Multiplex molecular infection diagnostic – Complete typing of human papilloma viruses

EUROIMMUN Medizinische Labordiagnostika AG

Topics of the talk

- Overview of current non-cultural methods for pathogen detection
- Development of a PCR based multiparameter assay

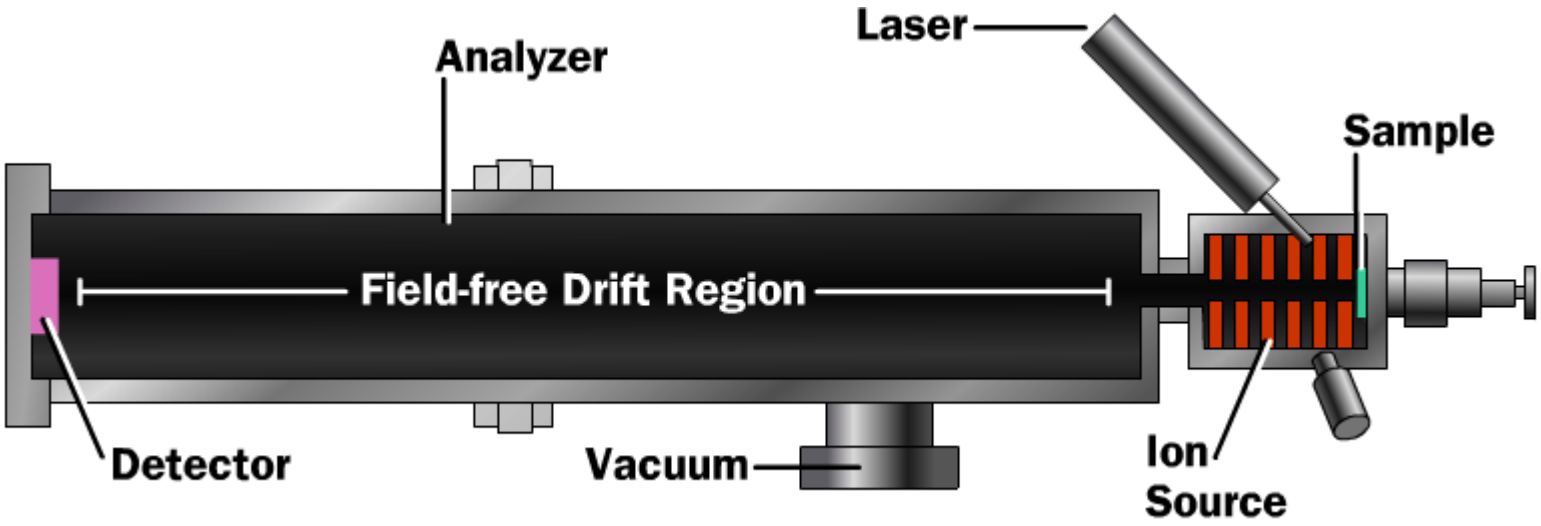
Non-cultural methods for pathogen diagnostics are fast and standardized

Short overview of current non-cultural methods for pathogen detection

- Maldi-Tof
- PCR-based Methods
  - Real-Time PCR
  - Sequencing
  - Microarray technology

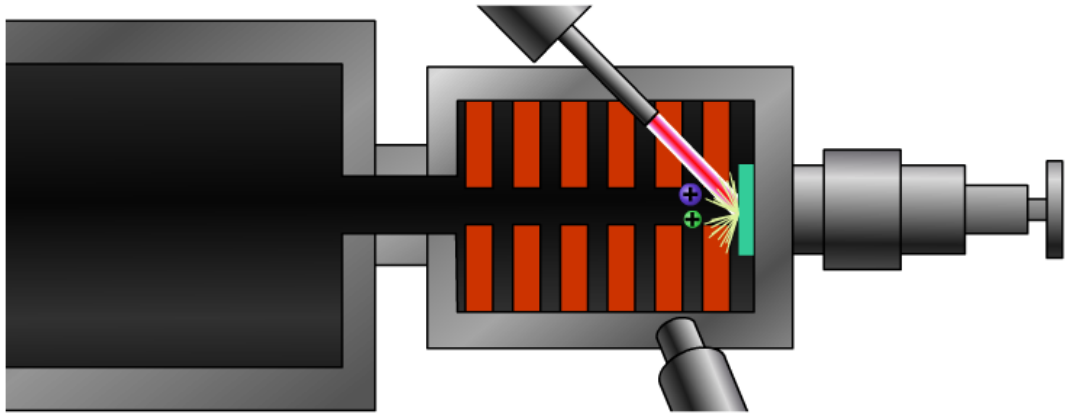
Maldi-Tof allows the analysis of biomolecules when ionized

- Maldi-Tof = Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer



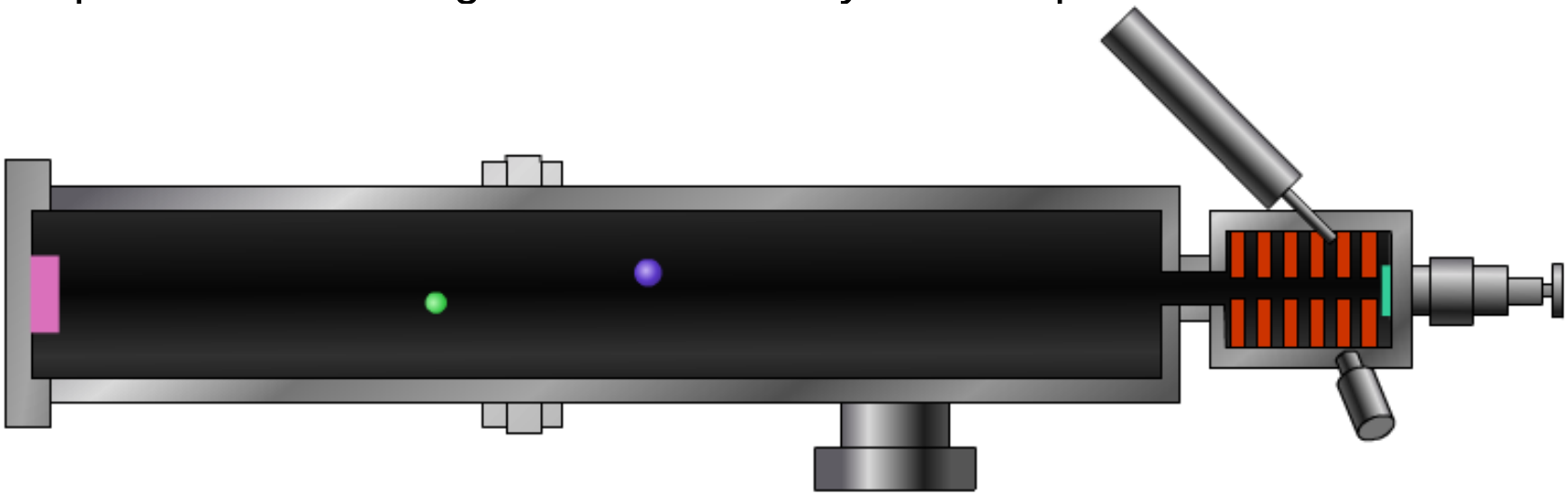
Biomolecules are separated based on their difference in mass

- The analyte will be co-crystallized with a matrix compound, usually a UV-absorbing organic acid
- The matrix absorbs UV-light and converts it to heat energy
- The matrix becomes ionized with a single positive charge, this positive charge is transferred to native sample proteins through their random collision in the gas phase
- Because all native sample proteins sample have an identical, single positive charge, the are separated based on their difference in mass



The sample will be identified in comparison to data of known organisms

- Heavier ions will travel through the mass analyzer at a slower velocity, compared to lighter ions
- An ion detector measures the time to impact
- Based on standards of known mass, the time to impact for each unknown analyte is converted into a mass-to-charge ratio
- Pattern of proteins will be compared with a database of Maldi-Tof spectra of know organisms to identify the sample

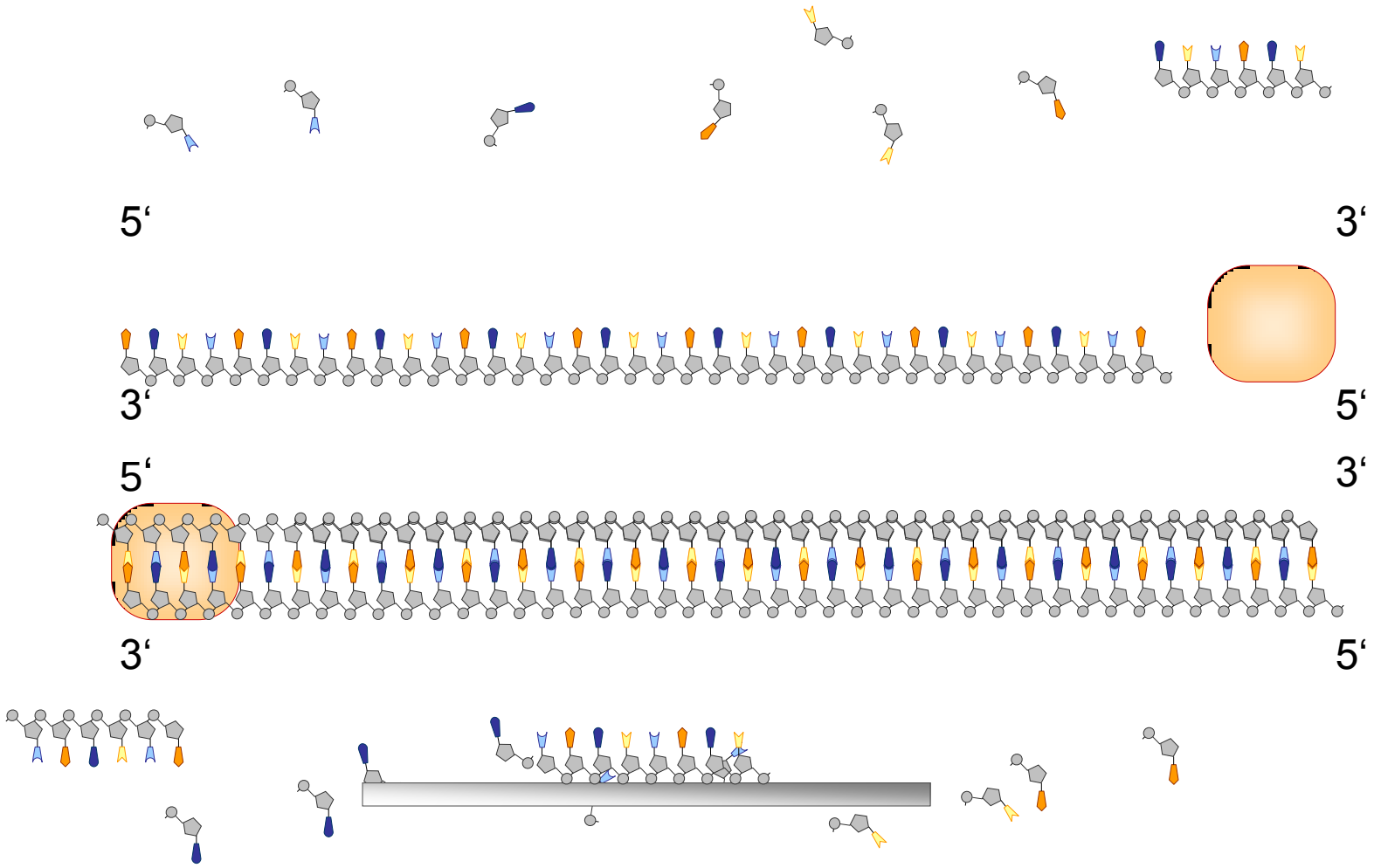


Advantages and disadvantages of Maldi-Tof analysis

- Advantages
  - Easy
  - Price/analysis is cheap

- Disadvantages
  - High investment costs
  - No resistance gene detection
  - Need of pure culture
  - Only protein patterns which are stored in a database may give an answer

PCR-based methods – Principles of Polymerase chain reaction



The selection of PCR-components is not unimportant

- There are various polymerase enzymes on the market
  - Speed
  - Quality of amplification
  - Stability
  - …
- The compounds shouldn’t be contaminated with the DNA which have to be amplified

PCR - one basic technique – different evaluation methods

Sequencing

Real-Time PCR

- Two common methods

  (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA while PCR

  (2) sequence-specific labelled DNA probes

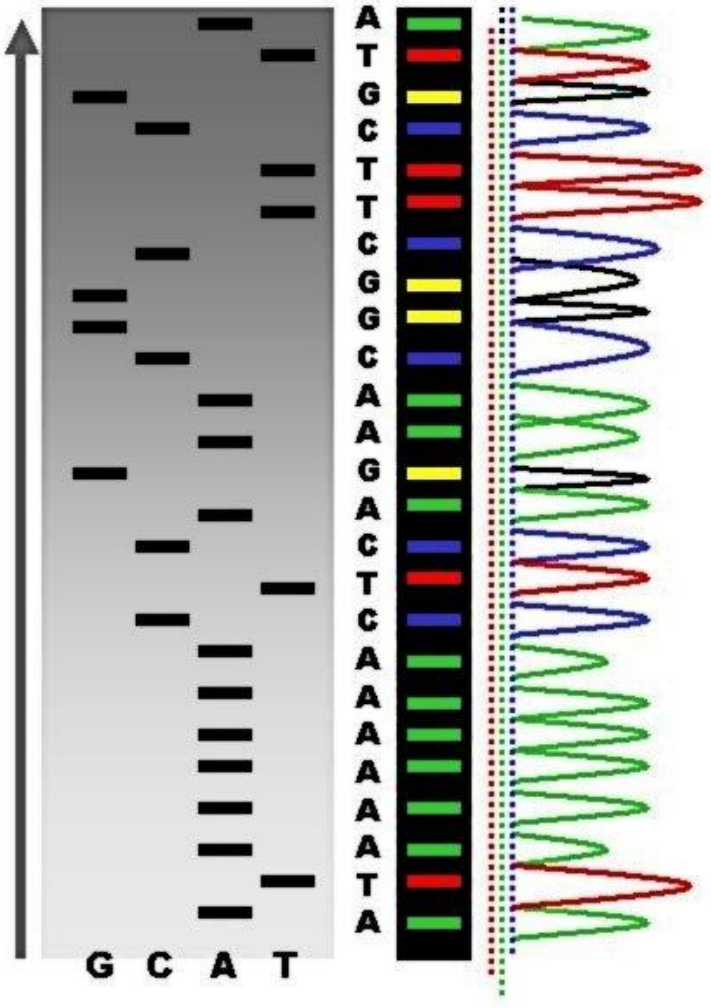
Microarray

- Amplification of the DNA by PCR
- Analysis via specific probe binding on an array

Sequencing is the process of determining the nucleotide order of a DNA fragment

Sanger Sequencing

- Chain termination method
- Uses sequence-specific termination of a DNA synthesis during the PCR
- The knowledge about the terminating modified nucleotide shows the sequence of the DNA fragment



