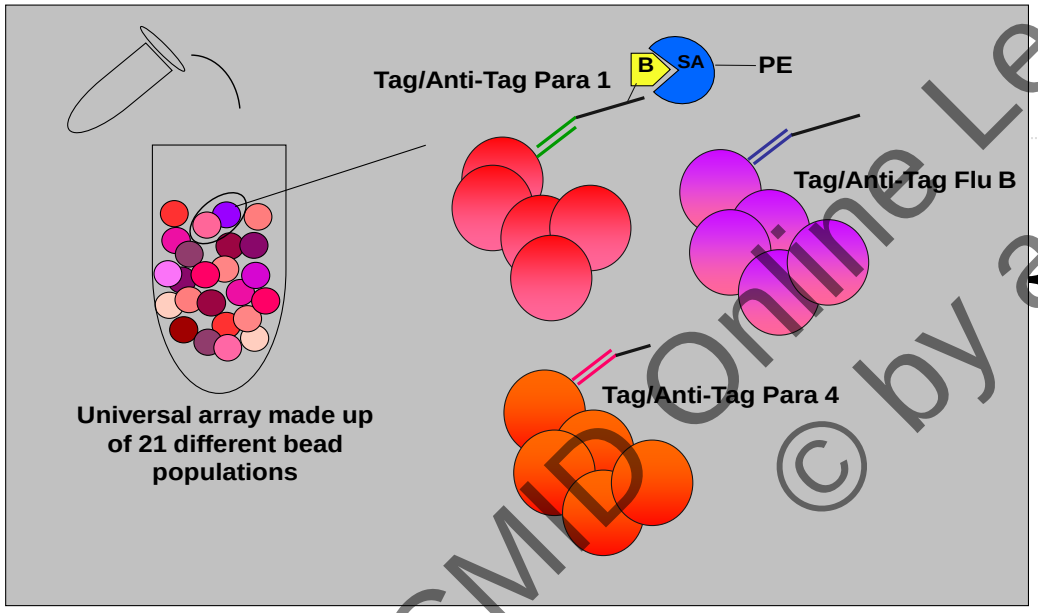
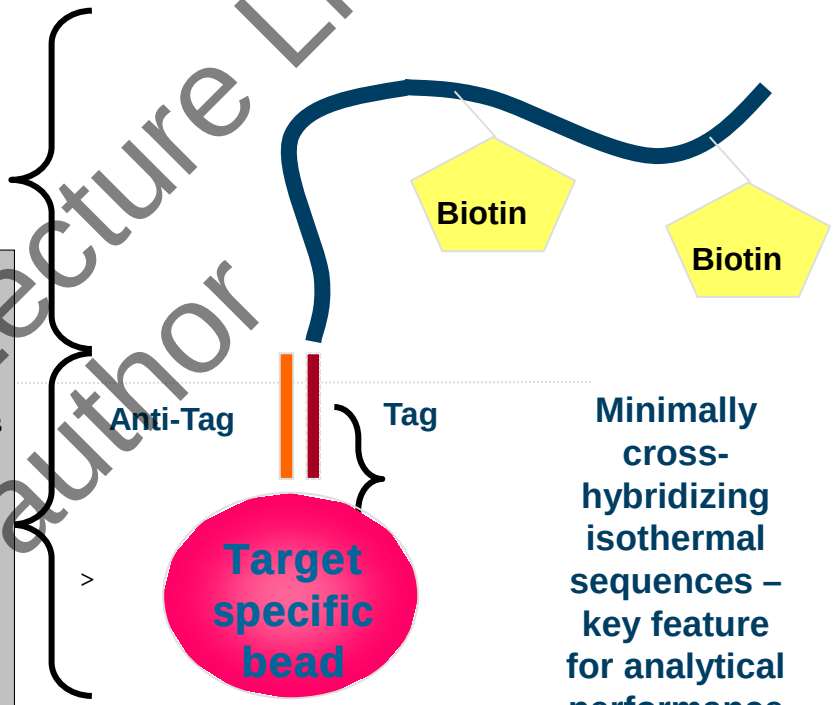
Luminex xTAG Universal Bead Array

Universal Array Sorting

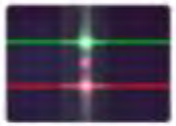
Oligo-coupled beads used with Tag-It products. Use MFI for every target-specific primer to determine presence or absence of each virus





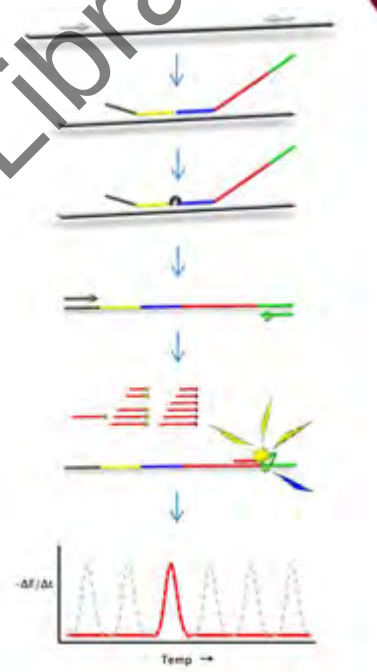
Minimally cross-hybridizing isothermal sequences – key feature for analytical performance

