LightMix® Universal Color Compensation Hexaplex Plus

Cat.-No. 40-0320-12 Roche 06296971001

Color Compensation Reagents for Roche 480 instruments (LightCycler® systems and cobas z 480 Analyzer). Reagent for use with LightMix® Modular Kits; use with other kits must be verified by the user.

Index:

1. Contents, Selection of Calibrator Dye and Storage

12 vials containing premixed primer, dark quenched Hydrolysis Probe and DNA target for 15 reactions:

Cap color	Tube Name	Fluorophore	Comment
Orange	500 N	Cyan500	**N**ormal reagent for Cyan500 assays.
Orange	500 S	Cyan500	**S**pecial reagent. Use if 500 signals are visible in the 530 channel.
Yellow	530 N	Fluorescein (FAM)	**N**ormal reagent for FAM and SimpleProbe assays.
Red	580 N	Rhodamine 6G	**N**ormal reagent for HEX, JOE, R6G (and VIC) assays.
Red	580 S	Rhodamine 6G	**S**pecial reagent. Use if crosstalk from channels 610/640 is seen.
Black	580 Y	Yakima Yellow	**S**pecial reagent for assays for YAK assays.
Lilac	610 N	LightCycler® Red 610	**N**ormal reagent for ROX, Texas Red and LC610 assays.
Lilac	610 S	LightCycler® Red 610	**S**pecial reagent to minimize crosstalk.
Blue	640 N	LightCycler® Red 640	**N**ormal reagent for LC640 assays.
Blue	640 S	LightCycler® Red 640	**S**pecial reagent. Use if crosstalk from channel 580 is seen.
Green	660 N	LightCycler® Red 670	**N**ormal reagent for Cy5 and Atto647 assays.
Salmon	700 N	LightCycler® Red 705	**N**ormal reagent Cy5.5 and IRD700 assays (for z 480 only).

Store lyophilized reagent at 4°C to 25°C. Once reconstituted store up to 30 days refrigerated at 2°C to 8°C in the dark, for long term storage frozen (until expiry of the kit). Do not combine / mix the reagents.

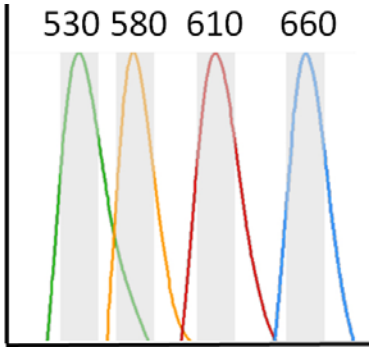
2. Additional Reagents and Materials Required:

LightCycler® FastStart DNA Master HybProbes	Cat. No. 03 003 248 001
or LightCycler® 480 Probes Master	Cat. No. 04 707 494 001
or LightCycler® Multiplex DNA Master	Cat. No. 07 339 585 001
or LightCycler® Multiplex RNA Virus Master	Cat. No. 06 754 155 001
LightCycler® 480 Multiwell Plate 96 (White)	Cat. No. 04 729 692 001
or LightCycler® 480 Multiwell Plate 384 (White)	Cat. No. 04 729 749 001

Notes: CC generated with Multiplex RNA master can be used for runs with DNA master and vice versa. The use of other polymerases have not been tested but is expected to work in the same way. Mastermix must not contain fluorescent dyes (e.g. ROX calibrator). We recommend use of the master-mix that is intended to be utilized for assay performance. Use white plates or strips. The use of clear multiwell plates is not recommended

3. Introduction and Description

Roche PCR instruments use a combination of a specific excitation light and specific filters to collect the fluorescence from one specific dye. Since the emission spectra of the fluorophores overlap with the neighboring channels the instrument collects some signals from the overlapping dye.



The Roche Color Compensation is an algorithm of the instrument software that corrects for signal read in the neighboring channel.

To create a Color Compensation file the instrument is calibrated with samples containing pure dye, performing a single dye Real-Time-PCR run and reading the fluorescence in the respective channel and the neighboring channels. The Roche Color Compensation is based on fluorescence data recorded in the melting step. The amplification plate may be used for the calibration of multiple instruments (repeat the melting step only).

The following channels are available in the different Roche Instruments:

MDx Channel ► and sample dyes ► **▼ Instrument**	**500 Cyan500**	**530 FAM**	**580 YAK HEX R6G**	**610 LC610 ROX TEXAS**	**640 LC640**	**660 LC670 Cy5**	**700 LC705 Cy5.5**
LightCycler® 480	450-500	483-533	523-568	558-610	558-640	615-670	•
LightCycler® 480 II	440-488	465-510	533-580	533-610	533-640	618-660	618-660
cobas z 480 Analyzer	•	465-510	540-580	540-610	540-645	610-670	610-700
LightCycler® 2.0	•	530	560	•	•	•	•

The generated Color Compensation file is specific for the respective dyes and master-mix used. For the most accurate CC file, perform CC procedure with the same Enzyme Master utilized in the experiments, that shall be compensated. The CC file may work for similar dyes or other master mixes, but this must be verified before use. To verify, run single positive samples or controls for all channels and check that there are no significant signals recorded in the neighboring channels.

