LightMix® Kit *human Adenovirus* (hAdV)

Cat.-No. 40-0303-32 **2015**: 32rxn/vial and IC changed to Extraction Control

Kit with reagents for the detection of *human Adenovirus* DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each.
Shipping at ambient temperature. Store protected from light at 4°C-25°C - do not freeze !

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 II Instrument / cobas z 480 Analyzer see pages 6-7

1. Introduction

Adenoviruses have a linear double stranded ~ 40 kb DNA genome, containing 30-40 genes. The virus affects membranes of the respiratory tract, eyes, intestines, and urinary tract. Symptoms of respiratory illness range from the common cold to pneumonia and bronchitis. Adenoviruses account for about 10% of acute respiratory infections in children and are a frequent cause of diarrhea. The disease is usually mild and requires at most a symptomatic treatment. Immune-compromised patients are vulnerable to severe complications of Adenovirus infections. Transmission is airborne or fecal route.

The 51 serotypes of human adenovirus (HAdV) are divided into six groups named from A to F. Respiratory diseases are mainly due to virus from groups B and C, conjunctivitis to groups C and D, while the causative virus for gastroenteritis is probably limited to group F viruses (types 40 and 41 only).

Group	Type
A	12, 18, 31
B	3, 7, 11, 14, 21, 34, 35
C	1, 2, 5, 6
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39
E	4
F	40, 41

Routine diagnosis is accomplished by antigen testing, serology, or PCR. Clinical samples can be swabs, feces, rectal swabs and tissue as well as blood, serum, plasma, or liquor.

2. Description

This kit provides a fast and accurate system to detect *Adenovirus* (AdV) genomic DNA from a nucleic acid extract; the kit includes a spiked extraction control working also as amplification control reaction. The kit has been verified to work with AdV types 1-51 and type 53. For a determination of the viral load in liquid samples please see section 10. Conversion Factor.

A 129 bp long fragment of the Hexon gene is amplified with several specific primers and detected with probes labeled with LightCycler® Red 640 (channel 640). The presence of the PCR product can be verified by running a melting curve, but without the capability of serotyping. If typing is required, the PCR product is ready to be analyzed using a Chipron LCD chip for serotype group determination.

The control reaction is based on a 278 bp long PCR fragment generated from Lambda DNA, detected with LightCycler® Red 690 labeled hybridization probes (channel 705). The control reaction is supplied in a separate vial and may be optionally run in a separate reaction instead of duplex PCR in order to achive a higher sensitivity, in particular as the AdV PCR is based on a complex mixture of primers.

The former internal control (IC) has been changed to a spiked extraction control (sEC) to monitor successful extraction and demonstrate the ability to run an amplification reaction (no PCR inhibition). We recommend to use the 'Extraction Control' procedure; for compatibility with the former procedure the use as IC is also described. The extraction control target ^{n}ECT contains Lamdba and PhHV DNA and may be used also for other LightMix kits, without adding the ECT provided with the other kit(s).

The use of a color compensation file generated with the ColorCompensation kit 40-0318 is a prerequisite to run is a prerequisite to detect the control reaction.

The supplied standard row (AdV group B) allows to determine the linear range and the detection limit of the device.

The kit must be used with 'LightCycler® FastStart DNA Master HybProbe' or "LightCycler® FastStart^{PLUS} DNA Master Hybridization Probes" (capillary and plate based LightCycler® Instruments).

3. Set Contents

3	Vials with **blue** cap containing premixed lyophilized primers and probes for 32 PCR rxns *HAdV*
3	Vials with **white** cap containing premixed primers and probes for each 32 control reactions
1	Standard row with 6 lyophilized plasmid standards of HAdV from 10^1 to 10^6 target equivalents / rxn
1	Sealing foil for the standard row
1	Vial with **white** cap with the universal Extraction Control Target (^{n}**ECT**): 4.8 x 10^6 copies (total)
1	Vial with **black** cap containing the stabilizer for the No Template Control (NTC)
1	Certicate of Analysis (CoA) with lot-specific data

4. Additional Reagents and Items Required

	Roche Diagnostics
ColorCompensation HybProbe order n° 40-0318-00	Cat.-No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
or LightCycler® FastStart DNA Master^{PLUS} HybProbe	Cat.-No. 03 515 575 001
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of 640 and F3 instead of channel 705. We recommend to upgrade to software version 4.10 or higher.