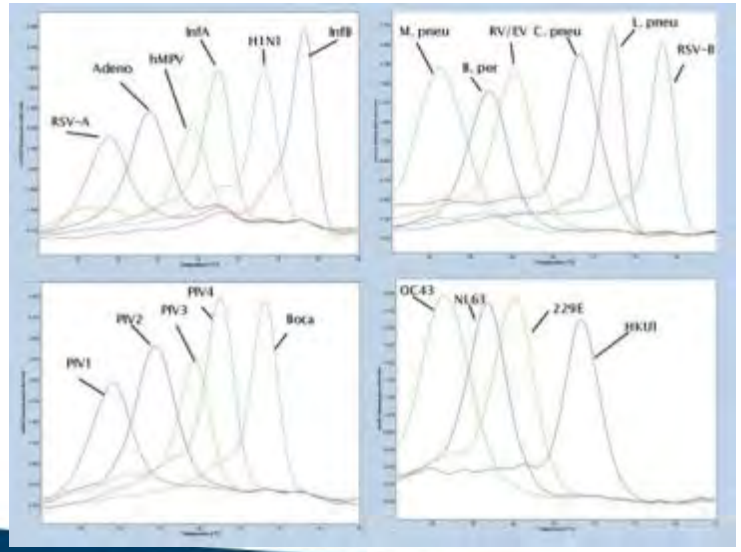
RVP 10-12 hr to RVP Fast 4-5 hr



RespiFinder Mx assay, Pathofinder

- Rapid, conventional & real-time PCR
- Multiplex capabilities expanding till up to 25 targets
- 18 viral + 4 bacterial pathogens in 1 RT assay
- Gene-specific Mx reverse transcription step
- 2 pathogen specific probes hybridized by ligation, amplified and detected by melting curve analysis
- Contains a competitive internal amplification control
- Diagnosis within 6 hours
- Validated on QCMD panels
  - CE-IVD labelled





MxDs: The Past, Today and the Future

The future

- Major advances in microfluidics and -electronics: “Biochips”, “Lab-on-a-chip” , “nanotechnology” or POCT

Miniaturization

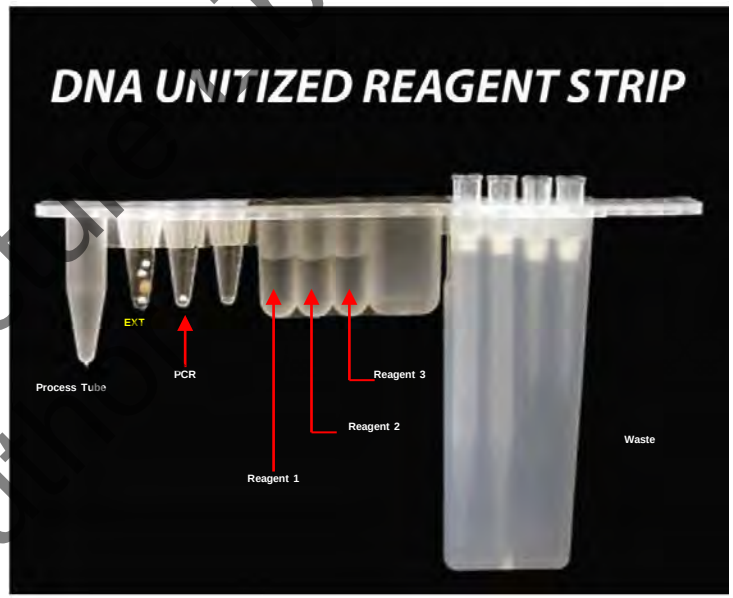
Change of scale

Cepheid GeneXpert System

Single use cartridge based test
TAT 1.5 to 2/5 hr
Up to 6 targets
RVP panel

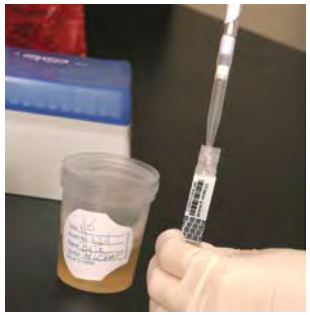
BD Max Jaguar System by Handylab

- Complete System: extraction, amplification and real time detection
- Reagent strip and PCR cartridge
- 1st generation: 2 colors
- 2nd generation: 6 colors
- BD developed assays
- Open system for LDTs



PCR Cartridge

Max Testing Process



One manual pipetting step

Load reagents and specimens

Place rack in Max

Load PCR cartridge

Place on Max

Create worklist and Close door to initiate run

2 positions with 12 slots per position

Idaho Technology Inc.
Innovation Amplified

- System integrates sample preparation, amplification, detection and analysis
- All reagents are freeze dried in 1 pouch
- Closed system prevents cross-contamination
- Internal controls for each step
- Advanced software runs the system and automatically analyzes and reports results
- Multiplexed testing analyzes up to 120 tests per sample
- Rapid results in 1 hr from sample injection