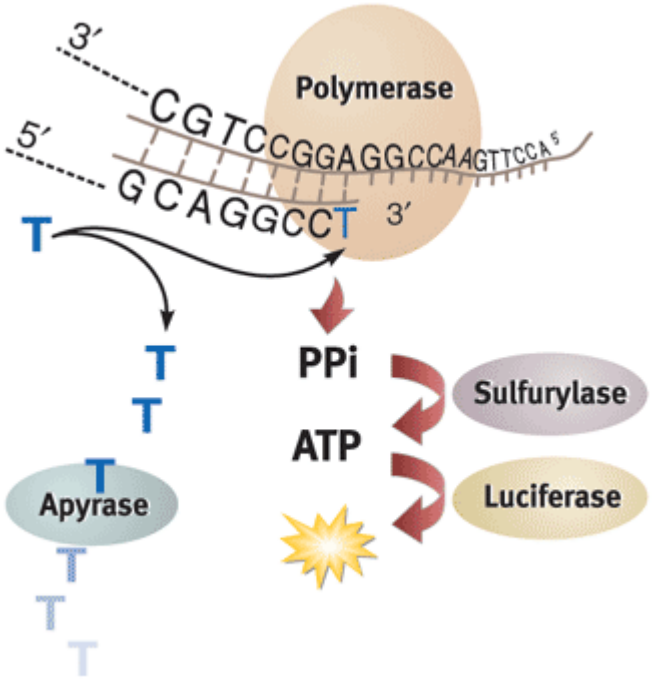
New sequencing technologies are gaining an increasing share of the sequencing market

Next-generation sequencing methods

Whole genomes can be sequenced in a single run with several times coverage

Example: Pyrosequencing

- DNA is annealed to beads and amplified via emulsion-based clonal amplification
- Free nucleotides are washed over the DNA
- ATP is generated when nucleotides join with their complementary base pairs
- Enzymes produce light in the presence of ATP
- The signal strength is proportional to the number of nucleotides, incorporated in a single nucleotide flow



Advantages and disadvantages of sequencing methods

- Advantages
  - All information in one step
  - Also new mutations are detected

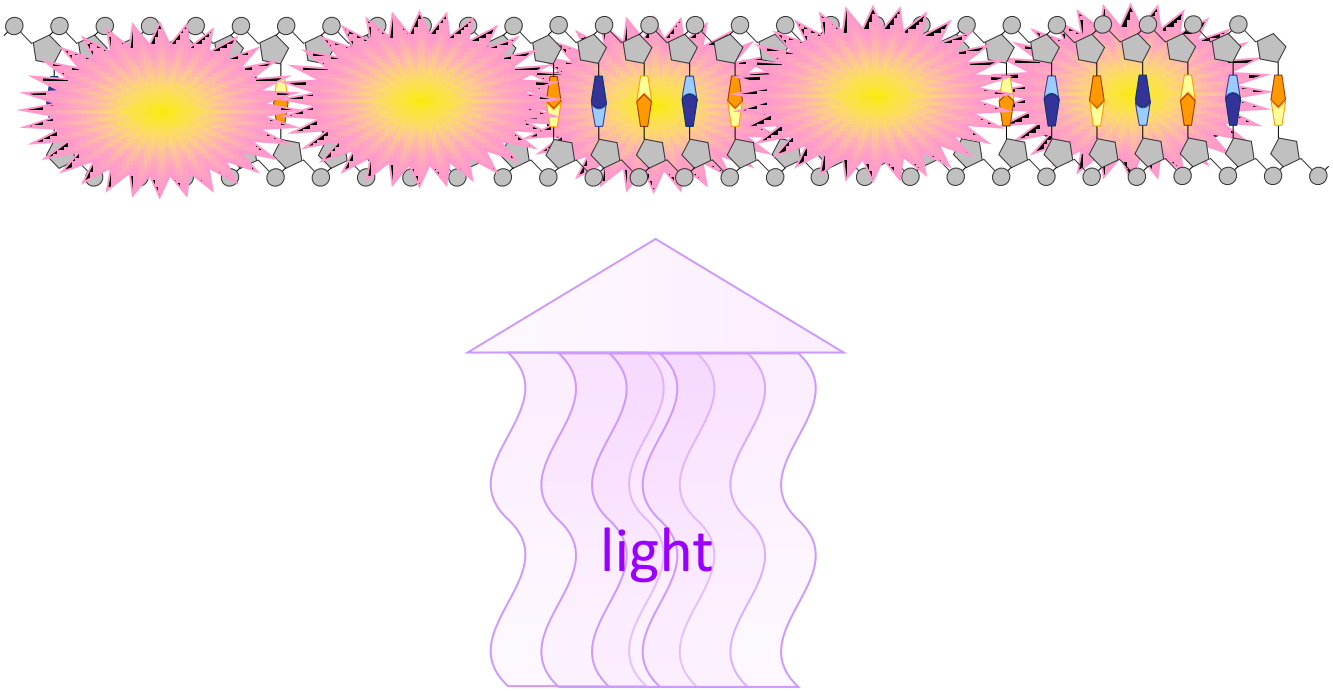
- Disadvantages
  - High investment costs
  - Expensive
  - Need of pure culture
  - Depending on the method high amount of data and more information then requested

Real-Time PCR monitors the amplification of a targeted DNA molecule during the PCR

- There are two ways for the direct detection of PCR products while RT-PCR
  - non-specific fluorescent dyes that intercalate with any double-stranded DNA while PCR
  - sequence-specific labelled DNA probes

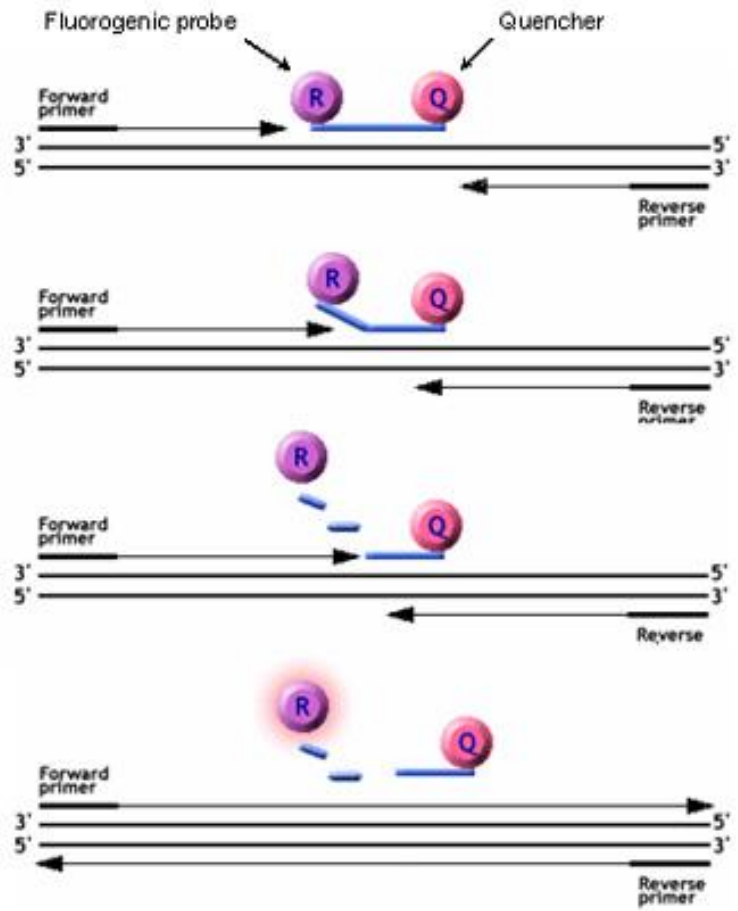
Easy but no additional specificity – intercalating dyes

- Intercalating dyes like SybrGreen give a fluorescent signal by activation with light



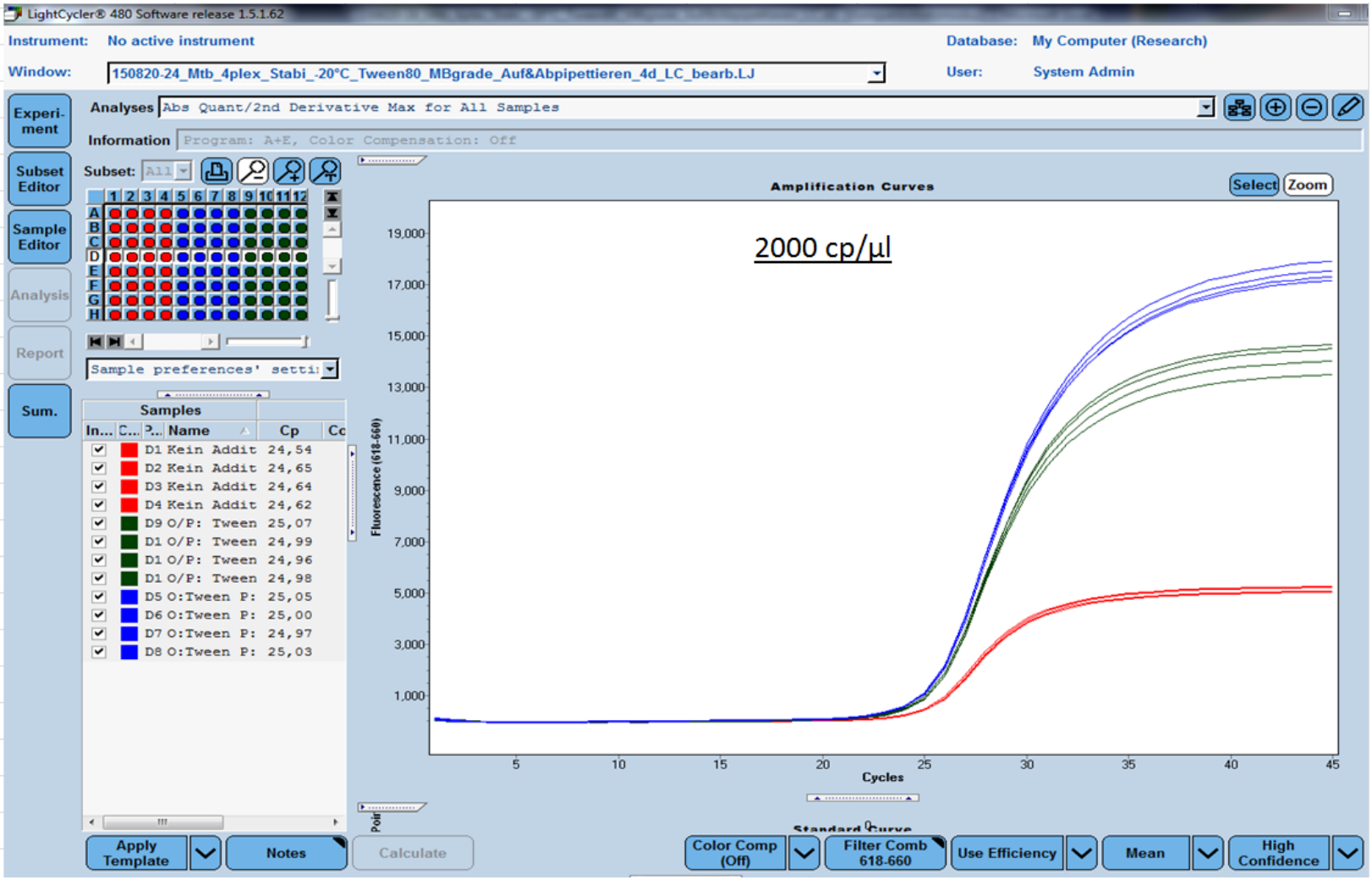
Real-Time PCR with dye-labeled probes – two step specificity possible

- Fluorescence of the reporter dye is prevented by the quencher
- Probe has to bind to its specific, complementary sequence
- As the DNA polymerase moves along the template, the probe is cleaved (broken) between the reporter and quencher
- This allows the reporter dye to emit fluorescence as it is no longer suppressed by the quencher dye
- Reporter fluorescence increases during each PCR cycle and is proportional to the amount of PCR product.



Hawrami, K and Brewer, J (1997) Journal of Medical Virology, 53 pp60-63

The typical outcome of a RT-PCR is an amplification curve



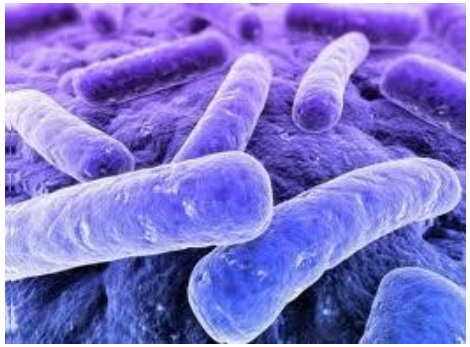
Advantages and disadvantages of RT-PCR

- Advantages
  - Amplifying and detection in one step

- Disadvantages
  - Quantification of DNA/RNA copies possible
  - Number of test parameters limited

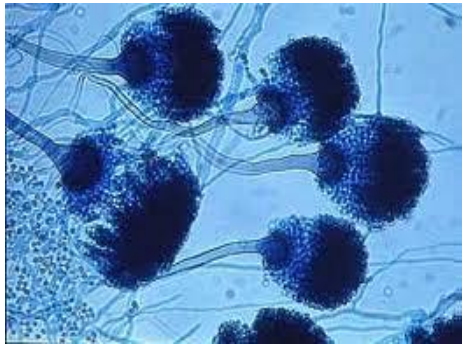
Challenges of the molecular infection diagnostics

- Direct analysis of pathogens and resistant gens by the detection of the DNA with µArray technology

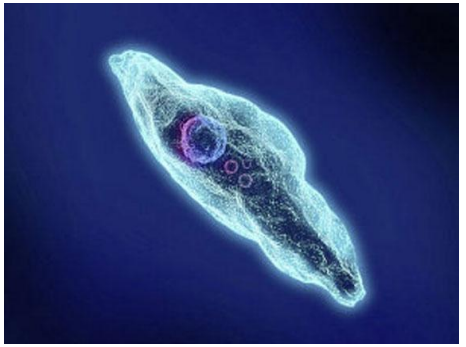


Bacteria

Virus



Fungus

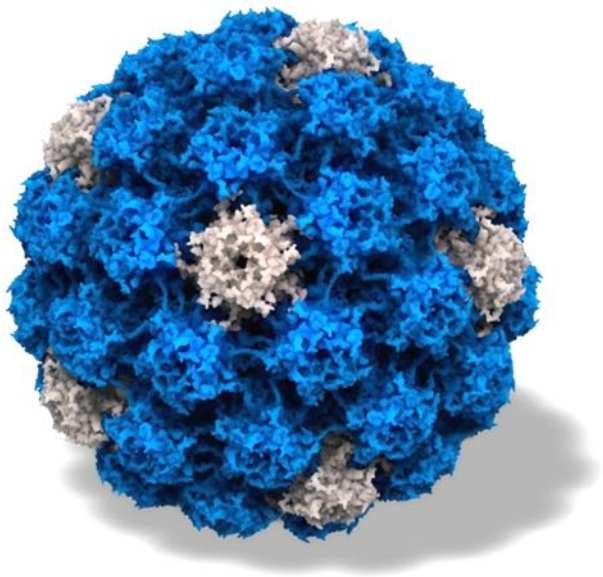


Parasites

Microarrys in the CE/IVD diagnostics - Development of an HPV Array

„There is a sufficient evidence that testing for human Papillomavirus infection as the primary screening modality can reduce cervical cancer incidence and mortality rates”