5. Product Characteristics

PCR results (activation, 50 cycles and melting curve) are obtained within 50 minutes with the capillary based LightCycler® 1.x / 2.0 Instruments and within 80 minutes with '480' plate based Instruments.

Sensitivity
Reagents detect 10 copies/reaction positive control target DNA using FastStart DNA Master HybProbe.

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of target genomic DNA.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at 4°C-25°C. See expiry date on the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved positive controls must be stored at -20°C. Avoid multiple freeze-thaw cycles.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).
- Dissolved reagents can be long-term stored frozen at -20°C. Avoid multiple thaw-freeze cycles.

6. Experimental Protocol

Start programming before preparing the solutions. See the Instrument operator's manual for details.

6.1. Preparation of Parameter-Specific Reagents (PSR):

One reagent vial with a **blue** cap contains primers and probes to run 32 reactions of *Adenovirus.*
One reagent vial with a **white** cap contains primers and probes to run 32 Control Reactions.

Check for the colored pellet, then **add 66 µl** PCR-grade water, mix (vortex) and spin down.

►**Use 2µl** reagent for a 20 µl PCR reaction.

This solution is stable at least ten days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of Extraction Control Target (ECT):

Add **1,200 µl** PCR-grade water to the vial with **white** cap containing the Extraction Control Target

6.3. Sample Preparation:

Before extraction add **10 µl** of ECT (vial **white** cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

6.4. Preparation of No Template Extraction (NTC):

Add **900 µl** PCR-grade water to the **NTC** (vial **black** cap) and add **100 µl** of ECT (vial **white** cap).

►Use **5 µl** No Template Control DNA for a 20 µl PCR reaction.

6.5. Preparation of the Standard Row

The target DNA is provided in 6 different quantities to yield from 10^1 to 10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. Add **40 µl** of **NTC** (vial **black** cap) to each well. Use water if the IC procedure is selected.Mix the target DNA by pipetting the solution up and down 10 times.

►**Use 5 µl** standard for a 20 µl PCR reaction.

This standard solution is not long-term stable. Use only fresh prepared solutions only. After adding the target DNA to the reaction mix, use the provided sealing foil to close the vials in order to avoid contaminations.

6.6. Preparation of the Reaction Mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled plus one additional reaction.

For use with the Roche FastStart^{PLUS} Master			For use with the Roche FastStart Master	
Single reaction IC	**Single reaction EC**	**Component**	**Single reaction EC**	**Single reaction IC**
6.5 µl	7.0µl	water, PCR-grade (colorless cap, provided with the Roche Master kit)	7.4 µl	6.9 µl
--	--	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	1.6 µl	1.6 µl
2.0 µl	2.0 µl	**PSR** mix (parameter specific reagents, see **6.1.**)	2.0 µl	2.0 µl
2.0 µl	2.0 µl	Primers and probe mix for the **IC/EC**	2.0 µl	2.0 µl
0.5 µl	**--**	**ECT** (white cap, DNA control target, see 6.2.)	--	0.5 µl
4.0 µl	4.0 µl	Roche Master (red cap, for preparation see Roche manual)	2.0 µl	2.0 µl
15.0 µl		**Volume of reaction mix**		**15.0 µl**

Table 1

Mix gently, spin down and **transfer 15 µl** of the reaction mix to a capillary or well.

Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to the multiwell plate and seal, and spin down.

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	0.2	20
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

Table 2

7.2. Data Analysis

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual Color Compensation Kit HybProbe 530/640/690.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.

We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *Adenovirus* data in channel 640, Quantification mode (LightCycler® 2.0 Instrument). The negative control (NTC) must show no signal.
For the identification of the PCR product view *Adenovirus* data in channel 640 Melting Curves mode.

For the Control Reaction view channel 705 data. The negative control and the low-concentrated *Adenovirus* DNA samples (10 to 1,000 copies) should show an amplification curve for the EC/IC with a Cp at approximately cycle 25-31.

The provided standard row of cloned DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Adenovirus* should have Cp values between cycles 18 and 36.