Viral: Adeno, Boca, Corona (229E, HKU1, OC43,NL63), Flu A, Flu A H1, Flu A H1 2009, Flu A H3, Flu B, hMPV, Para (1, 2, 3, 4), RSV, Rhino
Bacterial: *B. pertussis, C. pneumoniae, M. pneumoniae*

Some Technical, Scientific and practical hurdles

- For number of newest technologies: limited validation and little proven clinical applications
- Few FDA cleared
- Which pathogen?
- Which clinical specimen?
- Colonization or infection?
- Sample preparation
- Some methodologies: low throughput tests
- Some methodologies: need for multiple instruments
- Technically demanding and labor intensive
- Costly

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EQA panel to evaluate the performance of different real-time PCRs in GRACE

GRACE Sample Number	Sample Content	Conc. / Dilution (Ct)	Centre 2 Results	Centre 3 Results	Centre 4 Results
GRACE-01	HMPV-I	$1x10^{-6}$ (36)	**hMPV** Positive	**hMPV** Positive	**Negative**
GRACE-02	Influenza A virus Type H3	$1x10^{-7}$	**Negative**	**PIV 2/4** Positive	**Negative**
GRACE-03	Parainfluenza virus 2	-34	**PIV 2** Positive	**PIV 2/4** Positive	**PIV 2** positive
GRACE-04	Negative Transport Medium	0	**Negative**	**Negative**	**Negative**
GRACE-05	Negative Transport Medium	0	**Negative**	**Negative**	**Negative**
GRACE-06	Influenza B virus	$1x10^{-6}$	**INF B** Positive	**INF** Positive	**Negative**
GRACE-07	HMPV-II	$1x10^{-5}$ (35)	**hMPV** Positive	**hMPV** Positive	**Negative**
GRACE-21	RSVA	$1x10^{-5}$ (24)	**RSV** Positive	**RSV** Positive	**ADV + RSV** positive

Sample content	Conc. / Dilution	Centre 2	Centre 3	Centre 4
Influenza A	1x10-6	**Negative**	**INF** positive	**Negative**
Influenza B	1x10-6	**INF B** positive	**INF** positive	**Negative**
Influenza A	1x10-7	**INF A** positive	**Negative**	**Negative**
hMPV-II	1x10-5	**hMPV** positive	**hMPV** positive	**Negative**
RSV A	1x10-5	**RSV** positive	**RSV** positive	**RhV** positive

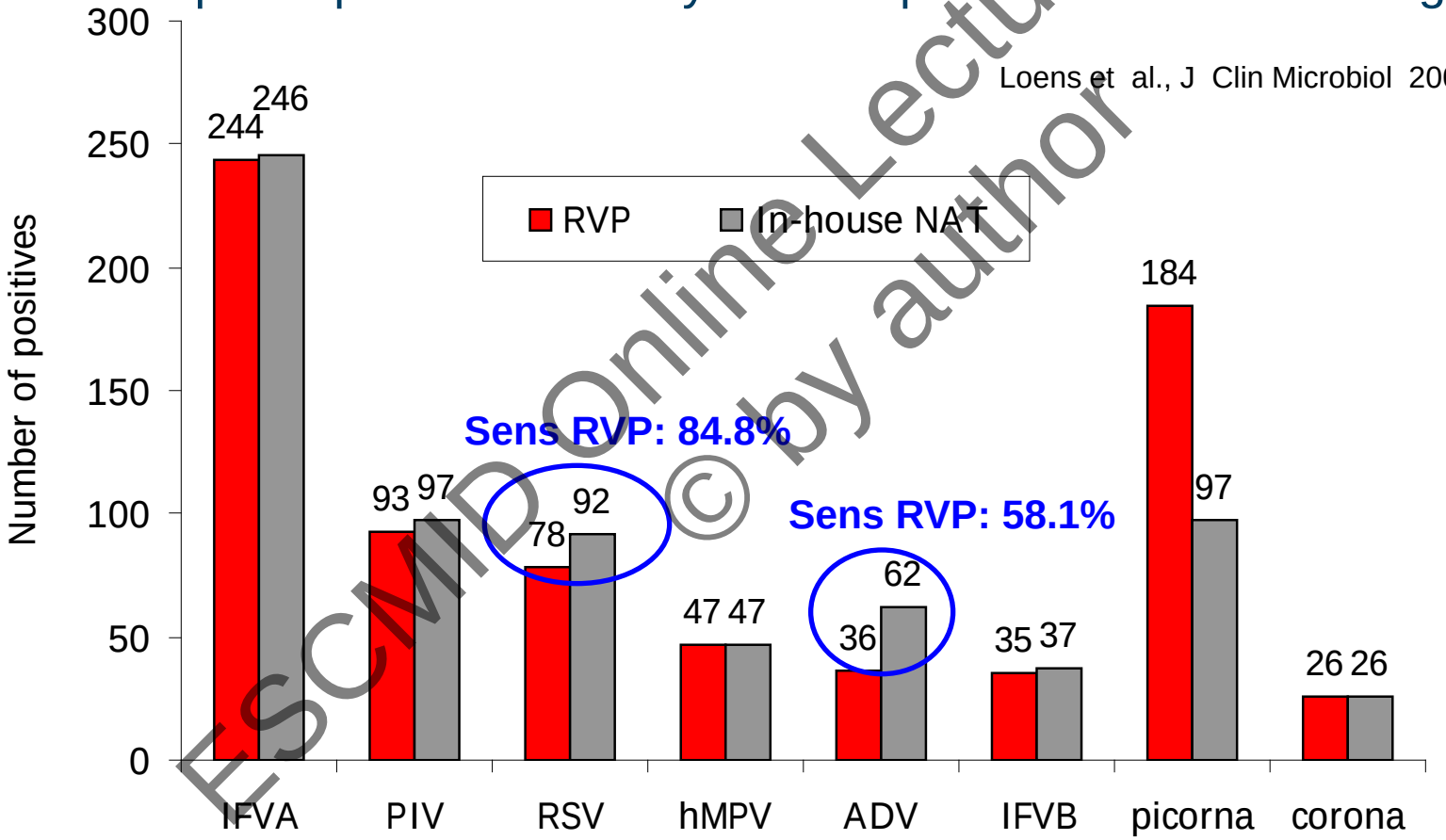
EQA panel to evaluate the performance of different real-time PCRs in GRACE