The dye signals can be influenced by a particular probe sequence and cause the Color Compensation to fail, generating very low signals in the neighboring channel that may be called as (false) positive results by the instrument software. The novel special reagents force the Color Compensation algorithm to make a subtle overcompensation; negative shape curves will not be called positive. Negative signals will be subtracted from the positive signals in this channel. As long as the negative signals are less than approx. 5% of the expected positive signals, no change in the calling of positive results is expected. However, there could be a shift of Cp values resulting in a change of quantitative results. The use of the special reagents must be tested before inclusion in quantitative multiplex PCR assays colour compensation file.

In our experience the CC files generated with Multiplex RNA Virus Master or LC480 Probes Master can be used interchanged, however this must be verified experimentally.

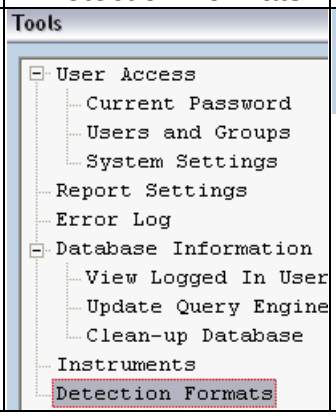
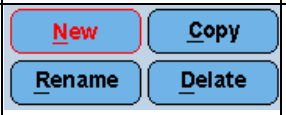
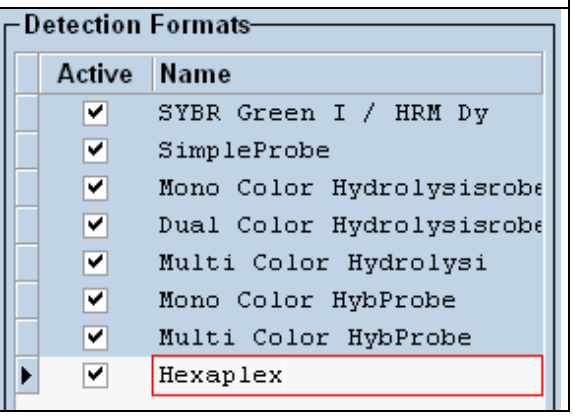
For use with LightCycler® 2.0 systems refer to the Roche manual chapter “7.2 Using Color Compensation”.

The CC file is generated utilizing the melt curve data collected during performance of a Real-Time-PCR experiment with hydrolysis (TaqMan) probes. In order to collect reliable data, we recommend running the reactions in triplicates – the respective procedure is described in this manual.

The manual describes the definition of a new format (Hexaplex) with Quant factors that differ from the default settings.

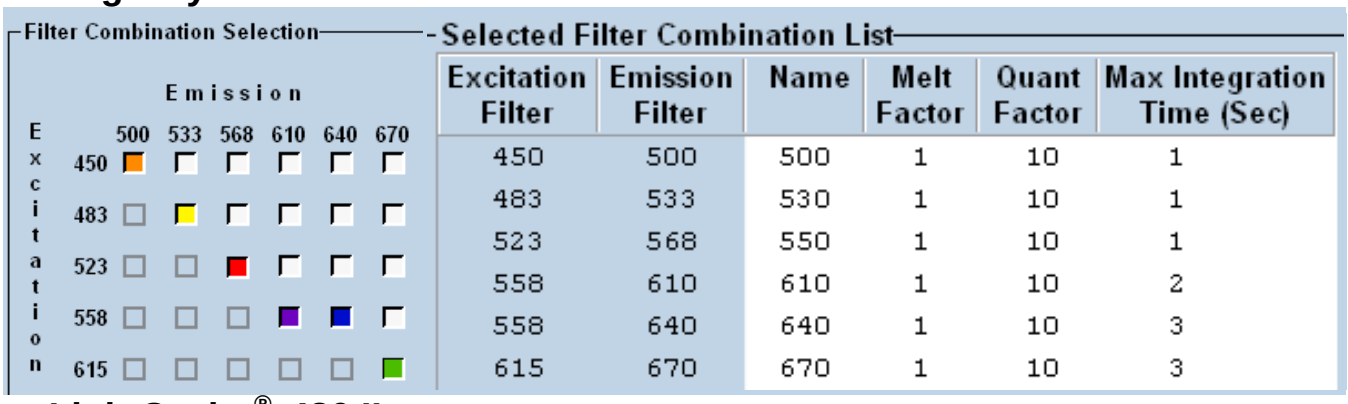
The CC can be included in a sample run however this requires creation of a distinct subset for the CC.