[International Agency for Research on Cancer – IARC, Handbooks of Cancer Prevention Cervical Cancer Prevention, Volume 10, IARC Press 2005]



HPV are the most common sexual transmitted virus

- HPV are the most common pathogens of STD
- An HPV infection very often occur already during the first sexual contacts
- The HPV prevalence vary in the population depending on the age, the social stratum, the culture group an the associated sexual behavior between 3% und 50%
  (Munoz et al., 1996; van den Brule et al., 1991; Schneider et al., 2000)

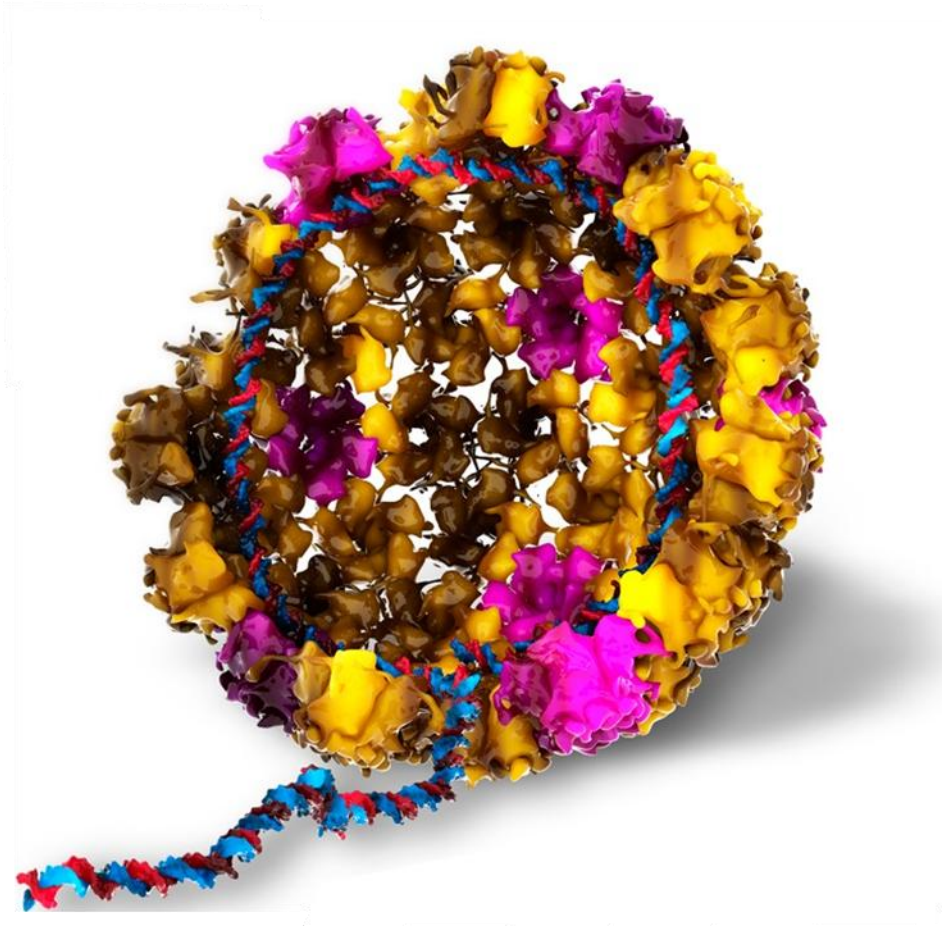
HPV - some backgrounds

- HPV are double stranded DNA virus
- The genome size is approximately 8000 bp
- ~210 humanpathogene HPV-subtypes are described (http://pave.niaid.nih.gov/#explore/reference_genomes/human_genomes)
- HPV infection is limited to the basal cells squamous epithelium of skin and mucosa
- The viral replication is only possible in fully differentiated squamous epithelium
- After the infection the viral DNA will be replicated extrachromosomal in the cell nucleus of the host cell

The oncogenic potential of the HPV is mediated by two genes

- The double stranded circular HPV DNA is organized in 9 genes
  - Variable number of early genes: e.g. **E6**, **E7**, E1, E2, E3, E4, E5
  - 2 late genes: L1, L2

- High-risk HPV and low-risk HPV have different abilities to influence the cell cycle by the inhibition or deactivation of cell cycles regulating proteins because of their variants of the E6 and E7 protein



The HPV genome is subdivided in a LCR, early and late gens

- Non-coding-long-control-region (LCR)
  - Promotor region for the control of the gen expression of the HPV gens (early-gens and late-gens)

- Early-gens
  - Not very conservative
  - Regulatory gen products
  - Necessary for the process of the malign degeneration of the host cell

- Late-gens
  - Conservative
  - Coding for the viral capsid proteins L1 and L2

- HPV genes will be transcribed as polycistronic RNA with overlapping open-reading-frames (ORF)

30 anogenital HPV are known

- 30 HPV are known which infect exclusively the skin and the mucosa of the anogenital region
- Anogenital HPV are sub-classified in two groups

(1) Low-risk-HPV subtypes[1]

- HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 und 89 (CP6108)

(2) High-risk-HPV subtypes[1,2]

- Identified in 99,7% of all cervical cancer tumors
- These days most of cervical carcinoma (~ 70%) provoked by high-risk HPV subtypes 16 and 18
- HPV 16, 18, 26, 31, 33 , 35, 39, 45, 51, 52 , 53, 56, 58, 59, 66, 68, 73 und 82

[1]Munoz,N., Bosch,F.X., de,S.S., Herrero,R., Castellsague,X., Shah,K.V., Snijders,P.J., and Meijer,C.J. (2003). Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med *348*, 518-527

Official classified as carcinogenic by the WHO,Cogliano,V., Baan,R., Straif,K., Grosse,Y., Secretan,B., and El,G.F. (2005). Carcinogenicity of human papillomaviruses. Lancet Oncol. 6, 204

HPV are able to induce cellular transformations

- Some HPV subtypes can induce (malign) cellular transformations
  - Cervical cancer
  - Vagina carcinoma
  - Penis carcinoma
  - Anal carcinoma
  - Carcinoma of the oral mucosa

Basis of PCR reactions – the primer and probe design

- Some key-proteins (and their nucleic acid sequences) stayed conserved thru evolution and differ in only few bp
  - For example: ribosomal subunits
  - With primers/probes for conserved DNA sequences from a bundle of organisms will be amplified

- Some proteins (and their nucleic acid sequences) are unique and only present in one organism
  - With primers/probes for unique regions DNA sequences from only one organism will be amplified

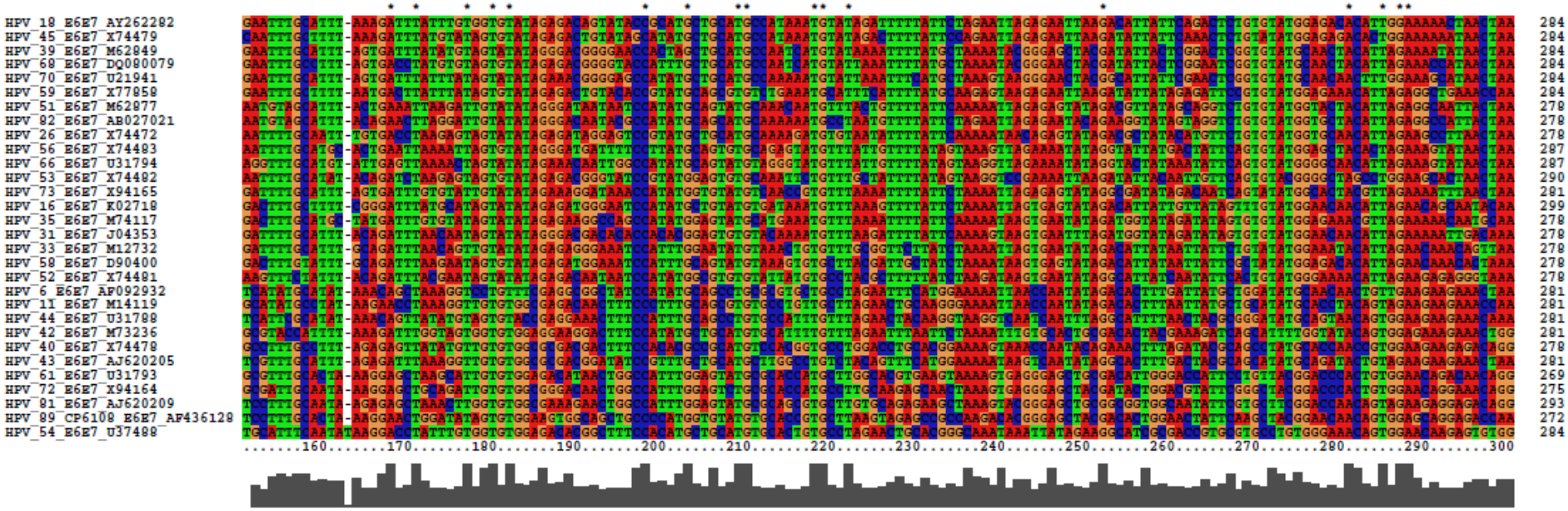
Depending on the pathogen panel different strategies for primers and probes are useful

- Conservative primers and probes
  - Detection of all subtypes as a group with only a few primer/probe-systems
  - No subtyping possible

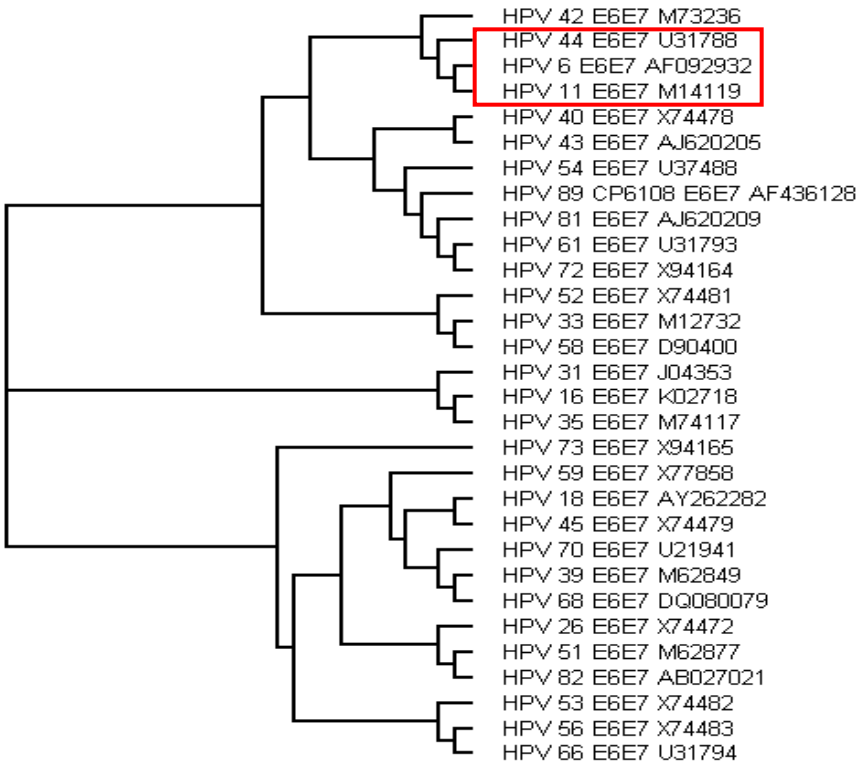
- Conservative primers, specific probes
  - Detection of all subtypes as a group with only a few primer
  - Specific probes give the chance for a limited subtyping

- Specific primers, specific probes
  - Detecting and full subtyping
  - Complex PCR-reactions with a lot of primers

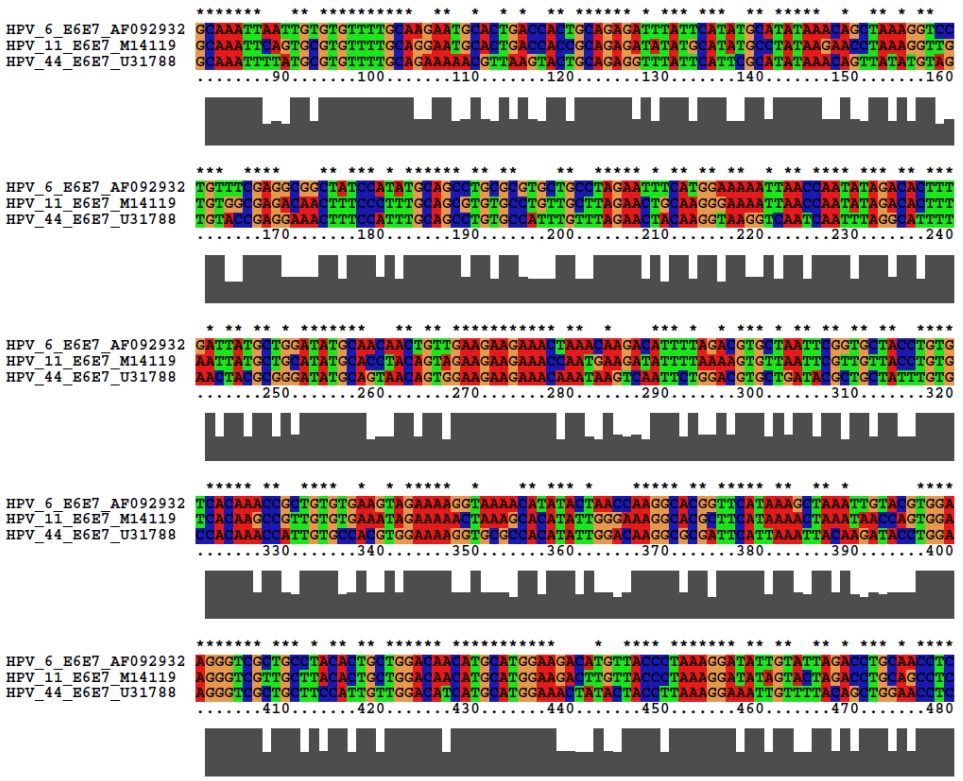
In-silico work on HPV E6/E7 no conservative primer/probe-systems