GRACE Sample Number	Sample Content	Conc. / Dilution (Ct)	Centre 2 Results	Centre 3 Results	Centre 4 Results
GRACE-22	Coronavirus NL63($2x10^{-6}$)	(35-37)	**Negative**	**hCoV** Positive	**Negative**

Sample content	Conc. / Dilution	**Centre 2**	**Centre 3**	**Centre 4**
hCoV NL63	2x10-6	**Negative**	**hCoV** positive	**Negative**
hCoV OC43	1x10-4	**hCoV** positive	**hCoV** positive	**Negative**
RhV 90	1x10-5	**RhV** positive	**RhV** positive	**Negative**
ADV 1	1x10-2	**ADV** positive	**ADV** positive	**Negative**

Single detection versus Mx testing?

- Both multiplex PCR and NASBA formats scored a smaller number of samples positive for *M. pn., C.pn., L. pn.* than monoplex reactions .
- One commercial Mx kit scored only 1/3 of all + samples positive despite reported sensitivity of 10 copies of DNA for each organism.



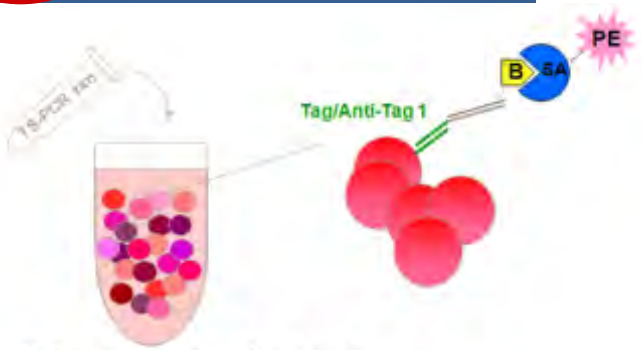
From Fox. J. ECCMID 2OO8

Sensitivity and specificity of two different commercial assays

- Evaluation of 2 CE labeled multiplex assays: RespiFinder (Pathofinder) and xTAG RVP (Luminex) on
- Compared with in-house PCRs used in GRACE

Virus	RespiFinder		xTAG RVP	
	Sensitivity	Specificity	Sensitivity	Specificity
INF	84.8%	98.4	72.3	96.8
HCoV	89.1	99.0	32.6	98.7
hMPV	100	98.5	96.6	99.4
HRV	95.2	98.3	88.7	99.6
RSV	88.5	100	71.4	99.1

- Both commercial assays are less sensitive than in-house PCRs used in GRACE
- RespiFinder (Pathofinder) is more sensitive than Luminex assay



Limited target detection versus multiplex detection

Limited target detection

- Usually ⇧ analytical sens.
- Lower cost
- Often lower TAT
- In outbreak situations
  - SARS Coronavirus
  - Influenza, H1N1
  - RSV, *L. pn*, *M.pn*
- As first approach
  - in high prevalence periods
  - if therapeutic implications
    - Influenza, *Legionella* spp, *Mycoplasma pn., B. pertussis*
- Outside normal lab working hrs

Multiplex detection

- In >90% similar results
- Expensive
- TAT usually > 4-6hours
- For epidemiological studies
  - Prevalence of respiratory etiologies
  - Role of respiratory viruses
- As add-on diagnostic test
  - In severely ill patients
  - In immunocompromised
- For virus discovery studies

Influenza H1N1 outbreak management

- Semiautomated and fully automated assays for detection of FluA (including H1N1) and FluB:

  Flu A sensitivities varying between 93% - 98%,

  specificities varying between 99 -100%

Sails AD et al. J Virol Methods 2009, 162: 88-90
Ginocchio CC et al J Clin Virol 2009; 45: 191-195
Casalegno JS et al Clin Microbiol Infect 2009; 16: 326-329
Beck ET, et al J Mol Diagnostics 2010; 12: 74-81

⇨ Automated **high throughput molecular system** allows clinicians and public health officials to **react quickly during outbreaks**

Impact on patient management: Need for speed! TAT is crucial!