4. Define the 'Hexaplex' Detection Format

1) Open Tools	2) Select Detection Formats	3) New	4) Set name Hexaplex
			

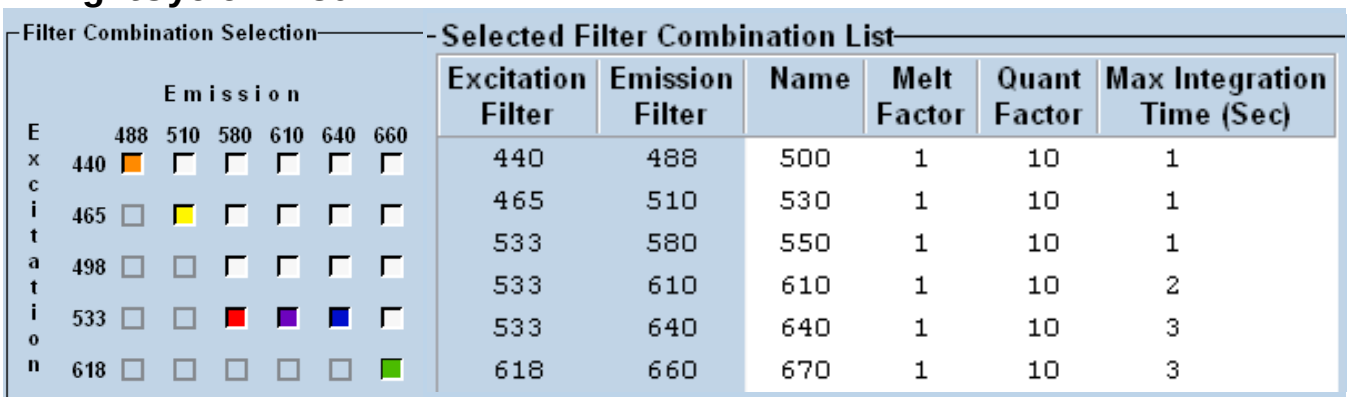
Select Filter Combination, edit Names, Melt Factor, Quant Factor and Max Integration Time:

LightCycler® 480



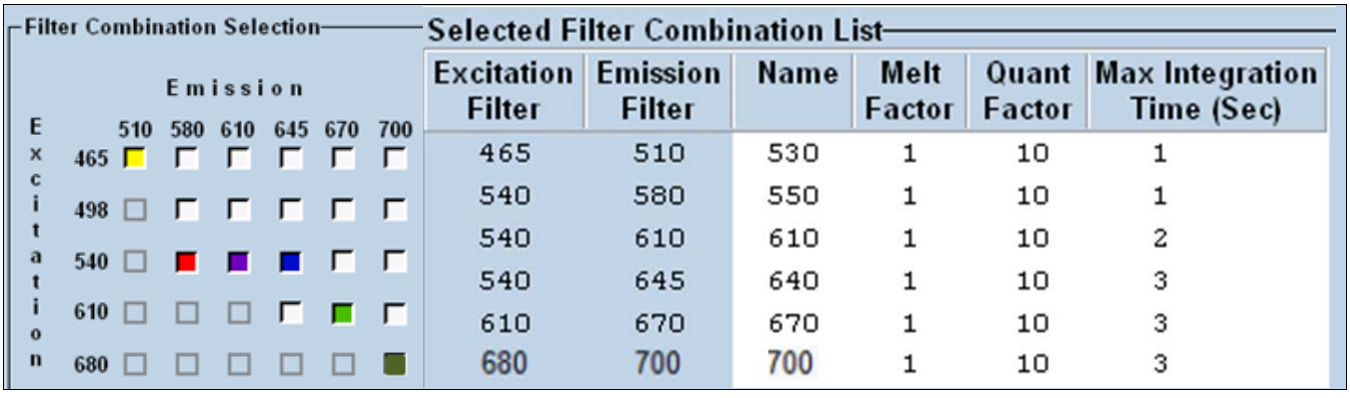
Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
450	500	500	1	10	1
483	533	530	1	10	1
523	568	550	1	10	1
558	610	610	1	10	2
558	640	640	1	10	3
615	670	670	1	10	3

LightCycler® 480 II



Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
440	488	500	1	10	1
465	510	530	1	10	1
533	580	550	1	10	1
533	610	610	1	10	2
533	640	640	1	10	3
618	660	670	1	10	3

cobas z 480



Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
465	510	530	1	10	1
540	580	550	1	10	1
540	610	610	1	10	2
540	645	640	1	10	3
610	670	670	1	10	3
680	700	700	1	10	3