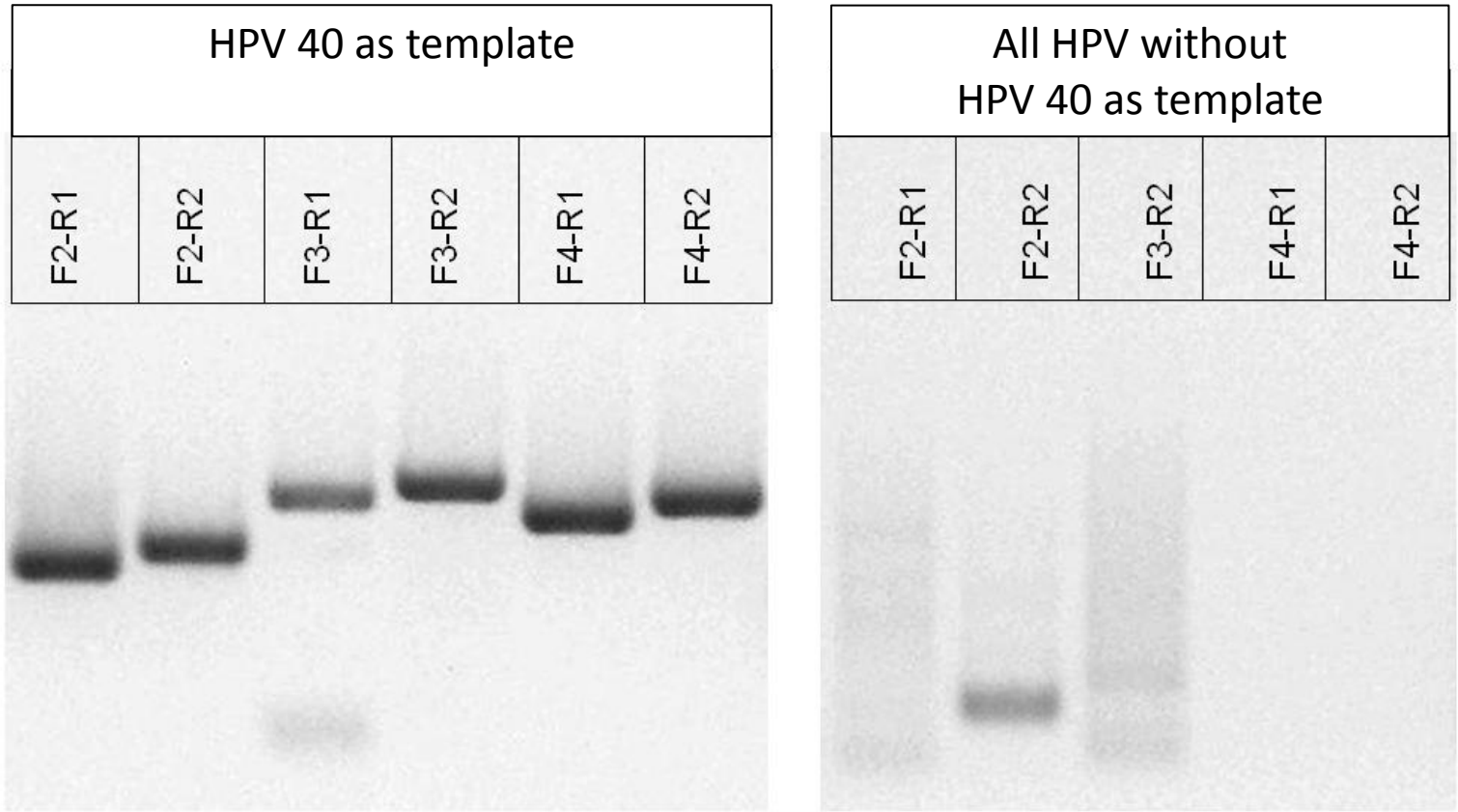
Smaler subgroups shows homologies - Selection of sequences for the development



Alignment der HPV Gene E6-E7

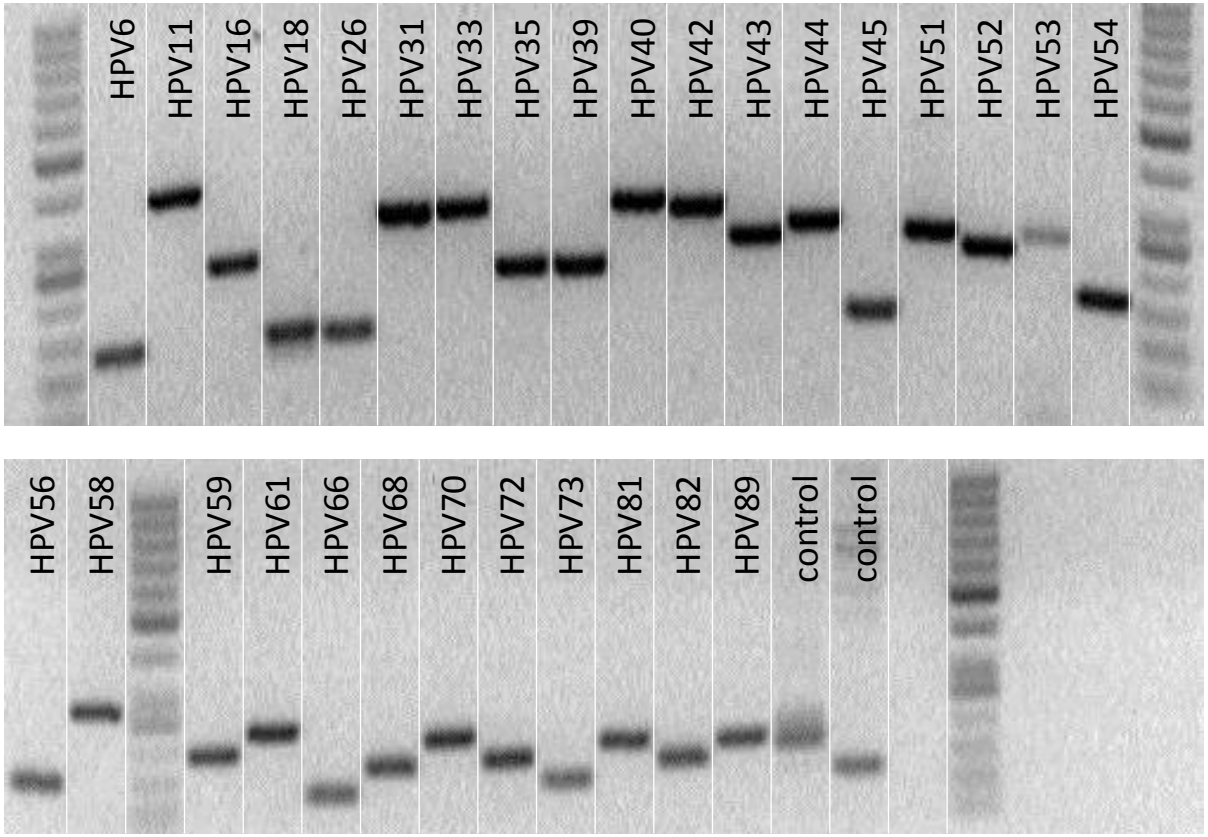


Example: development of a primer system for the HPV 40 detection

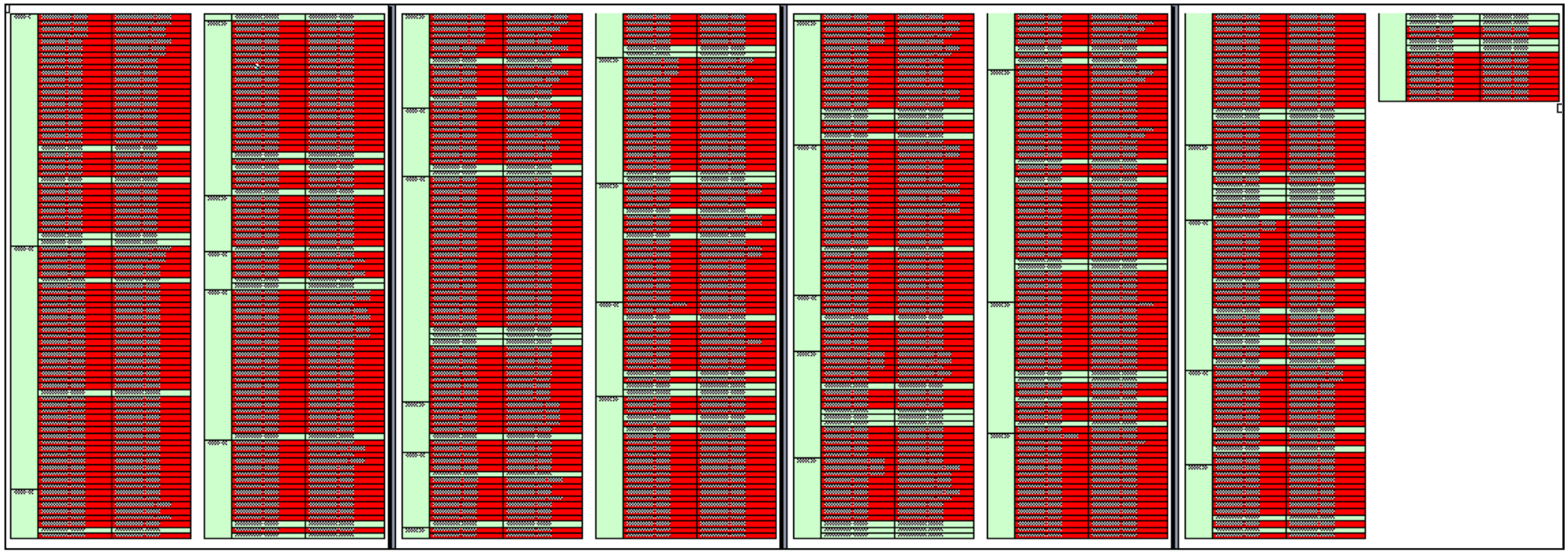


Optimizing of PCR-sytems against cross reactions with single primer systems

Primer: single primer-system + Template: all 30 E6-E7 HPV-sequences + human genomic DNA

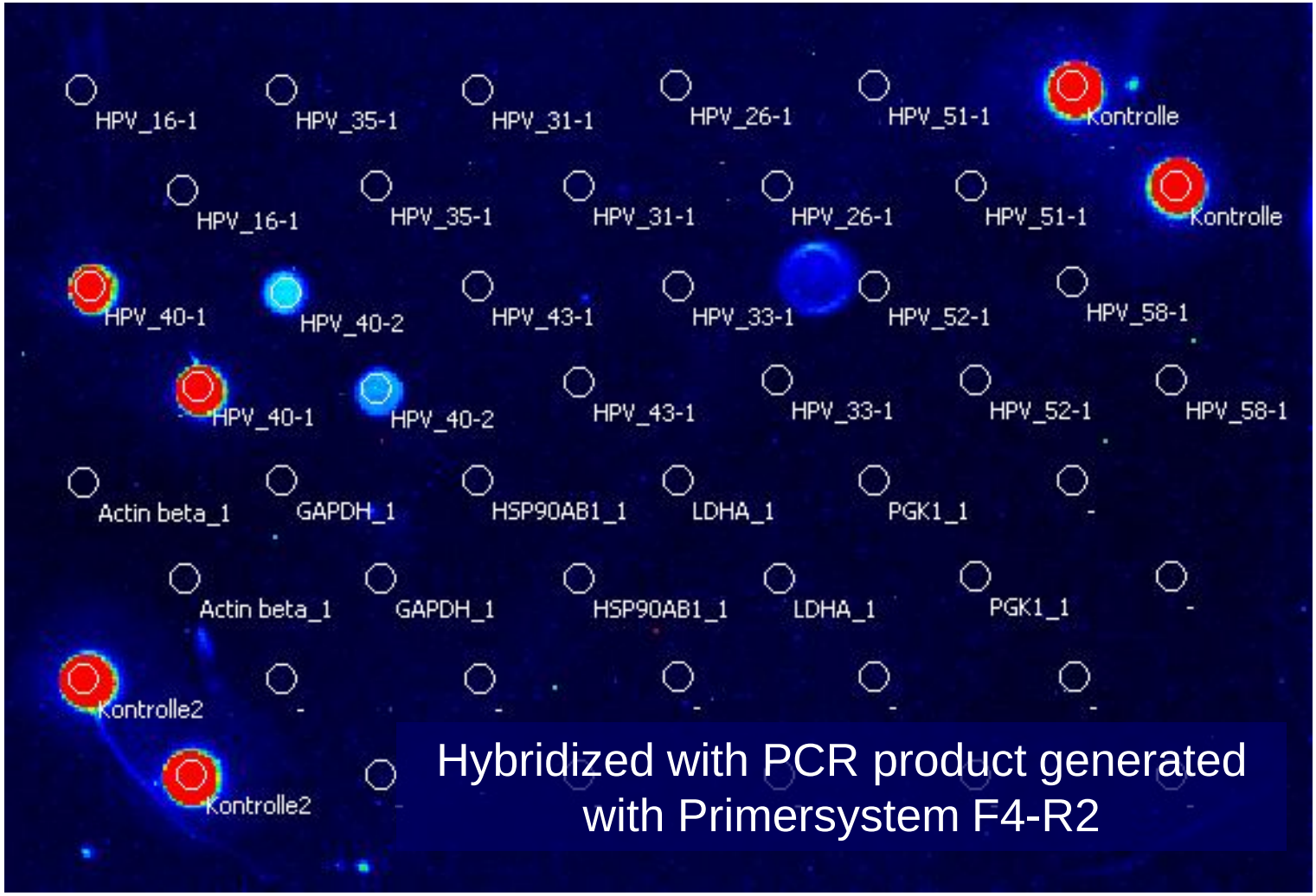


For a PCR with 31 primer pairs over 700 primer systems were developed an characterized



Over 700 tested primersystems

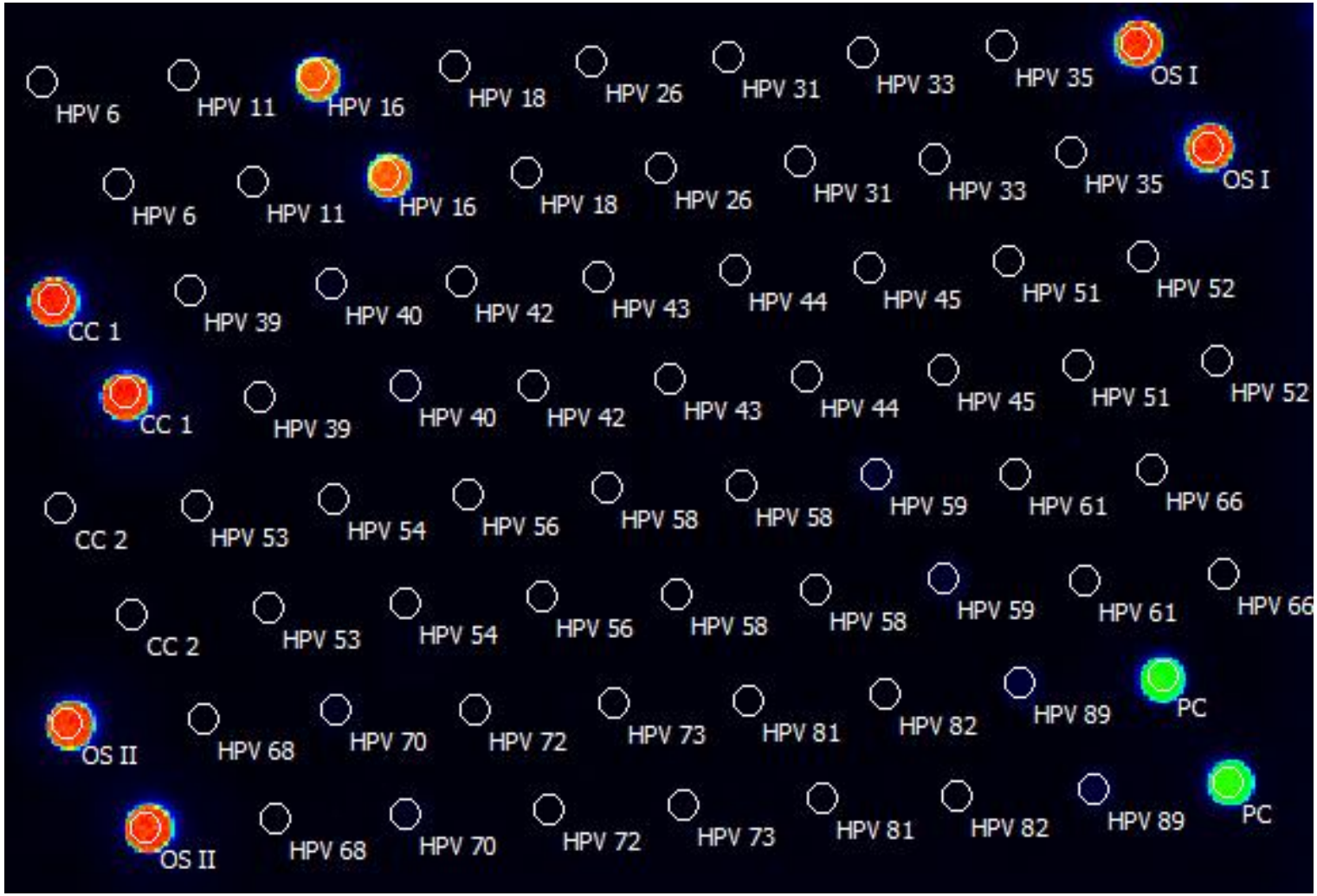
Example from the development: Selection of probes for HPV 40