- Evaluation of 2 real-time RT-PCR assays (Cepheid, Sunnyvale, USA) on SmartCycler **(TAT < 1hour)**
  - RSV Analyte Specific Reagent (ASR) bead
  - Influenza A/B ASR bead
- Comparison with “in-house” multiplex real-time PCR on +/-100 respiratory samples for Influenza and RSV

- Results: facilitates urgent testing outside batched runs or
  - RSV: sens: 98.2%, spec: 100%
  - Influenza A/B: sens: 96.5%, spec: 100%

⇒ Compared to “in house” multiplex: significant in ⇓ TAT

⇒ facilitates urgent testing outside batched runs or normal working day

Sails AD et al. J Virol Methods 2009, 162: 88-90

GRACE: Etiologic diagnosis of LRTI in primary care: impact of RT-PCR

- EU FP6 Network of excellence
  16 PCN in 11 countries
- **From 10/2007 – 12/04/2010**
  - 3102 adult patients with LRTI
  - 2984 controls
  - 16 PCN in 12 countries
- Blood & respiratory samples taken, transported to Antwerp

⇒ Presence viral etiologic agent by RT-PCR: **53.7%**

⇒ In total: etiology found in **> 70%** in LRTI in community

Significance of etiologic agents?

Target	Patient with LRTI prevalence n/total (%) first visit (n=3059)	Patient with LRTI prevalence n/total (%) follow up visit (n=2566)	*P*-value	Matched Control subject prevalence n/total (%) (n=1678)	*P*-value
Parainfluenza 1-4	73 (2.4)	12 (0.5)	< 0.00001	8 (0.5)	< 0.00001
Rhinovirus	589 (19.3)	112 (4.5)	< 0.00001	72 (4.3)	< 0.00001
Human MPV	126 (4.1)	7 (0.3)	<0.00001	3 (0.2)	< 0.00001
Human AV	38 (1.2)	42 (1.6)	0.26	24 (1.4)	0.68
Bocavirus	13 (0.4)	12 (0.5)	0.96	16 (1.0)	**0.04**
RSV	143 (4.7)	13 (0.5)	< 0.00001	11 (0.7)	< 0.00001
Influenza A/B	300 (9.8)	10 (0.4)	< 0.00001	6 (0.4)	< 0.00001
Coronaviruses	208 (6.8)	70 (2.7)	< 0.00001	27 (1.6)	< 0.00001
Polyomavirus WU	45 (1.5)	54 (2.1)	0.09	39 (2.3)	**0.04**
Polyomavirus KI	27 (0.9)	28 (1.1)	0.51	17 (1.0)	0.77
M. pneumoniae	0/809 (0)	0/653 (0)	NS	0/492 (0)	NS

Ieven M et al on behalf of GRACE ECCMID 2010

[1]:Only data of first winter season

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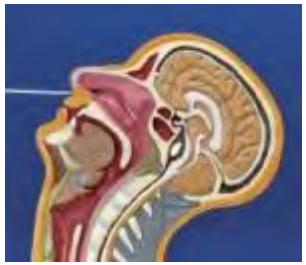
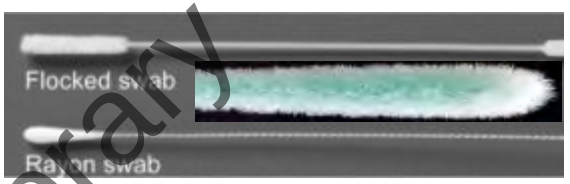


CA-LRTI: comparison of samples/organism



Pathogen	Sample ranking	Method	Age (yr)	Total no. of specimens/ no. of patients	Reference
M. pneumoniae	Sputum > TW > NPS > OPS	PCR	20-93	552/144	(1)
	OPS > NPS	PCR	NSp	132/66	(2)
	OPS > BAL > Sputum	PCR	NSp	325/197	(3)
	Sputum > OPS	Gene-probe test	>18	160	(4)
	Sputum > NPA	Ag-EIA	>18	102/51	(5)
	Sputum > OPS	Culture, PCR, NASBA	NSp	302/180	(6),(7)
	NPS = OPS	PCR	NSp	63	(8)
	Sputum > NPA = OPS	PCR	22-29	96/32	(9)
	OPS > NPA	PCR	NSp	102	(10)

(1) J. Clin. Microbiol. 2001, 39: 1184-6
(2) Scan. J. Infect. Dis. Suppl. 1997, 104: 11-2
(3) J. Clin. Microbiol. 2000, 38: 1382-4
(4) J. Infect. Dis. 1990, 162: 70-5
(5) Eur. J. Clin. Microbiol. Infect. Dis. 1993, 12: 872-5
(6) J. Microbiol. Methods 2008, 73: 257-62
(7) J. Clin. Microbiol. 2008, 46: 185-91
(8) J. Clin. Microbiol. 2004, 42: 3339-41
(9) J. Med. Microbiol. 2005, 54: 287-91
(10) Eur. J. Clin. Microbiol. Infect. Dis. 1995, 14: 58-61