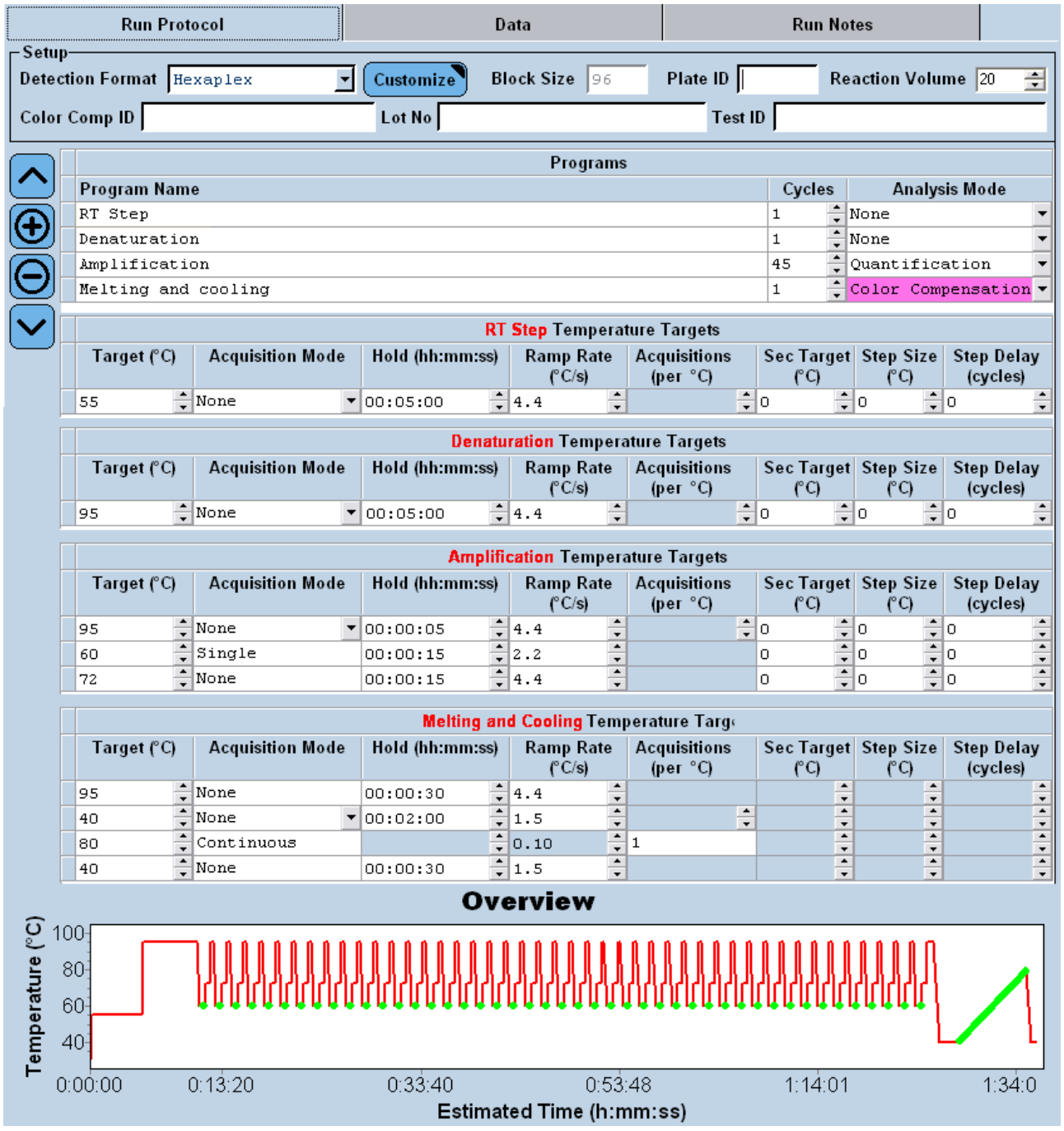
5. Instrument Programming

Color Compensation for Hydrolysis (TaqMan) Probes requires performance of a PCR experiment with single dyes and water as a blank reference. The probes are cleaved during the amplification; the instrument records the signals during the melting analysis, to enable compensation of the crosstalk at different temperatures.

Program the amplification followed by a temperature gradient or melting curves program and select 'Color Compensation' in the Analysis Mode field.