7.3. Interpretation of Data

Sample 640 *Adenovirus*	Sample 705 Ctrl Reaction	Channel 640 PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 37[+]	not relevant	amplification	negative	**Positive for *Adenovirus***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Table 3. Typical analysis results (LightCycler® 2.0 Instrument, Roche Master: FastStart)

[+] The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies

7.4. Sample Data – Typical Results

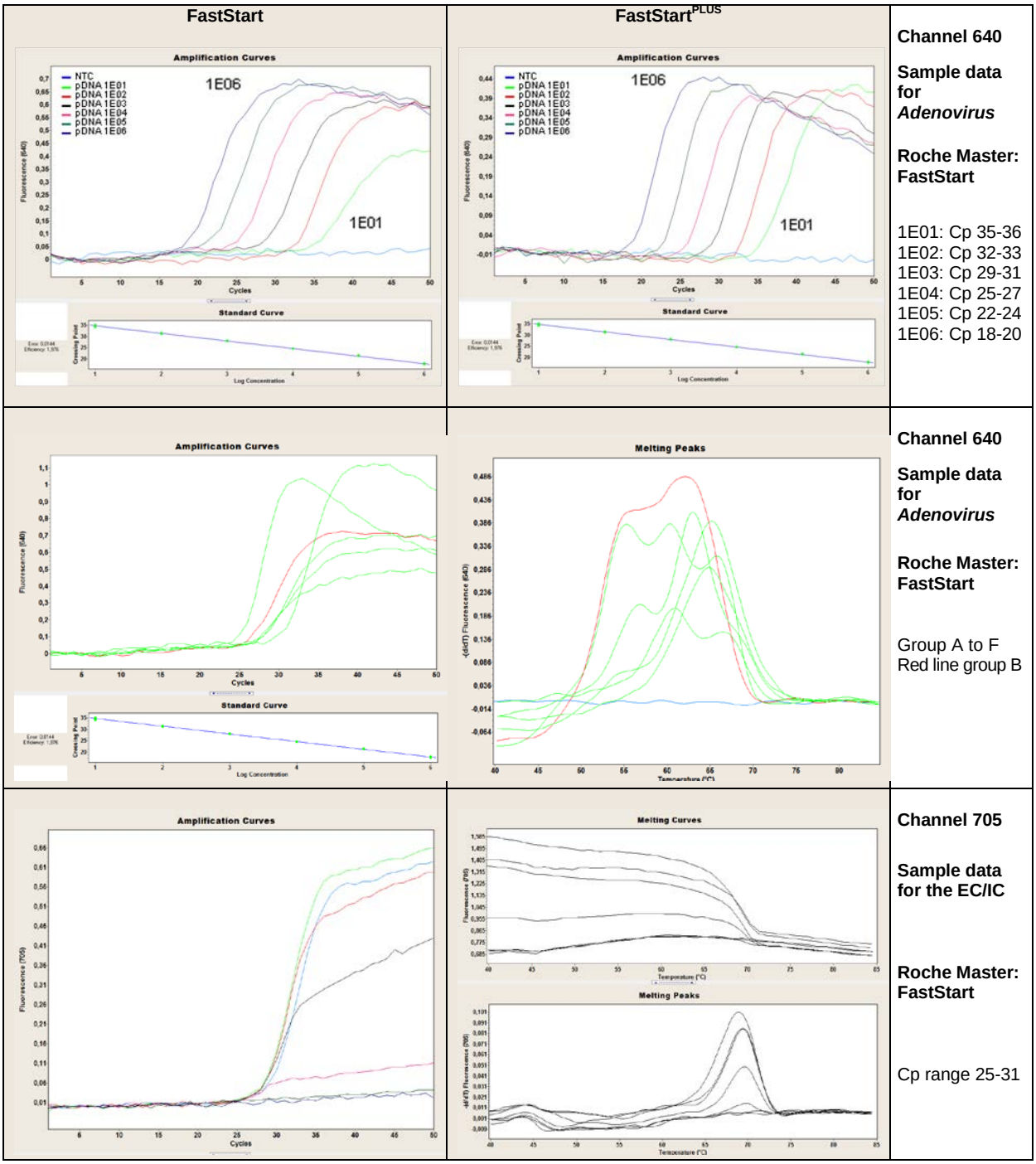

Fig.1. LightCycler® 2.0 sample data for the Adenovirus **detection system.**

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) with amplification curve for AdV.
Middle panels:. Left panel channel 640 quantification mode (Second Derivative Maximum) for the samples.
Right panel channel 640 melting peaks for Adenovirus groups A to F.
Lower panels: Left panel channel 705 quantification mode (Second Derivative Maximum) with amplification curves for the control reaction. 705 melting analysis for the control reaction (not relevant for detection).