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- Costly

TheraEDGE Project

An Integrated Platform Enabling Theranostic Applications at the Point of Primary Care

- 4 year project
- Budget: 11M€
- 9 different countries

Theranostics & Personalized Medicine

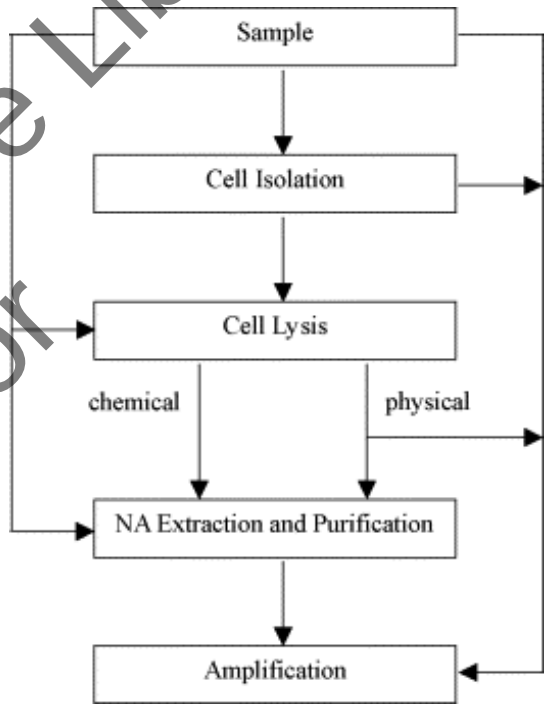
TheraEDGE

www.theraedge.org

A Big Bottleneck in Developing POCTs: Sample Preparation

- Off-chip (macroscale) sample prep
  - Laborious
  - Refrigerated/frozen reagents
  - Large sample volumes
  - Requires centrifuges, bead beaters, several machines
  - Few hours

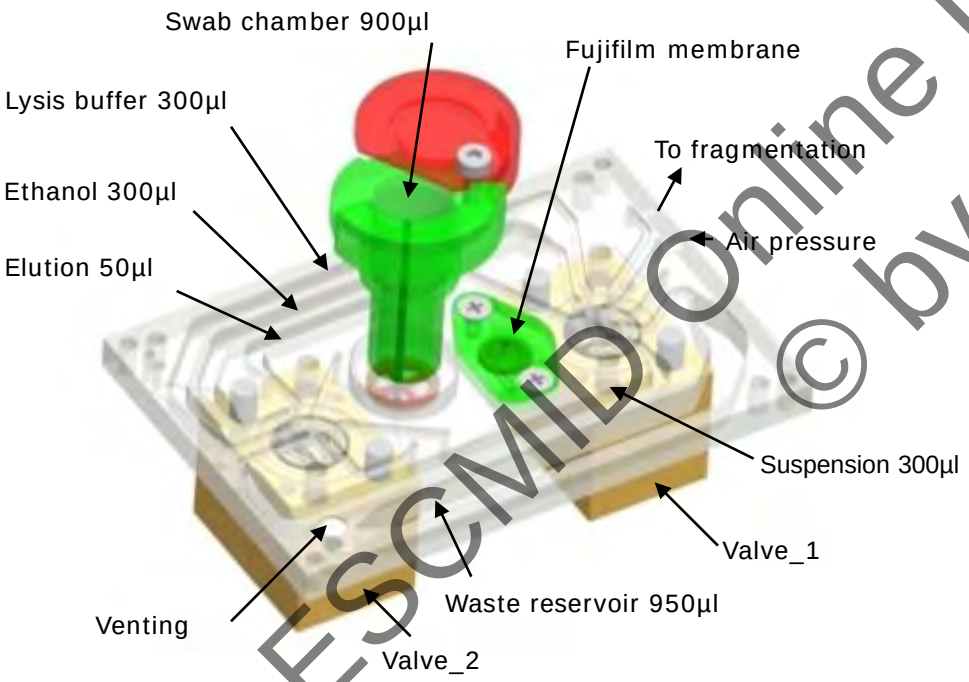
- On-chip sample prep
  - Room temperature stable reagents (disposable chips with on-chip storage)
  - Microliter volumes
  - Few minutes!!