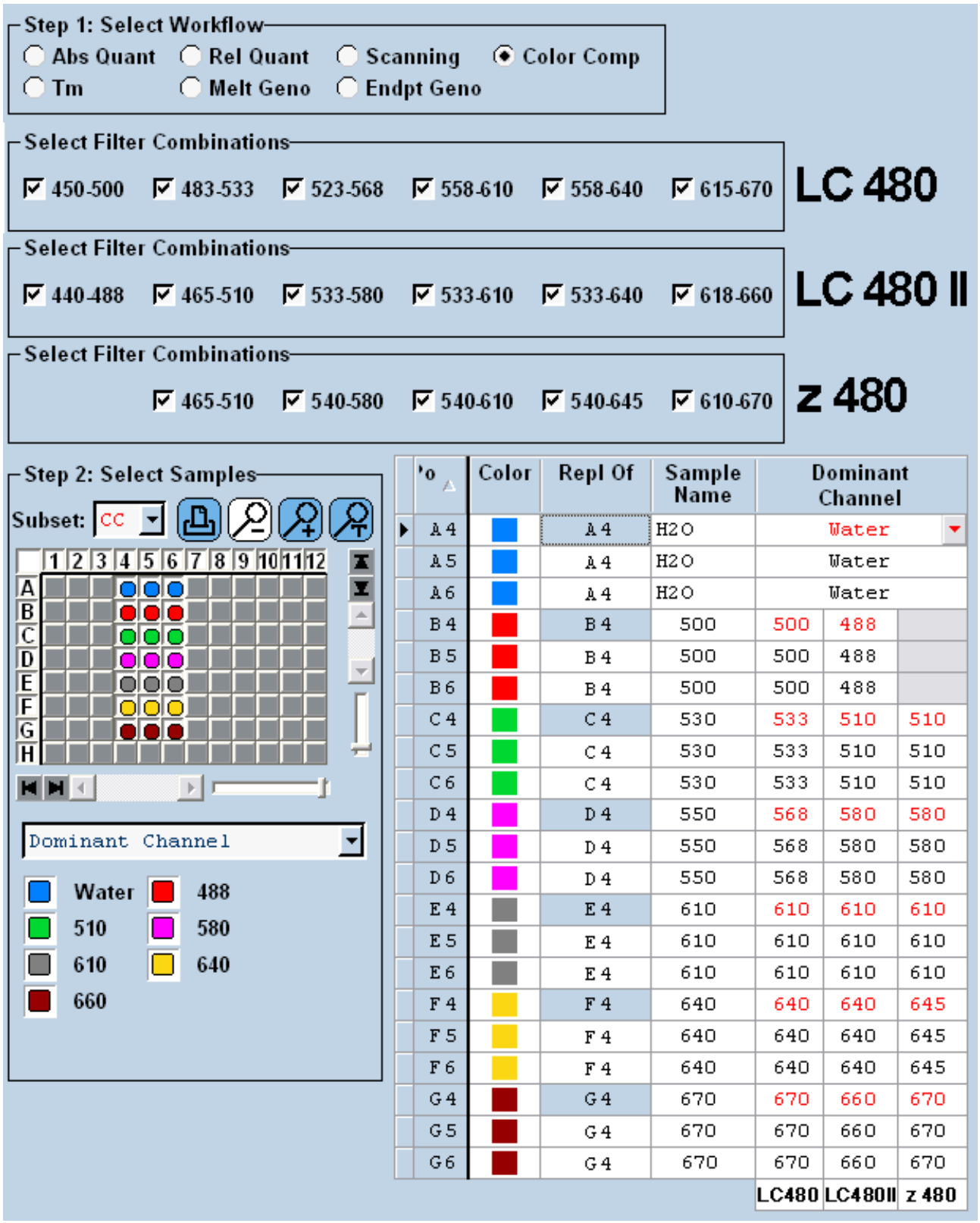
The Color Compensation can be included in a run with routine samples, but the positions used for the Color Compensation must be included in one subset with no other samples.

Do programming before preparing the solutions.



6. Sample Editor - Define the Dominant Channel

Select the Workflow 'Color Comp', then the filter combinations, and last the Dominant Channels:



Upper part of the figure channel setting for LightCycler® 480, LightCycler® 480 II and cobas z 480 analyzer. Set “Repl of”, “Sample Name” and “Dominant Channel” - in this example run the Normal (N) reference dyes have been placed on columns 4 to 6.
Lower part left: Colors in the subset are set automatically and can be not chosen!
Lower part right side: Dominant channel names for LightCycler® and cobas instruments.
See the LightCycler® operator’s manual of the specific LightCycler® Instrument for details.

7. Reagent Preparation

Spin down the tubes. Reconstitute each tube in 75 µl water. Vortex and allow to resuspend for 5 minutes. Vortex and spin down. Do not mix reagents.

Cap color	Tube Name	Fluorophore	PCR-grade H_2O
Orange #	**500 N and/or 500 S***	Cyan500	75.0 µl
Yellow	**530 N**	Fluorescein (FAM)	75.0 µl
Red	**580 N and/or 580 S***	Rhodamin 6G	75.0 µl
Black	**580 Y**	Yakima Yellow	75.0 µl
Lilac	**610 N and/or 610 S***	LightCycler® Red 610	75.0 µl
Blue	**640 N and/or 640 S***	LightCycler® Red 640	75.0 µl
Green	**660 N**	LightCycler® Red 670	75.0 µl
Salmon $	**700 N**	LightCycler® Red 705	75.0 µl

* Consider including both N and S version to create different Color Compensation files from one run.
Use of 500 S, 530 S, 580 S, 610 S, 640 S can cause subtle overcompensation in the neighboring channel.
Not for use with cobas z 480 analyzer $ For use with cobas z 480 analyzer only.