* **Note:** Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8. LightCycler® 480 II Instrument / cobas z 480 Analyzer

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660
cobas z 480 Analyzer: 465-510, 498-645, 498-700

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0.5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	**1**	-

Table 4

8.2. Data Analysis

Note: cobas z 480 Analyzer signal levels are about 50% compared to LightCycler® 480 II results.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual 'LightMix® Kit – Color Compensation HybProbe.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.
We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically.

View *Adenovirus* data with Filter Combination 498-640, Quantification mode. The negative control (NTC) must show no signal. For the identification of the PCR product view *Adenovirus* data with Filter Combination 498-640 Melting Curves mode.

If the control reaction is used, view data with Filter Combination 498-660 Quantification mode. The negative control and the low-concentrated *Adenovirus* DNA samples (10 to 1,000 copies) should show an amplification curve for the EC/IC with a Cp at approximately cycle 25-31.

The provided standard row of cloned and purified DNA with concentrations in the range from 10^6 copies/rxn to 10^1 copies/rxn of *Adenovirus* should have Cp values between cycles 18 and 36.

8.3. Interpretation of Data

Sample 640 *Adenovirus*	Sample 660 Ctrl Reaction	Channel 640 PositiveControl	Negative Control (NTC)	Result (warnings)
no amplification	detectable	amplification	negative	**Negative (not detectable)**
Cp < 36[+]	not relevant	amplification	negative	**Positive for *Adenovirus***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Table 5. Typical analysis results (LightCycler® 480 Instrument, Roche Master: FastStart)

[+] The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than observed for 10 copies coresponds to ~5 copies