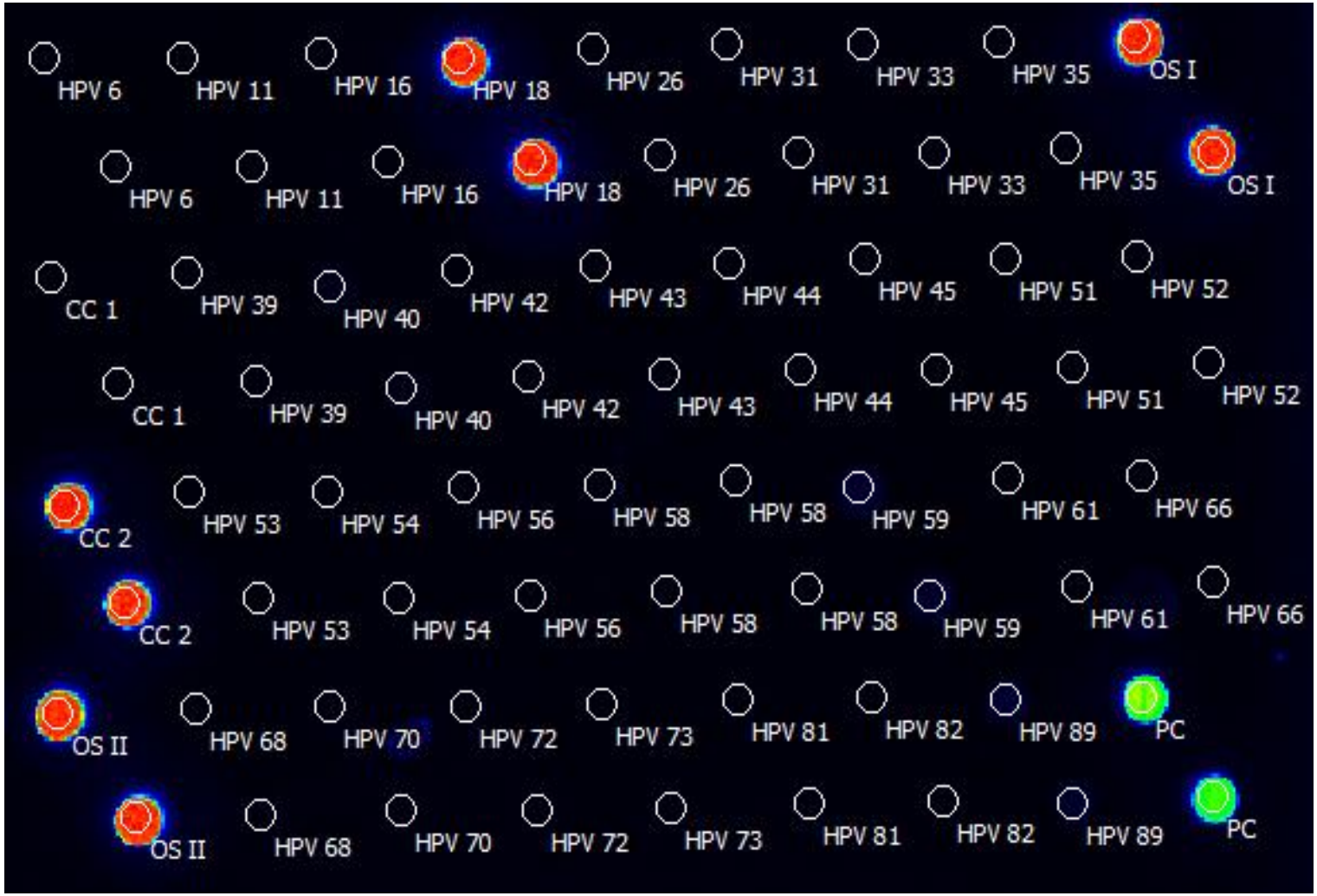
One primersystem for all HPV-subtypes + controll = 62 primers - it works !!!

Template	HPV 6	HPV 11	HPV 16	HPV 18	HPV 26	HPV 31	HPV 33	HPV 35	HPV 39	HPV 40	HPV 42	HPV 43	HPV 44	HPV 45	HPV 51	HPV 52	HPV 53	HPV 54	HPV 56	HPV 58	HPV 59	HPV 61	HPV 66	HPV 68	HPV 70	HPV 72	HPV 73	HPV 81	HPV 82	HPV 89	PDM	PDM	DNA	NTC
©	150	200	150	50	100	50	100	100	50	500	50	200	150	600	200	50	50	50	50	50	100	150	500	300	600	100	200	400	50	999	LoD	20x LoD	100 ng	
Patienten-ID	HPV6	HPV11	HPV16	HPV18	HPV26	HPV31	HPV33	HPV35	HPV39	HPV40	HPV42	HPV43	HPV44	HPV45	HPV51	HPV52	HPV53	HPV54	HPV56	HPV58	HPV59	HPV61	HPV66	HPV68	HPV70	HPV72	HPV73	HPV81	HPV82	HPV89	PK 1xLoD	PK 20xLoD	PK hum. DNA	NTC
	OT 1 Feld A	OT 1 Feld B	OT 1 Feld C	OT 1 Feld D	OT 1 Feld E	OT 2 Feld A	OT 2 Feld B	OT 2 Feld C	OT 2 Feld D	OT 2 Feld E	OT 3 Feld A	OT 3 Feld B	OT 3 Feld C	OT 3 Feld D	OT 3 Feld E	OT 4 Feld A	OT 4 Feld B	OT 4 Feld C	OT 4 Feld D	OT 4 Feld E	OT 5 Feld A	OT 5 Feld B	OT 5 Feld C	OT 5 Feld D	OT 5 Feld E	OT 6 Feld A	OT 6 Feld B	OT 6 Feld C	OT 6 Feld D	OT 6 Feld E	OT 7 Feld A	OT 7 Feld B	OT 7 Feld C	OT 7 Feld D
HPV 6	38406	0	96	16	32	0	81	0	0	128	96	64	0	96	81	0	0	32	0	64	64	16	0	0	33	0	0	0	0	19644	59404	0	64
HPV 6	43011	161	16	32	16	0	64	81	64	144	0	193	32	112	16	0	128	64	0	0	32	112	48	64	32	64	144	32	96	370	20880	59244	96	80
HPV 11	0	2969	0	65	0	0	80	0	161	145	48	97	0	0	48	16	96	80	0	32	0	32	16	16	48	48	32	32	241	64	2568	12727	0	32
HPV 11	0	3130	80	16	32	64	80	81	0	32	0	16	80	128	0	32	32	97	64	48	0	48	64	0	32	48	96	112	16	64	2648	11330	16	16
HPV 16	80	0	2439	16	16	32	0	0	0	16	32	112	48	16	0	0	0	32	32	16	97	32	65	32	0	64	80	0	48	32	2167	33206	32	97
HPV 16	80	0	2681	48	225	16	0	65	0	0	0	64	0	16	16	113	80	80	0	48	97	16	96	48	81	65	48	48	49	80	2343	35116	80	0
HPV 18	321	337	288	41502	321	192	369	289	192	385	225	224	145	145	176	289	337	241	289	209	112	224	240	224	305	321	257	128	32	1476	10945	59468	193	17
HPV 18	433	385	402	31537	369	209	369	273	225	305	273	224	225	128	193	337	273	273	289	193	225	225	337	289	337	304	257	145	209	321	10335	59404	209	0
HPV 26	386	208	401	578	18504	289	353	241	304	337	514	176	160	112	80	305	337	257	193	241	129	273	272	289	353	257	192	192	224	289	8603	50105	177	49
HPV 26	401	305	386	497	18152	273	273	225	289	256	337	160	225	209	257	321	192	256	241	193	225	273	273	321	273	305	177	273	177	305	7960	43412	177	32
HPV 31	305	224	257	321	225	5103	257	257	144	257	321	305	128	161	305	417	257	369	257	241	257	209	272	386	273	321	288	289	257	337	2696	33462	304	176
HPV 31	241	256	272	337	305	5168	161	289	240	208	209	321	256	289	321	353	337	288	305	401	241	337	241	289	305	289	273	305	305	273	3001	35901	257	113
HPV 33	112	96	128	96	80	64	9245	113	96	48	161	96	0	256	96	96	48	128	177	80	81	49	80	96	144	128	64	112	97	32	12036	59597	16	80
HPV 33	48	113	113	144	128	96	8940	16	113	112	128	112	16	80	97	160	128	129	144	96	64	128	129	145	48	96	96	128	161	113	12679	59597	80	161
HPV 35	0	64	64	112	0	16	129	7142	0	0	96	48	0	161	80	32	33	144	80	64	48	80	32	96	16	128	64	80	304	16	6355	40395	64	48
HPV 35	0	0	0	0	64	0	0	6885	0	0	48	0	0	0	0	0	0	0	0	32	0	16	0	33	0	0	0	0	289	0	6018	36704	0	0
HPV 39	0	128	81	225	48	16	321	0	13819	32	16	80	0	65	32	161	144	0	112	176	80	240	80	208	97	96	224	32	112	112	6339	33061	0	16
HPV 39	80	128	113	257	144	33	176	129	13834	113	64	145	32	161	65	145	112	16	144	193	113	128	96	192	128	161	192	48	144	128	6307	30526	48	80
HPV 40	160	177	129	193	96	97	161	65	145	11057	193	144	64	81	80	225	160	65	144	112	129	144	160	160	128	177	176	128	144	112	9004	59581	16	16
HPV 40	224	129	113	241	128	113	144	193	225	11651	64	112	48	16	113	193	193	129	144	144	161	144	128	224	161	257	160	48	80	144	9132	59485	0	64
HPV 42	225	97	16	48	289	97	80	97	97	32	4654	80	48	64	145	0	129	96	0	64	80	144	80	112	64	0	80	144	97	145	2376	23014	128	64
HPV 42	0	240	81	97	48	0	112	113	81	16	3194	0	112	0	113	48	65	64	417	80	48	128	64	128	64	48	64	81	48	96	2359	26305	113	112
HPV 43	144	48	145	81	80	65	80	48	81	65	48	6917	128	48	113	0	128	0	96	96	96	160	112	32	65	113	161	144	0	129	5810	49977	96	0
HPV 43	128	49	49	80	0	64	64	129	16	32	32	5232	96	48	81	401	32	80	0	0	48	96	96	144	17	145	96	0	418	161	5970	49046	80	32
HPV 44	96	112	145	161	112	33	96	0	65	16	129	96	3354	48	81	113	16	129	112	16	16	32	16	112	192	97	48	96	97	129	3515	27556	177	0
HPV 44	16	145	97	81	128	129	0	80	32	192	81	80	2873	128	32	80	144	97	48	0	80	80	64	0	177	145	97	161	96	96	3146	22485	96	16
HPV 45	144	145	81	17	16	16	48	16	16	81	0	80	97	4975	16	64	96	49	80	48	64	80	113	145	241	48	80	112	16	48	4413	24924	0	80
HPV 45	49	16	48	32	0	0	0	48	0	32	48	32	64	3755	16	65	48	0	0	65	64	32	32	32	33	64	64	48	0	32	3434	22099	64	16
HPV 51	0	48	64	32	32	16	0	16	0	0	48	32	80	16	1412	65	48	128	32	65	64	96	128	48	48	16	112	32	64	96	1220	10416	48	48
HPV 51	80	0	64	16	97	0	209	48	0	48	32	64	64	0	1075	96	64	32	160	49	16	112	48	16	64	0	0	80	16	112	1204	11940	16	96
HPV 52	193	96	96	160	176	0	128	209	129	80	96	129	145	0	97	9485	96	177	96	48	64	129	129	177	96	97	145	128	192	161	4976	41085	112	96
HPV 52	129	176	64	112	32	49	0	64	17	32	144	17	65	16	33	8731	192	209	0	129	129	0	97	129	144	209	161	224	176	96	4494	38213	193	144
HPV 53	65	0	0	0	321	321	0	16	0	32	0	0	16	0	0	0	5328	32	0	112	64	0	80	0	0	0	0	0	0	0	7961	38421	48	32
HPV 53	32	16	0	48	96	0	0	16	0	33	16	0	80	0	80	16	5505	113	0	65	112	48	64	0	112	96	48	80	0	64	7768	34264	48	0
HPV 54	144	0	113	0	112	97	64	49	129	97	16	96	112	65	32	96	32	10415	128	81	48	32	112	80	112	112	48	48	48	128	8891	59629	0	16
HPV 54	16	97	16	81	32	64	64	64	16	112	0	112	16	0	321	32	113	9533	193	16	48	16	16	96	97	49	32	32	16	128	9646	59533	80	112
HPV 56	16	16	337	16	0	48	80	33	49	80	0	80	0	64	32	32	129	80	3739	112	48	112	96	209	48	81	80	96	65	96	2359	15969	225	80
HPV 56	96	65	48	32	0	32	80	33	48	48	0	80	32	16	0	32	81	96	4269	112	65	96	128	112	161	113	64	64	16	96	2520	18585	48	48
HPV 58	0	64	97	32	64	17	96	112	96	80	49	0	80	96	16	209	80	145	209	3579	81	48	16	32	81	97	112	32	48	80	4751	36206	112	16
HPV 58	97	64	0	161	32	129	64	65	112	144	65	0	97	192	48	144	112	353	80	3964	32	160	64	128	16	97	112	49	81	97	4622	40155	64	64
HPV 59	160	145	225	113	160	161	80	113	161	128	145	160	177	128	81	160	160	177	48	112	3274	160	96	112	161	177	97	80	145	145	2504	13529	96	112
HPV 59	177	209	225	161	113	161	161	129	193	96	209	192	209	161	113	144	80	193	112	96	3579	160	160	160	224	177	161	128	177	161	2729	13561	96	112
HPV 61	16	64	64	0	81	32	64	0	0	0	0	0	16	49	48	144	64	112	0	33	48	6965	0	0	48	48	32	0	16	0	5585	4349	48	32
HPV 61	0	64	0	16	0	0	0	0	0	32	0	0	64	16	48	0	32	0	0	0	113	8009	32	0	48	32	0	0	0	0	6323	3900	0	0
HPV 66	64	96	97	32	49	32	0	0	80	0	128	16	80	0	64	161	144	144	32	97	129	80	13626	80	81	257	80	160	96	144	13112	59661	96	96
HPV 66	0	0	0	0	0	0	0	0	0	0	0	0	369	0	0	129	0	112	0	0	0	96	12293	48	0	144	17	16	64	64	12631	59709	0	64
HPV 68	0	0	0	0	0	0	0	0	0	0	321	0	0	0	0	0	0	0	289	49	0	16	32	27717	0	0	0	0	97	0	25133	55112	16	0
HPV 68	96	16	32	0	32	0	0	16	0	48	16	0	0	16	16	0	32	32	0	0	0	0	32	26256	64	0	48	0	0	48	26963	56220	0	17
HPV 70	161	145	112	113	96	128	113	160	49	64	49	96	80	225	113	193	160	144	80	128	128	208	160	112	6420	193	160	145	80	48	5729	35147	145	80
HPV 70	225	144	112	112	177	96	129	160	128	113	64	145	64	96	80	0	128	209	209	145	161	64	241	0	6082	96	225	128	32	177	5376	33430	129	209
HPV 72	129	112	289	256	241	225	241	192	256	289	225	161	273	257	224	321	240	321	225	225	240	225	225	0	176	7864	273	305	161	225	4172	37394	161	209
HPV 72	240	209	273	273	241	257	273	176	224	321	209	208	273	193	112	225	225	225	144	257	225	193	225	193	241	6821	272	289	112	176	5136	36014	193	241
HPV 73	145	144	80	209	96	144	81	128	0	160	161	145	16	160	128	145	144	80	129	80	80	16	193	49	208	80	3932	145	97	81	3130	44568	129	177
HPV 73	144	80	160	80	161	80	64	128	160	80	16	0	80	161	144	257	160	128	32	225	128	128	145	96	112	128	3900	112	48	129	3306	32772	0	193
HPV 81	129	128	160	160	0	176	97	64	129	273	128	161	161	48	128	112	160	64	97	160	177	176	144	177	160	129	177	7126	97	225	5809	18537	97	144
HPV 81	161	48	96	96	145	145	177	64	144	145	161	161	160	161	224	129	145	112	144	129	129	193	129	192	129	112	192	6997	32	160	6018	15793	176	145
HPV 82	177	208	256	209	192	288	112	305	272	225	128	257	209	257	160	176	177	112	65	32	144	160	209	129	112	80	161	256	4606	177	3146	14075	193	0
HPV 82	176	256	225	96	96	144	192	176	224	257	177	273	128	225	32	145	193	112	96	96	144	64	49	177	129	192	224	160	4526	224	3226	15167	160	81
HPV 89	273	418	288	402	256	401	369	337	354	241	177	209	289	225	208	305	353	256	257	305	176	257	257	337	337	304	257	192	192	6210	5634	43685	97	241
HPV 89	305	96	96	305	225	369	241	289	289	193	209	225	273	208	337	241	176	321	418	80	289	209	241	160	225	161	224	145	225	6179	5634	45868	112	305
PC	58457	58907	58859	58730	58730	58859	58827	58843	58746	58602	58682	58682	58730	58923	58666	59164	59035	59244	59003	59180	59260	59501	59260	59244	59035	59308	59372	59292	59388	59356	59115	59035	59099	80
PC	58040	58843	58650	58714	58650	58762	58185	58441	58602	58345	58345	58441	58650	58714	58538	59228	59003	59260	58955	59147	59051	59260	59276	59147	59035	59228	59372	59276	59420	59324	58762	59196	59180	160