Plate based instruments require a melting curve witheach well run in triplicate. See the instrument manual for details on the generation of a CC file. Reagents needed for one reaction:

Components for one reaction $	FastStart DNA Master 03 003 248 001		LC480 Probes Master 04 707 494 001		Multiplex RNA Virus Master 06 754 155 001		Multiplex DNA Master 07 339 585 001	
Water, PCR-grade	10.6 µl		5.0 µl		10.9 µl	5.45 µl	11.0 µl	5.5 µl
Amplification Master	2.0 µl		10.0 µl		4.0 µl	2.0 µl	4.0 µl	2.0 µl
RT Enzyme solution	----		----		0.1 µl	0.05 µl	----	----
$MgCl_2$ solution 25 mM	2.4 µl		----		----	----	----	----
Fluorophore (contains DNA)	5.0 µl		5.0 µl		5.0 µl	2.5 µl	5.0 µl	2.5 µl
Final volume	20.0 µl		20.0 µl		20.0 µl	10.0 µl	20.0 µl	10.0 µl

$ For use with 384 well plates prepare 10 µl reactions.

When using six dyes plus water prepare total 22 (7x3 +1 excess) reactions, if using all12 dyes prepare 40 reactions:

Components for 20 µl reactions	FastStart DNA Master 03 003 248 001		LC480 Probes Master 04 707 494 001		Multiplex RNA Virus Master 06 754 155 001		Multiplex DNA Master 07 339 585 001	
Number of 20 µl reactions	**22 rxns**	**40 rxns**	**22 rxns**	**40 rxns**	**22 rxns**	**40 rxns**	**22 rxns**	**40 rxns**
Water, PCR-grade	233.2 µl	424.0 µl	110.0 µl	200.0 µl	239.8 µl	436.0 µl	241.0 µl	440.0 µl
Amplification Master	44.0 µl	80.0 µl	220.0 µl	400.0 µl	88.0 µl	160.0 µl	88.0 µl	160.0 µl
RT Enzyme solution	----	----	----	----	2.2 µl	4.0 µl	----	----
$MgCl_2$ solution 25 mM	52.8 µl	96.0 µl	----	----	----	----	----	----
Final volume	**330.0 µl**	**600.0 µl**	**330.0 µl**	**600.0 µl**	**330.0 µl**	**600.0 µl**	**330.0 µl**	**600.0 µl**

Mix gently and spin down. Transfer 15 µl of the reaction mix per well.

Components for 10 µl reactions $	FastStart DNA Master 03 003 248 001		LC480 Probes Master 04 707 494 001		Multiplex RNA Virus Master 06 754 155 001		Multiplex DNA Master 07 339 585 001	
Number of 20 µl reactions	**22 rxns**	**40 rxn**	**22 rxns**	**40 rxn**	**22 rxns**	**40 rxn**	**22 rxns**	**40 rxn**
Water, PCR-grade	116.6 µl	212.0 µl	55.0 µl	100.0 µl	119.9µl	218.0 µl	120.5 µl	220.0 µl
Amplification Master	22.0 µl	40.0 µl	110.0 µl	200.0 µl	44.0 µl	80.0 µl	44.0 µl	80.0 µl
RT Enzyme solution	----	----	----	----	1.1 µl	2.0 µl	----	----
$MgCl_2$ solution 25 mM	26.4 µl	48.0 µl	----	----	----	----	----	----
Final volume	**165.0 µl**	**300.0 µl**	**165.0 µl**	**300.0 µl**	**165.0 µl**	**300.0 µl**	**165.0 µl**	**300.0 µl**

Mix gently and spin down. Transfer 7.5 µl of the reaction mix per well

8. Pipetting the Plate

8.1 Use six reference dyes plus water.

Transfer 15 µl (384well 7.5 µl) of the reaction mix per well and add 5 µl (2.5) µl fluorophore:

Well position	Tube Name	Cap color	LC480	LC480 II	cobas z 480
A4, A5, A6	**H_2O**		**5.0 µl**	**5.0 µl**	**5.0 µl**
B4, B5, B6	**700 N**	**Salmon**	**----**	**----**	**5.0 µl**
B4, B5, B6	**500 N or S**	**Orange**	**5.0 µl**	**5.0 µl**	**----**
C4, C5, C6	**530 N**	**Yellow**	**5.0 µl**	**5.0 µl**	**5.0 µl**
D4, D5, D6	**580 N or S**	**Red**	**5.0 µl**	**5.0 µl**	**5.0 µl**
E4, E5, E6	**610 N or S**	**Lilac**	**5.0 µl**	**5.0 µl**	**5.0 µl**
F4, F5, F6	**640 N or S**	**Blue**	**5.0 µl**	**5.0 µl**	**5.0 µl**
G4, G5, G6	**660 N**	**Green**	**5.0 µl**	**5.0 µl**	**5.0 µl**