8.3. Sample Data – Typical Results

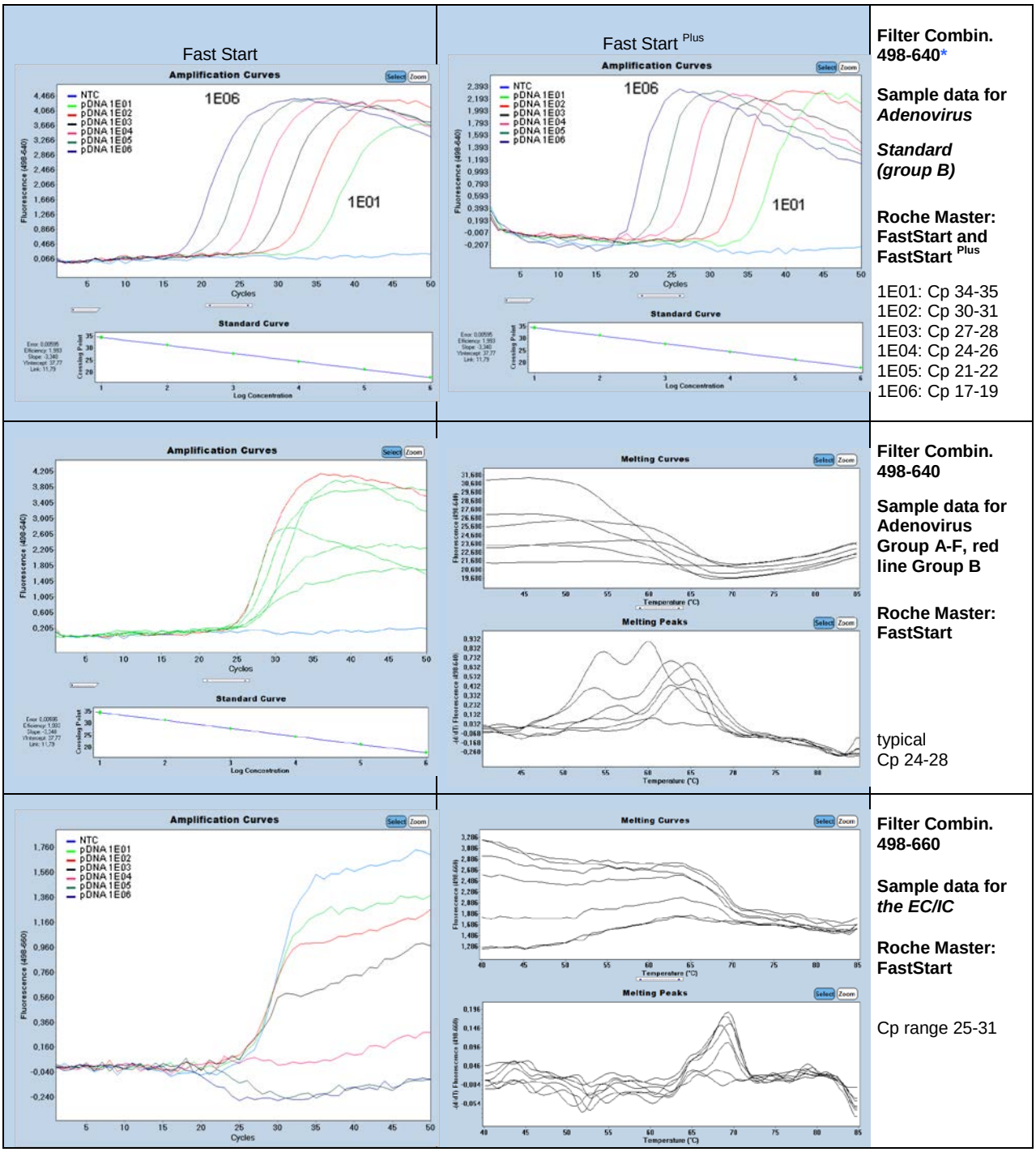

Fig.2. LightCycler® 480 II sample data for the *Adenovirus* detection system.

Upper panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for Adenovirus (Fast Start). Right panel Filter Combination 498-640 amplification curves for Adenovirus (Fast Start Plus).
Middle panels: Left panel Filter Combination 498-640 quantification mode (Second Derivative Maximum) with amplification curves for Adenovirus (Group A-F). Right panel Filter Combination 498-640 melting peaks for Adenovirus (Group A-F).
Lower panels: Left panel Filter Combination 498-660 amplification curves (Second Derivative Maximum)) for the control reaction. Right panel Filter Comb. 498-660 melting analysis and melting) for the control reaction (not relevant for detection).

* **Note:** Fluorescence levels may vary. The characteristics of the curve (shape and trend) should be similar to the curve shown. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values may vary between instruments by up to 2 cycles, while the delta Cp between dilution steps should be constant. The Cp values described in this manual (chart text) have been obtained using the supplied standard row.

9. Conversion Factor

The amount of virus per sample (Viral Load) is usually reported in copies / ml while PCR reports copies per reaction. The conversion factor between both depends on sample and extraction volumes. Extraction starts usually from less than one milliliter and PCR does not use the total extracted volume.

The viral load (VL) can be calculated using the following general formula:

VL [copies/ml] = MV (Measured Value) x EVF x SVF

Example: Extracting 200 µl clinical sample results in an extraction volume factor (EVF) of **5**. Using 5 µl sample from a total eluation volume of 100 µl results in a sample volume factor (SVF) of **20**, resulting in a conversion factor of **100**:

Viral Load [copies/ml] = Measured Value [copy number per reaction] **X 100**

10. References (Use of this kit)

Adenovirus Disease after Kidney Transplantation: Course of Infection and Outcome in Relation to Blood Viral Load and Immune Recovery. Watcharananana et al. (2011)

Swabbing for resp. viral infections in older patients: a comparison of rayon and nylon flocked swabs. Hernes et al. (2011)

Alt Solunum Yolu Enfeksiyonu Olan Çocuklarda Adenovirusların Araştırılması. Saglic et al. Mikrobiyol Bul (2013)

11. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a MSDS.

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

12. Version History

Red notes mark changes in procedures, blue modification of sequences

V111208	Correction of Cp values for the IC in LC 2.0
V130813	z 480 included, Conversion Factor, manual condensed to 8 pages
V150505	Kit changed from 6 vials 16 rxns to 3 vials 32 rxns Internal control changed to extraction control (EC) Kit equipped with the universal ^{n}ECT extraction control target
V160330	Cp values control reaction corrected, Storage conditions, Section 9 wording

Roche SAP order n° 05552451001

Notice to Purchaser

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.