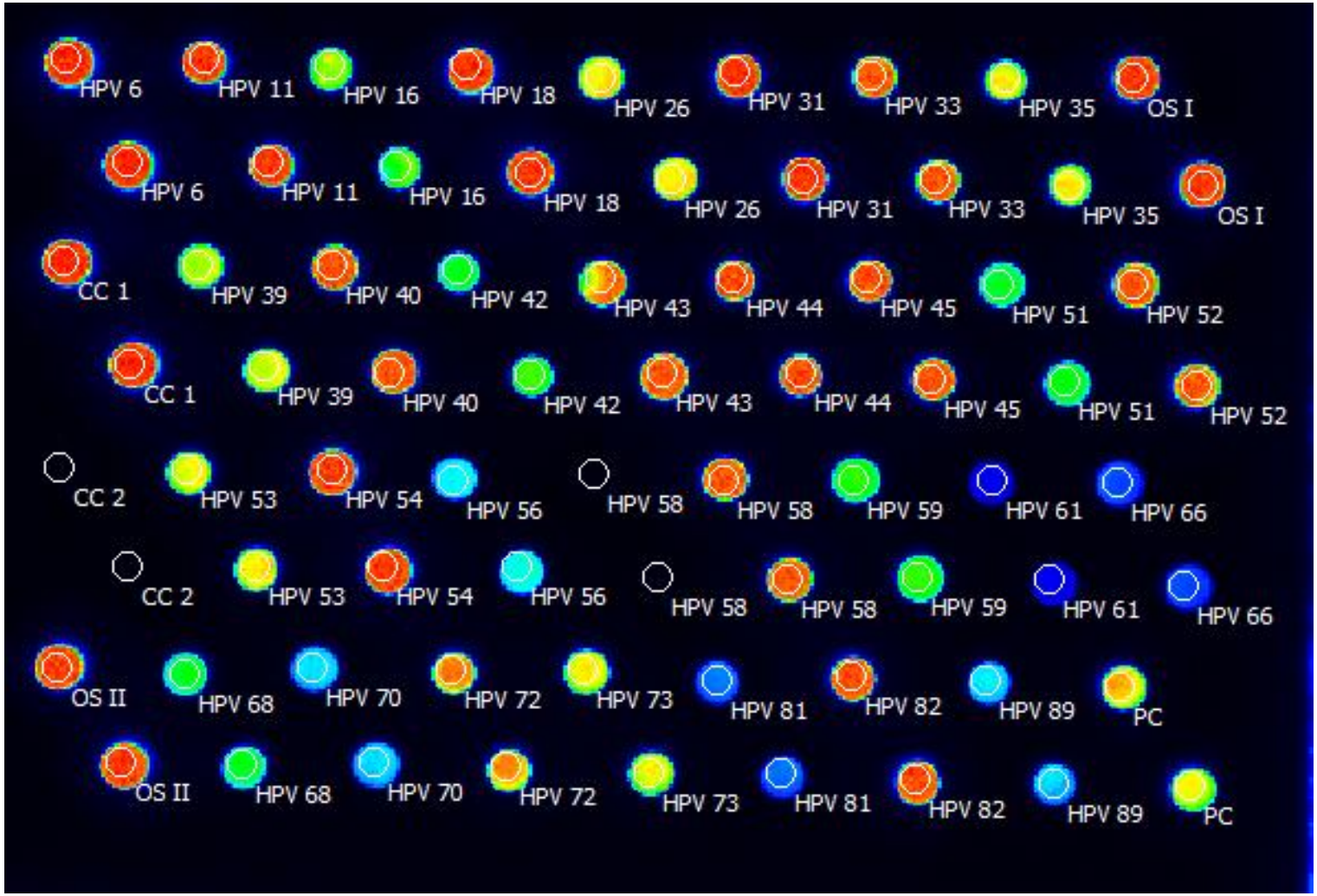
After various optimization: High sensitivity and specificity for 30 HPV