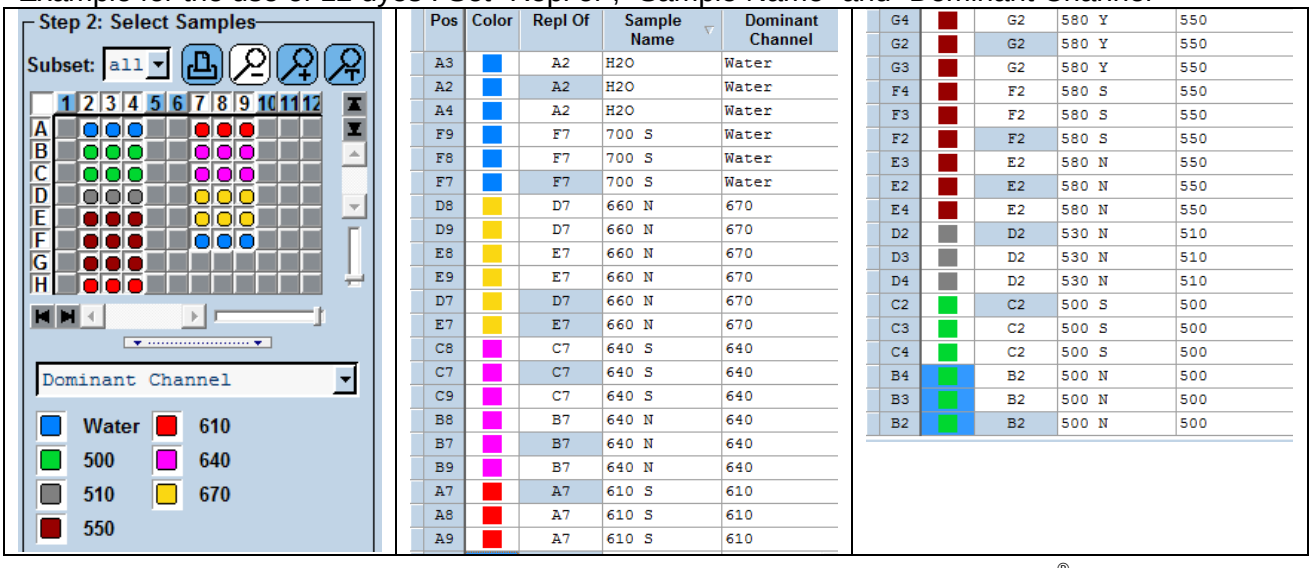
Note: The complete reaction used for the Color Compensation contains polymerase and cannot be stored.

8.2 Use twelve reference dyes plus water.

Use a maximum of twelve dyes with LC480 and cobas z480 systems (one plate can be applied to multiple instruments) and select the required dye combinations in distinct subsets.

Well position	Tube Name	Cap color	Twelve	LC480 II	cobas z 480
A2, A3, A4	**H_2O**		**5.0 µl**	**5.0 µl**	**5.0 µl**
B2, B3, B4	**500 N**	**Orange**	**5.0 µl**	**5.0 µl**	**----**
C2, C3, C4	**500 S**	**Orange**	**5.0 µl**	**5.0 µl**	**----**
D2, D3, D4	**530 N**	**Yellow**	**5.0 µl**	**5.0 µl**	**5.0 µl**
E2, E3, E4	**580 N**	**Red**	**5.0 µl**	**5.0 µl**	**5.0 µl**
F2, F3, F4	**580 S**	**Red**	**5.0 µl**	**5.0 µl**	**5.0 µl**
G2, G3, G4	**580 Y**	**Black**	**5.0 µl**	**5.0 µl**	**5.0 µl**
H2, H3, H4	**610 N**	**Lilac**	**5.0 µl**	**5.0 µl**	**5.0 µl**
A7, A8, A9	**610 S**	**Lilac**	**5.0 µl**	**5.0 µl**	**5.0 µl**
B7, B8, B9	**640 N**	**Blue**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C7, C8, C9	**640 S**	**Blue**	**5.0 µl**	**5.0 µl**	**5.0 µl**
D7, D8, D9	**660 N**	**Green**	**5.0 µl**	**5.0 µl**	**5.0 µl**
F7, F8, F9	**700 N**	**Salmon**	**5.0 µl**	**----**	**5.0 µl**

Example for the use of 12 dyes : Set “Repl of”, “Sample Name” and “Dominant Channel”



Pos	Color	Repl Of	Sample Name	Dominant Channel
A3		A2	H2O	Water
A2		A2	H2O	Water
A4		A2	H2O	Water
F9		F7	700 S	Water
F8		F7	700 S	Water
F7		F7	700 S	Water
D8		D7	660 N	670
D9		D7	660 N	670
E8		E7	660 N	670
E9		E7	660 N	670
D7		D7	660 N	670
E7		E7	660 N	670
C8		C7	640 S	640
C7		C7	640 S	640
C9		C7	640 S	640
B8		B7	640 N	640
B7		B7	640 N	640
B9		B7	640 N	640
A7		A7	610 S	610
A8		A7	610 S	610
A9		A7	610 S	610