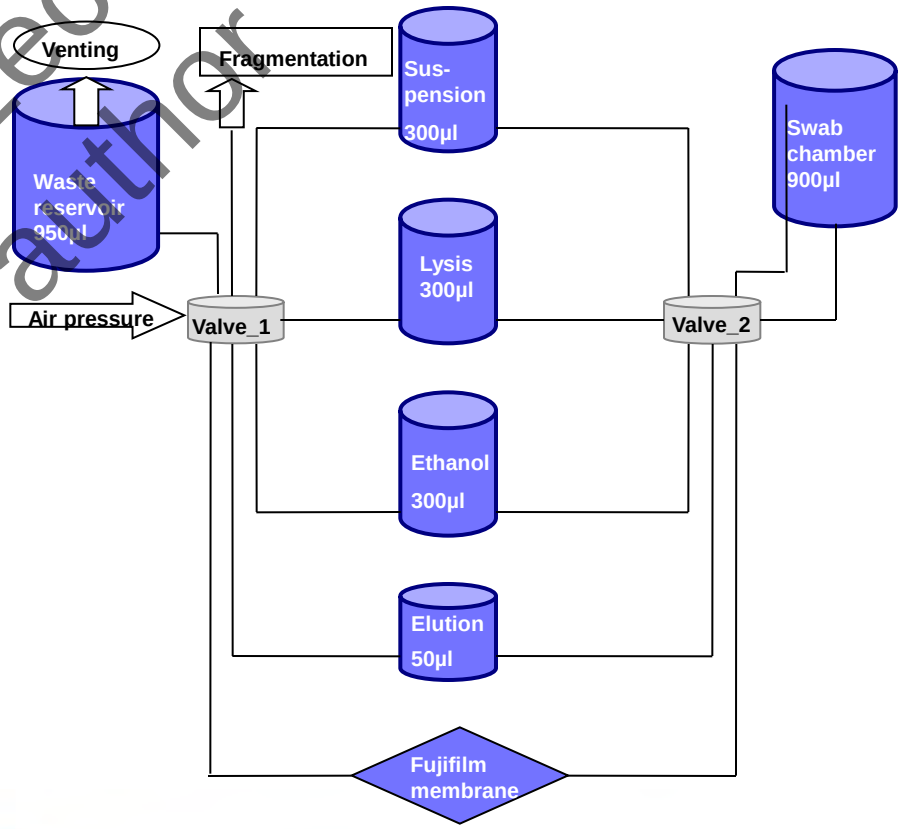
On-chip Bacterial Lysis and DNA Purification

Development of a proprietary bacterial lysis and DNA purification protocol and its successful application on a prototypal microfluidic chip for a CA-LRTI assay

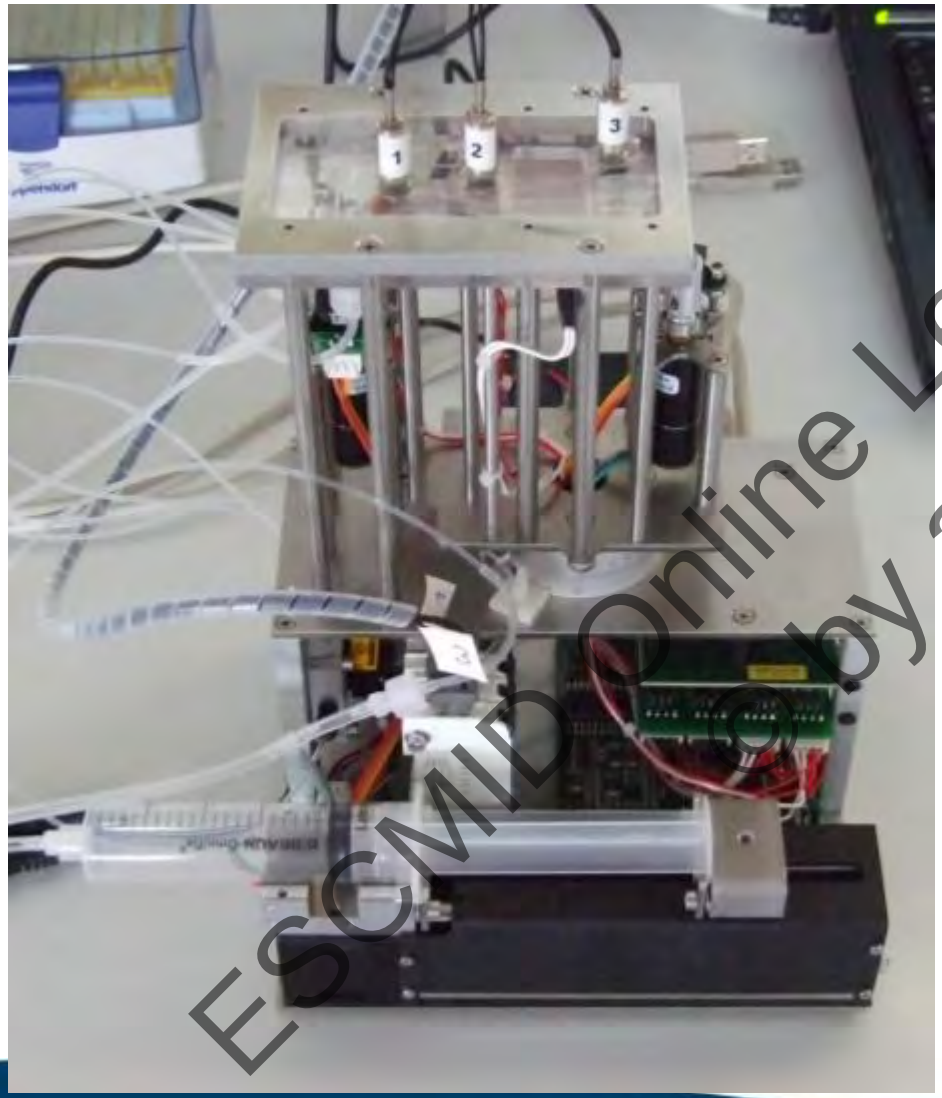
Chip design



Reaction steps



Semi-automated demonstrator setup



ESCMID EUROPEAN SOCIETY OF CLINICAL MICROBIOLOGY AND INFECTIOUS DISEASES

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RAPP-ID: Public Private Partnership

www.rapp-id.eu

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PROJECT WEBSITE: www.rapp-id.eu