EUROArray HPV - High sensitivity and specificity for 30 HPV types



Detection and Typing of all 30 anogenital HPV at the LoD in one reaction



With various primer-probe-systems a high sensitivity can be achieved

Template	NTC	PDM	Ohne 6	Ohne 11	Ohne 16	Ohne 18	Ohne 26	Ohne 31	Ohne 33	Ohne 35	Ohne 39	Ohne 40	Ohne 42	Ohne 43	Ohne 44	Ohne 45	Ohne 51	Ohne 52	Ohne 53	Ohne 54	Ohne 56	Ohne 58	Ohne 59	Ohne 61	Ohne 66	Ohne 68	Ohne 70	Ohne 72	Ohne 73	Ohne 81	Ohne 82	Ohne 89
Kopien		100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000	100.000
	OT 1 Feld A	OT 1 Feld B	OT 1 Feld C	OT 1 Feld D	OT 1 Feld E	OT 2 Feld A	OT 2 Feld B	OT 2 Feld C	OT 2 Feld D	OT 2 Feld E	OT 3 Feld A	OT 3 Feld B	OT 3 Feld C	OT 3 Feld D	OT 3 Feld E	OT 4 Feld A	OT 4 Feld B	OT 4 Feld C	OT 4 Feld D	OT 4 Feld E	OT 5 Feld A	OT 5 Feld B	OT 5 Feld C	OT 5 Feld D	OT 5 Feld E	OT 6 Feld A	OT 6 Feld B	OT 6 Feld C	OT 6 Feld D	OT 6 Feld E	OT 7 Feld A	OT 7 Feld B
HPV 6	0	58285	0	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 6	0	58285	0	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 11	0	58285	58171	0	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 11	0	58285	58171	0	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 16	0	58285	58171	58415	0	58382	58268	58138	58398	57256	58333	58203	58350	58382	50167	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 16	0	58285	58171	58415	0	58382	58268	58138	58398	56248	58333	58203	58350	56280	57959	56931	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 18	16	58285	58171	58415	58171	0	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 18	130	58285	58171	58415	58171	0	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 26	130	58285	58171	58415	58171	58382	0	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 26	65	58285	58171	58415	58171	58382	0	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 31	552	58285	58171	58415	58171	58382	58268	0	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 31	390	58285	58171	58415	58171	58382	58268	0	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 33	32	58285	58171	58415	58171	58382	58268	58138	0	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 33	0	58285	58171	58415	58171	58382	58268	58138	0	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 35	325	58285	58171	58415	58171	58382	58268	58138	58398	0	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 35	276	58285	58171	58415	58171	58382	58268	58138	58398	0	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 39	0	58285	49599	50705	58171	49404	49241	56540	58398	57500	0	48542	56508	54492	48607	51501	57776	58317	53907	58301	49306	39633	51940	54752	53858	51290	51468	49583	53175	29799	49875	54134
HPV 39	0	58285	54590	52444	55370	52931	50298	56979	58084	56866	0	48769	55013	50916	52720	51273	58512	58317	53110	53078	53614	44429	48705	49696	55467	51322	52216	56476	56995	47063	51160	53809
HPV 40	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	0	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 40	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	0	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 42	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	0	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 42	48	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	0	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 43	16	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	0	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 43	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	0	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 44	97	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	0	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 44	48	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	0	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 45	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	0	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 45	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	0	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 51	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	0	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 51	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	0	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 52	292	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	0	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 52	130	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	0	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 53	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	0	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 53	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	0	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 54	32	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	0	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
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HPV 56	178	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	0	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
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HPV 58	48	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	0	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
HPV 58	97	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	0	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
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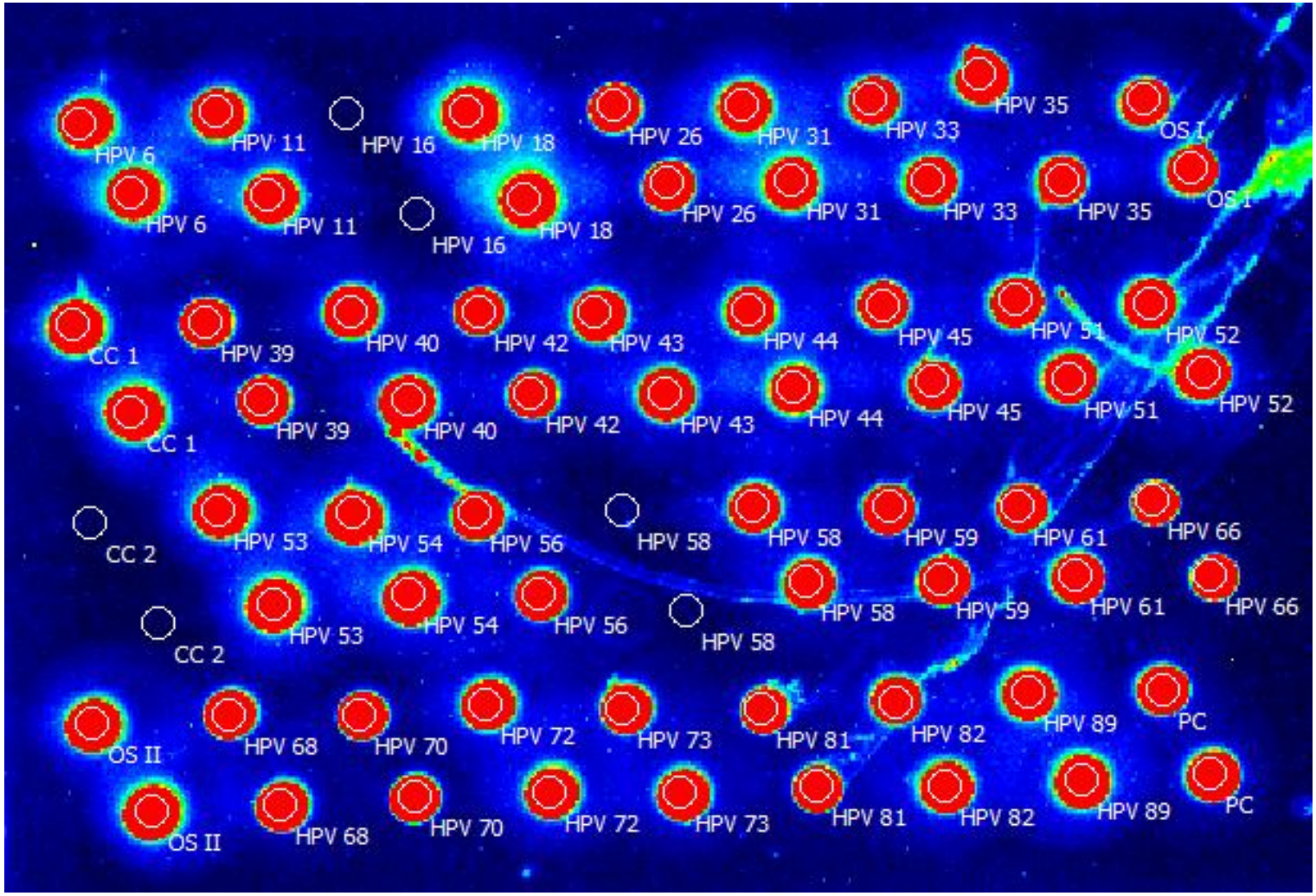
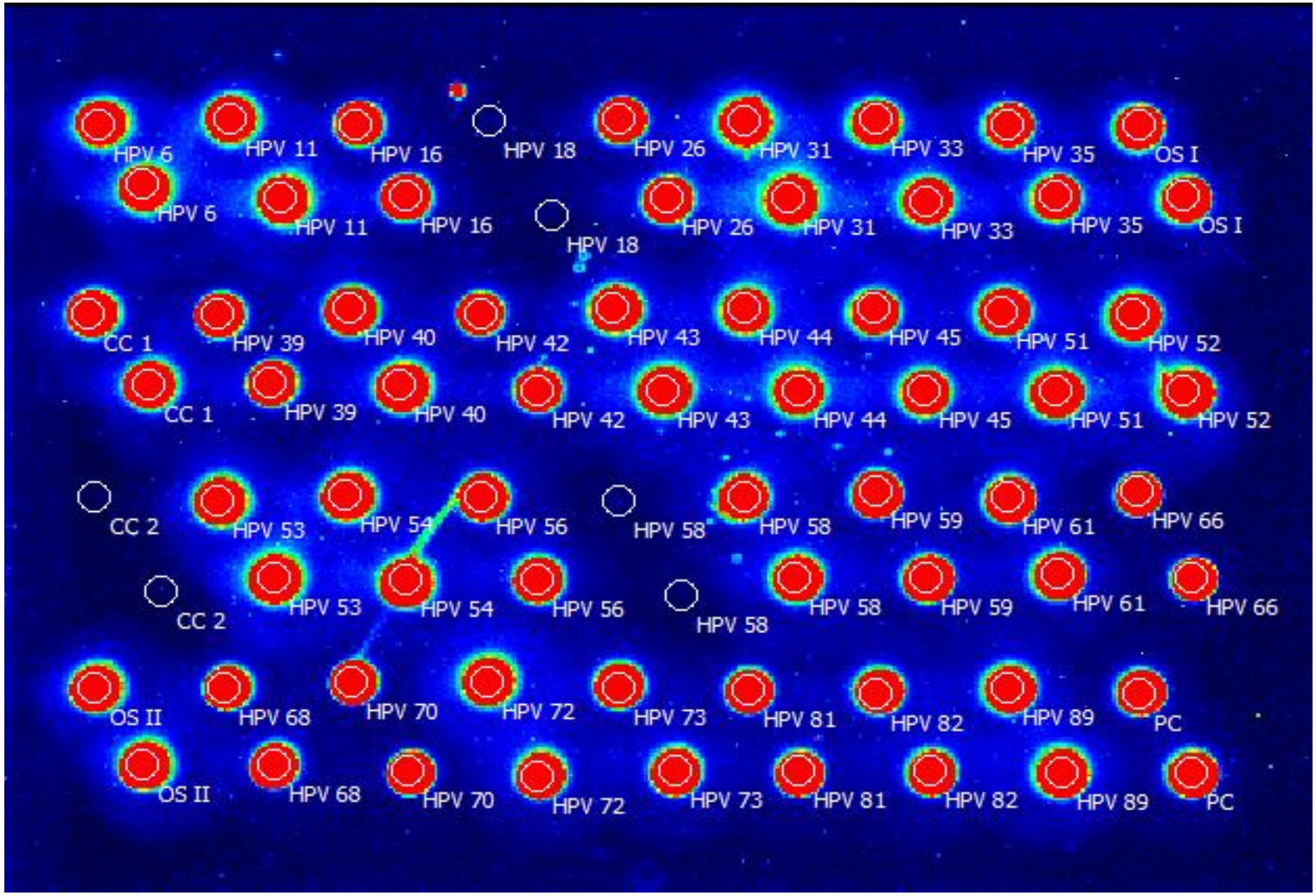
HPV 59	130	58285	58171	58415	48201	58382	58268	58138	57889	58171	58333	58203	58350	52671	57353	58366	58512	58317	58268	58301	58268	58463	0	57337	56361	58350	58366	58415	58138	58155	58236	58268
HPV 61	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	40625	58366	58512	53874	50802	58301	58268	58463	58480	0	58333	58350	58366	58415	58138	58155	58236	58268
HPV 61	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	45274	58366	58512	58317	48639	58301	58268	58463	58480	0	58333	58350	58366	58415	58138	58155	58236	58268
HPV 66	0	45616	25751	25052	17980	24986	22336	26888	27717	26125	26303	17882	28108	25409	26010	27620	29424	25441	24612	27799	35764	31472	28206	26254	0	27588	24531	33944	28042	29555	29132	29782
HPV 66	0	44121	30091	25474	24190	25832	22466	26758	28367	17070	27083	23507	23637	24856	26530	28937	29408	25994	25019	28433	29814	31326	27328	25214	0	27832	29116	29067	28416	28482	28498	29522
HPV 68	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	56182	53208	57174	52378	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	0	58366	58415	58138	58155	56459	58268
HPV 68	113	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	57759	58350	58382	57959	58366	58512	58317	54801	58301	58268	58463	58480	58333	58333	0	58366	58415	58138	58155	58236	58268
HPV 70	130	45031	44364	29165	31896	28904	38560	39113	45225	41812	34789	18776	34367	38674	44835	55549	48640	46656	37406	36480	54817	41064	39813	49599	44998	41715	0	39488	38642	39829	40853	49745
HPV 70	227	43682	28205	30465	32595	22060	39682	40170	50298	43844	37715	27246	36253	41308	52980	57548	51761	44803	40836	38057	40267	42332	43617	45599	43697	42349	0	46462	40820	40544	43145	51403
HPV 72	97	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	0	58138	58155	58236	58268
HPV 72	195	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	0	58138	58155	58236	58268
HPV 73	113	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	0	58155	58236	58268
HPV 73	162	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	0	58155	58236	58268
HPV 81	113	44592	51908	58415	42007	58382	47485	46916	42982	53647	45079	38788	55484	53874	57959	29977	43405	42413	58268	55646	56166	55548	54330	58333	46575	53143	49176	58415	39259	0	48185	51956
HPV 81	146	44218	41991	58415	35797	51517	47843	45209	47973	52428	45258	36951	55923	54004	57959	31912	46770	51029	58268	56427	57158	58463	55663	58333	44413	55972	49778	58415	39845	0	46868	53305
HPV 82	48	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	0	58268
HPV 82	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	0	58268
HPV 89	487	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	0
HPV 89	341	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	0
PC	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268
PC	0	58285	58171	58415	58171	58382	58268	58138	58398	58171	58333	58203	58350	58382	57959	58366	58512	58317	58268	58301	58268	58463	58480	58333	58333	58350	58366	58415	58138	58155	58236	58268

High specificity - EUROArray with all HPV but without HPV 16



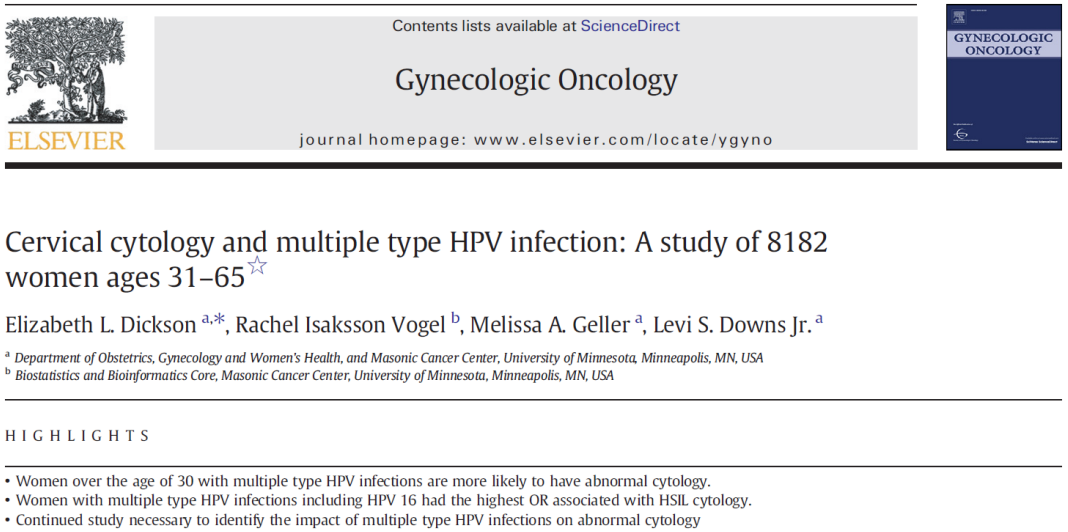
High specificity EUROArray HPV PDM without HPV 18



Multiplex analysis make a detection and typing of HPV in one step possible

- Detection of all anogenital high-risk HPV
  - Multiple HPV- infections are common

    (Menton et al., 2009; Insinga et al., 2008)
- Detection of all anogenital low-risk HPV
  - Differentiation between high-risk / low-risk induced CIN II
  - low-risk HPV may act as additional risk-factor for the development of cervical cancer



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Cervical cytology and multiple type HPV infection: A study of 8182 women ages 31–65☆

Elizabeth L. Dickson [a,*], Rachel Isaksson Vogel [b], Melissa A. Geller [a], Levi S. Downs Jr. [a]

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HIGHLIGHTS

- Women over the age of 30 with multiple type HPV infections are more likely to have abnormal cytology.
- Women with multiple type HPV infections including HPV 16 had the highest OR associated with HSIL cytology.
- Continued study necessary to identify the impact of multiple type HPV infections on abnormal cytology

Reasons for HPV-subtyping

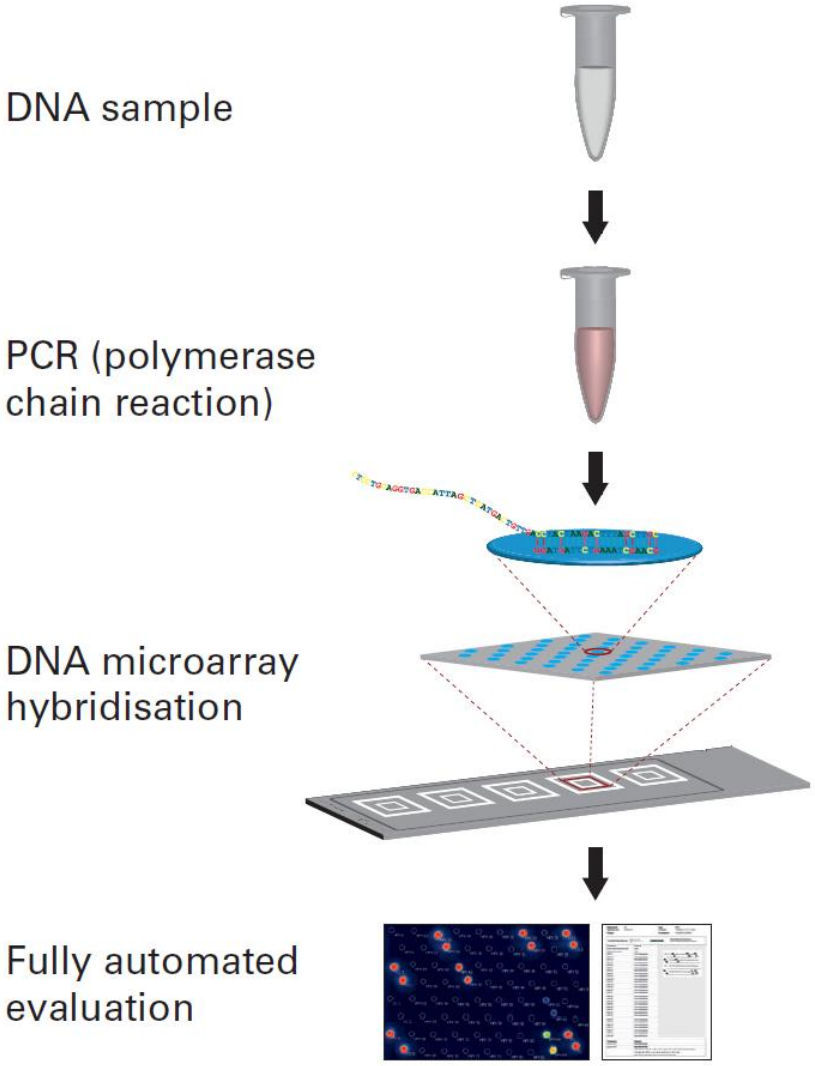
- Detection and typing in one step
  - Different HPV-subtypes come along with different risks
  - It is possible to discriminate between new and persistent infections
  - HPV-typing can be used as progression marker
  - The risk to develop cervical cancer is higher with multiple Infections, only with a typing test multiple infections are visible