Development of Rapid Point-of-Care Test Platforms for Infectious Diseases

RAPP-ID will develop a Point-of-Care Test (POCT) for rapid (hospital <2h, primary care <30min) detection of **bacteria, mycobacteria, fungi**, as well as **viruses** and **host biomarkers** by combining novel specific probes, novel methods of sample preparation, and demonstrated ultra-high sensitive detection methods. The platforms will also determine **resistance** to antimicrobial drugs

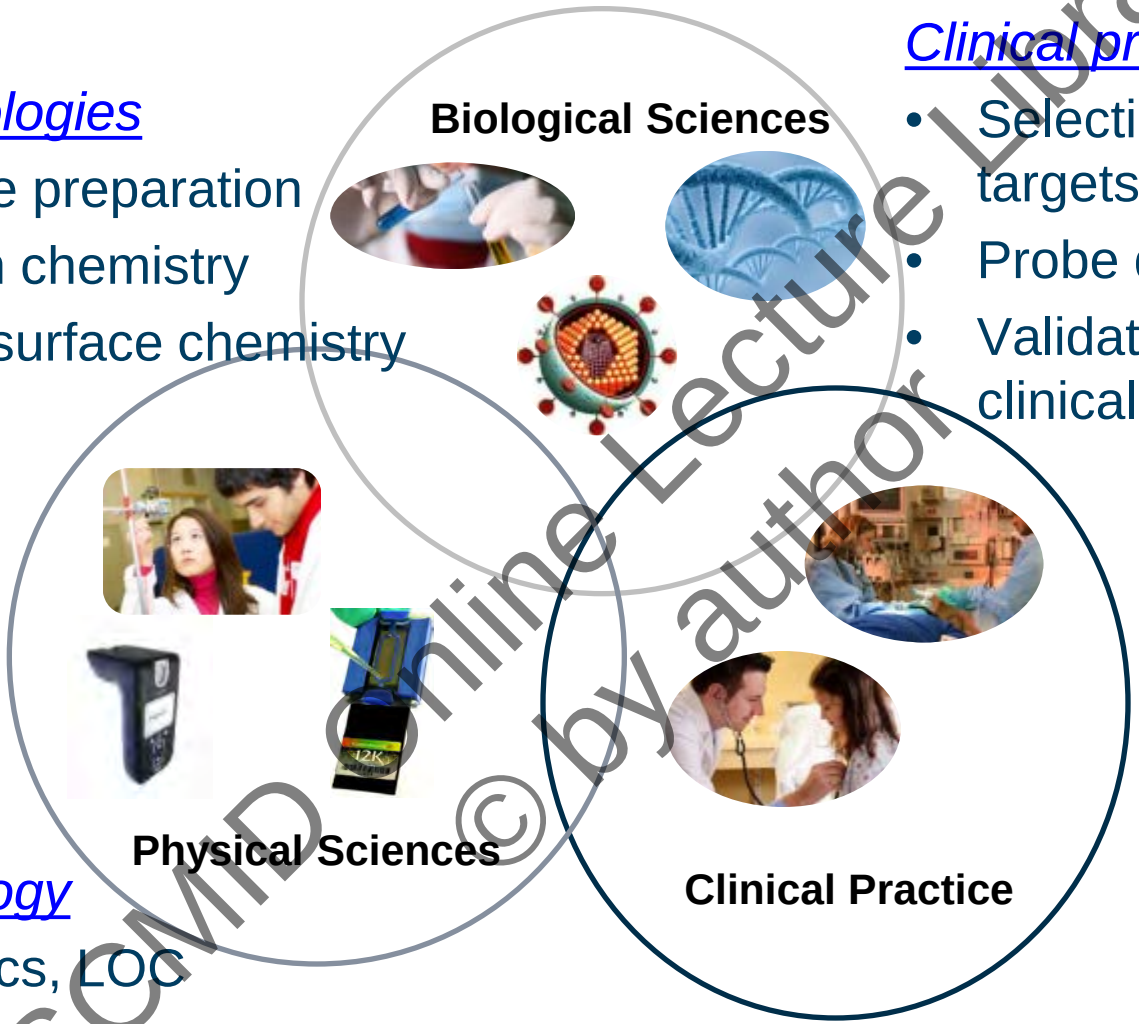
A plea for an integrated approach !!

Biotechnologies

- Sample preparation
- Protein chemistry
- Novel surface chemistry

Clinical practice

- Selection of relevant targets and applications
- Probe design
- Validation of analytical, clinical performance



Microtechnology

- Microfluidics, LOC
- Photonics
- biosensors

Innovative Medicines Initiative

RAPP-ID

FP7 projects

inTopSens

TheraEDGE