Some questions only can be answered with muliplex parameter platforms

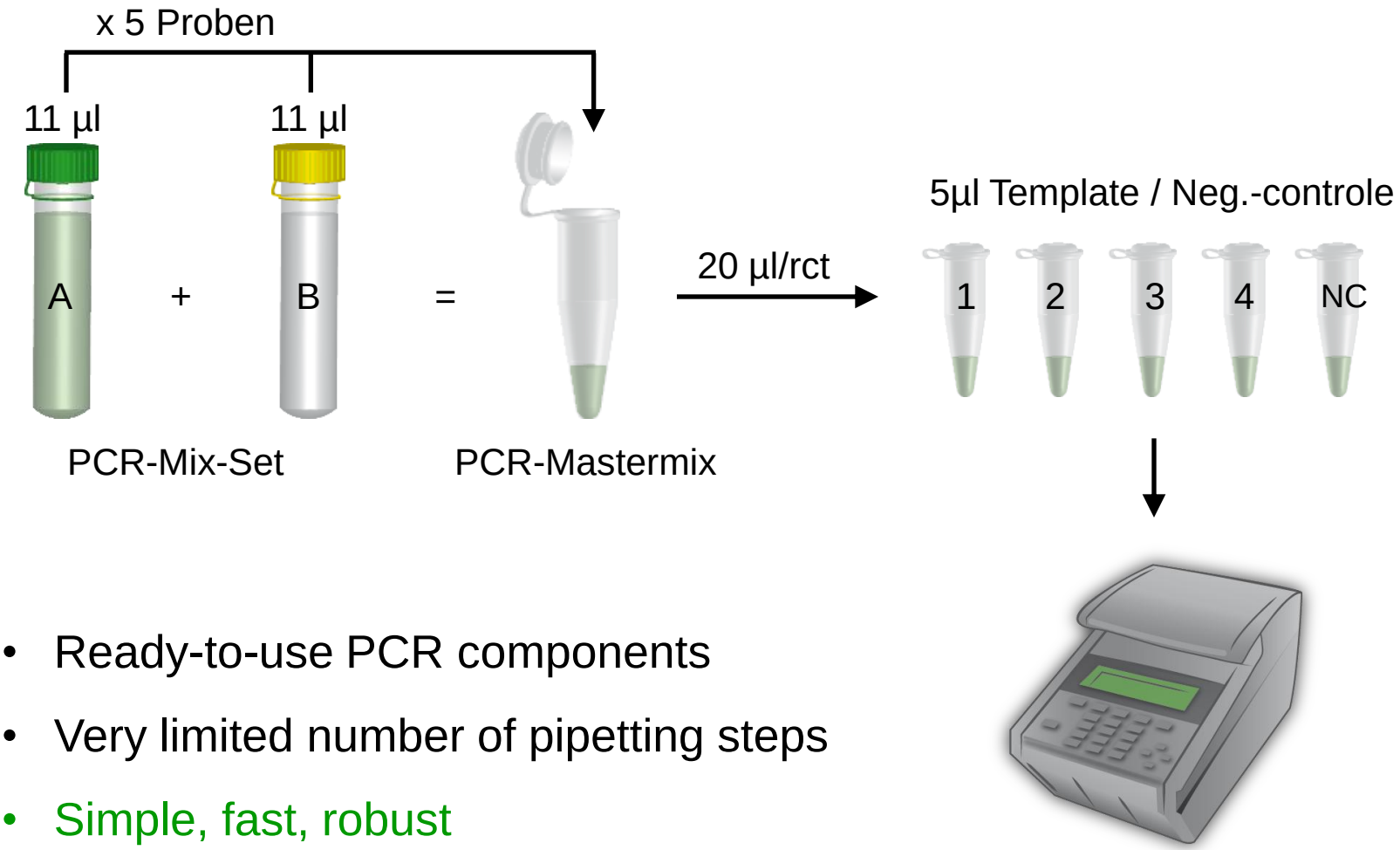
Detection of E6-E7 genes

- Not very conservative genes
- Detection of the oncogenes itself not of the capsid genes
- One primer-system for each HPV
- One specific probe for each HPV
- Detection also if the viral DNA is integrated in the host genom
  - A requirement for the malign transformation of the cell is the integration of the HPV-DNA into the human host- genom
    (Hopman et al., 2004; Durst et al., 1985; Cullen et al., 1991; Hopman et al., 2006)

EUROArray Workflow

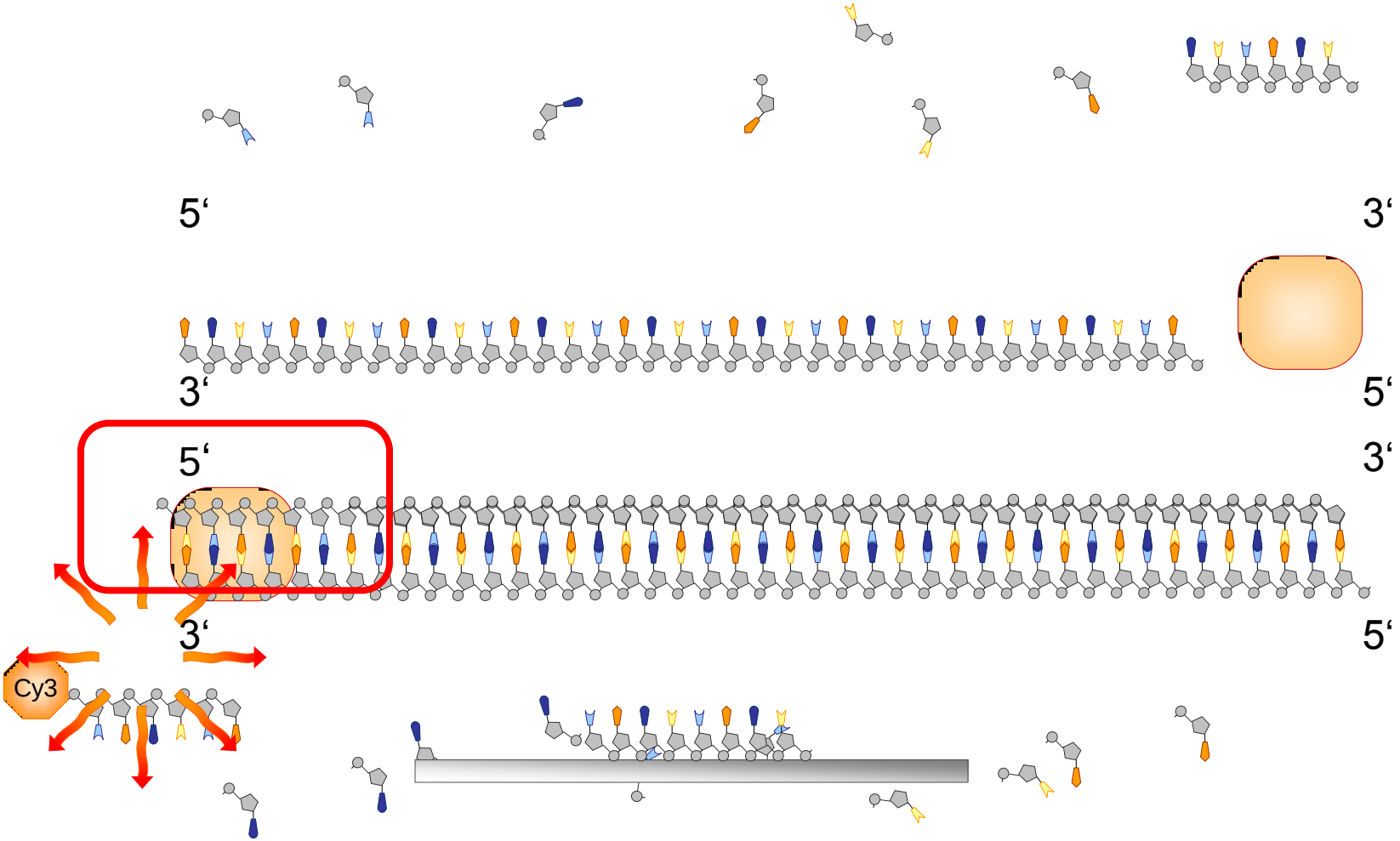


EUROArray system - Polymerase chain reaction



- Ready-to-use PCR components
- Very limited number of pipetting steps
- Simple, fast, robust

The PCR products are labeled during the reaction



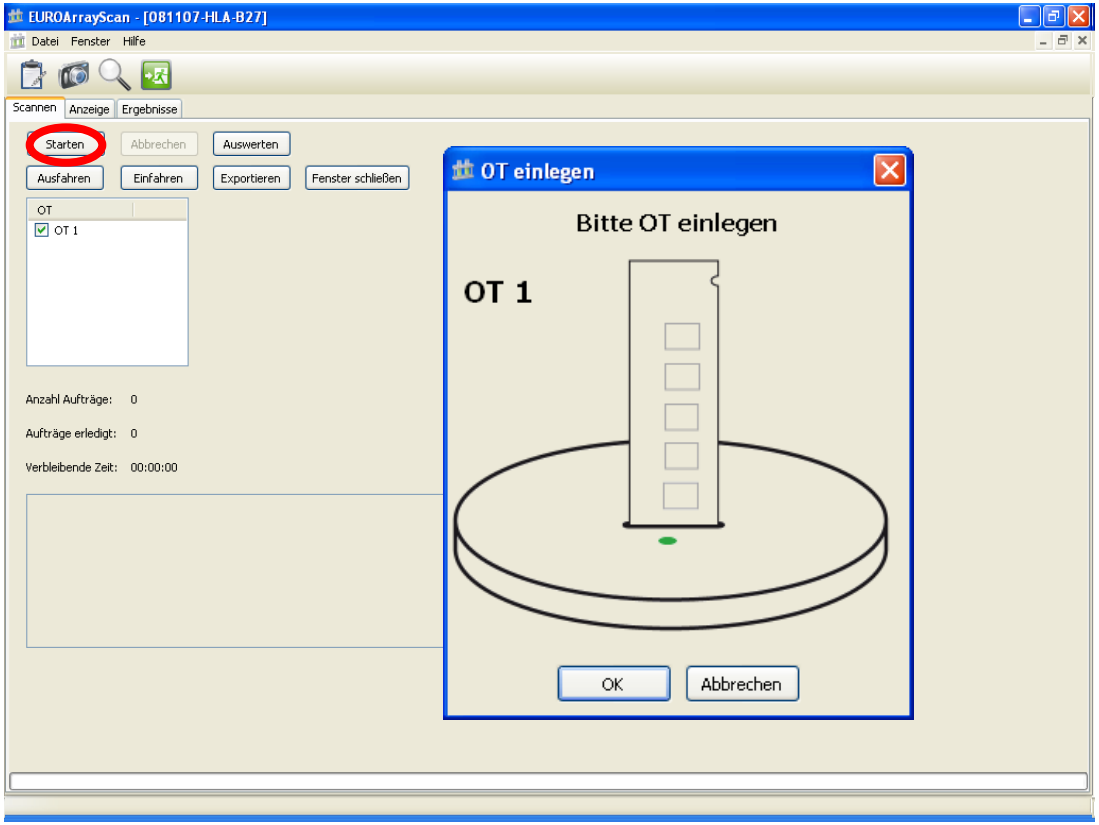
Microarray hybridization

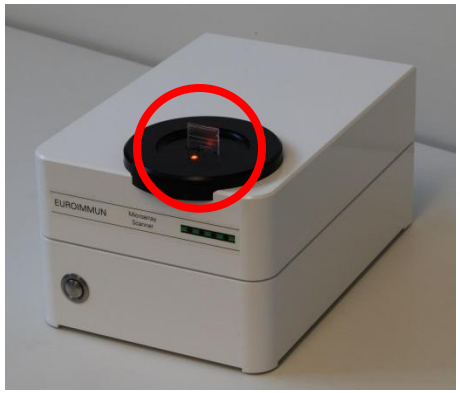
- Reproducible, simple handling

Scanning and evaluation

Fully automated standardized evaluation, interpretation and archiving of results

- Opening of the protocol and start
- Insertion of the µArray slide

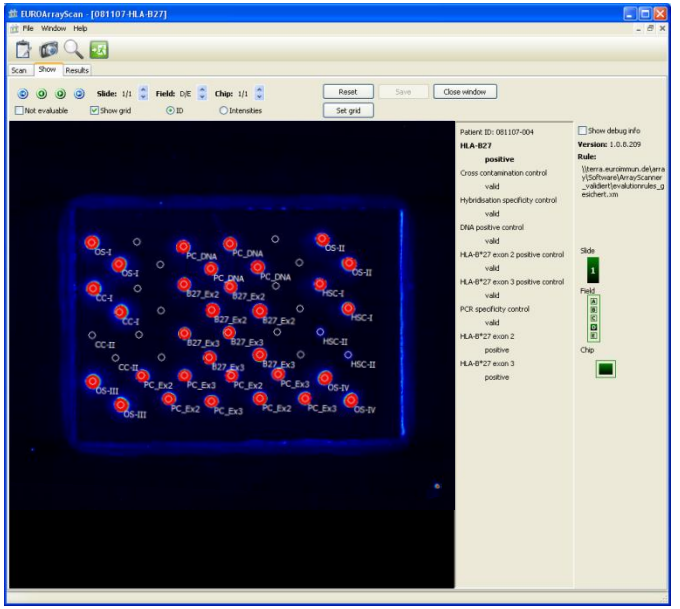




EUROArray system - Scanning and evaluation

Microarray Scanner

Software



- Complete process from receipt of samples to issuing of results is IVD validated and CE labelled (DNA extraction, test reagents, microarray scanner, software)

Patienten ID :	Thinprep 23 ohne waschschritte	**Test :**	HPV
Ergebnis vom :	22.10.2013	**Protokoll :**	131021JG_HPV
Druckdatum :	15.01.2014 15:01:03		

EUROIMMUN Medizinische Labordiagnostika AG

Automatische Auswertung
mit der EUROArrayScan-Software

Teilergebnis	Ergebnis
Kreuz-Kontaminationskontrolle	valide
DNA Positivkontrolle	valide
HPV 6**	nicht nachgewiesen
HPV 11**	nicht nachgewiesen
HPV 16*	nicht nachgewiesen
HPV 18*	nicht nachgewiesen
HPV 26*	nicht nachgewiesen
HPV 31*	nicht nachgewiesen
HPV 33*	nicht nachgewiesen
HPV 35*	nicht nachgewiesen
HPV 39*	nicht nachgewiesen
HPV 40**	nicht nachgewiesen
HPV 42**	nicht nachgewiesen
HPV 43**	nicht nachgewiesen
HPV 44**	nicht nachgewiesen
HPV 45*	nicht nachgewiesen
HPV 51*	nicht nachgewiesen
HPV 52*	nicht nachgewiesen
HPV 53*	nicht nachgewiesen
HPV 54**	nicht nachgewiesen
HPV 56*	nicht nachgewiesen
HPV 58*	NACHGEWIESEN
HPV 59*	nicht nachgewiesen
HPV 61**	nicht nachgewiesen
HPV 66*	nicht nachgewiesen
HPV 68*	nicht nachgewiesen
HPV 70**	nicht nachgewiesen
HPV 72*	nicht nachgewiesen
HPV 73*	nicht nachgewiesen
HPV 81**	nicht nachgewiesen
HPV 82*	nicht nachgewiesen
HPV 89**	nicht nachgewiesen

OT 1 Feld C Chip 1

Testergebnis	Ergebnis
high-risk HPV*	**NACHGEWIESEN**
low-risk HPV**	**nicht nachgewiesen**
	*high-risk HPV: HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68,73,82. **low-risk HPV: HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, 89. Nach N Engl J Med 348:518-527 and Lancet Oncol 6(4):204.

Unterschrift : _____

EUROIMMUN Medizinische Labordiagnostika AG

HPV microarray (IVD) – complete typing of human papilloma viruses

- Detection and typing of all 30 relevant anogenital HPV subtypes in one reaction
- Direct detection of the viral oncogenes E6/E7 provides highest possible sensitivity
- Significant results even in very early stages of the infection
- Distinction between high-risk and low-risk types of HPV
- Reliable identification of multiple infections

EUROArray HPV

(order no. MN 2540-####)

####: For detailed information about available test kit formats see our product catalogue or visit www.euroimmun.com.

- Well-established EUROArray technology
- Simple test performance – no in-depth molecular biology knowledge required!
- Ready-to-use PCR components, integrated controls
- Fully automated and standardised evaluation, interpretation and archiving of results (EUROArrayScan system)

EUROIMMUN AG · D-23560 Luebeck (Germany) · Seekamp 31 · Tel +49 451 58550 · Fax 5855591 · E-mail euroimmun@euroimmun.de
MV_2540_I_UK_A02, 11.2013

Molecular diagnostics for HPV screening: sensitive, fast , reliable

- Alternative: molecular diagnosis
  - Sensitive
  - Fast
  - Typing of HPV subtypes is possible
  - Objective
  - Detection of the pathogen in a very early stage of the infection possible
  - Extremely sensitive
  - Sub-typing is possible

- A combination of PAP-Testing and HPV-Testing as cervical cancer prevention is recommended by the FDA and national and international associations of gynecologists