G4	G2	580 Y	550	
G2	G2	580 Y	550	
G3	G2	580 Y	550	
F4	F2	580 S	550	
F3	F2	580 S	550	
F2	F2	580 S	550	
E3	E2	580 N	550	
E2	E2	580 N	550	
E4	E2	580 N	550	
D2	D2	530 N	510	
D3	D2	530 N	510	
D4	D2	530 N	510	
C2	C2	500 S	500	
C3	C2	500 S	500	
C4	C2	500 S	500	
B4	B2	500 N	500	
B3	B2	500 N	500	
B2	B2	500 N	500	

Sample Editor (**left**): The colors for the instrument channels are assigned by the LightCycler® software and do not correlate with the dye appearance nor with the label of the reagents!

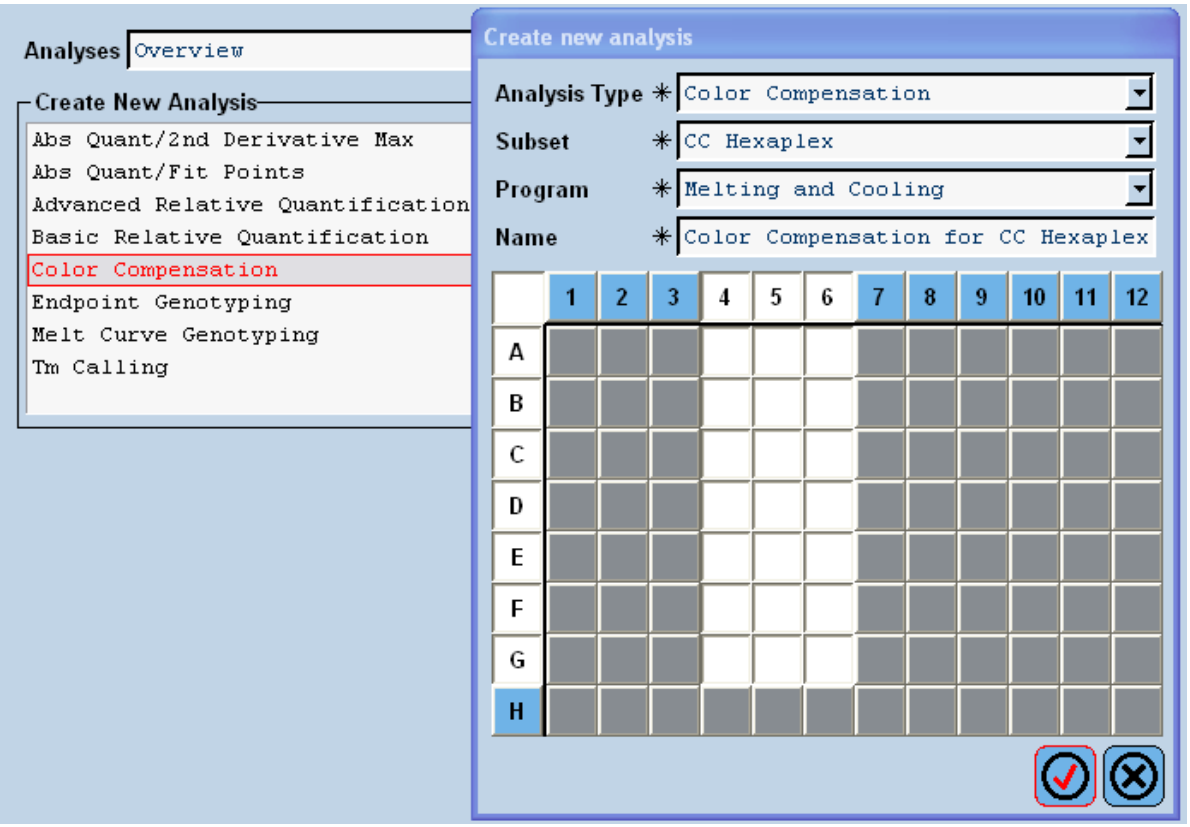
9. Start Run.

Seal the plate.
Centrifuge the plate.
Start run.

Save run as: **Hexaplex YYYY-MM-DD** (e.g., “Hexaplex 2019-08-08”).

10. Define Subsets for the Analysis

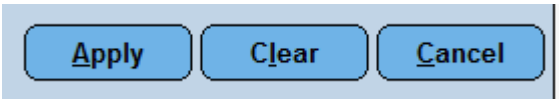
Create a New Analysis, select 'Color Compensation', select defined subset e.g. Hexaplex CC
If using six dyes the subset will be identical with the positions of the reactions (see 8.1):



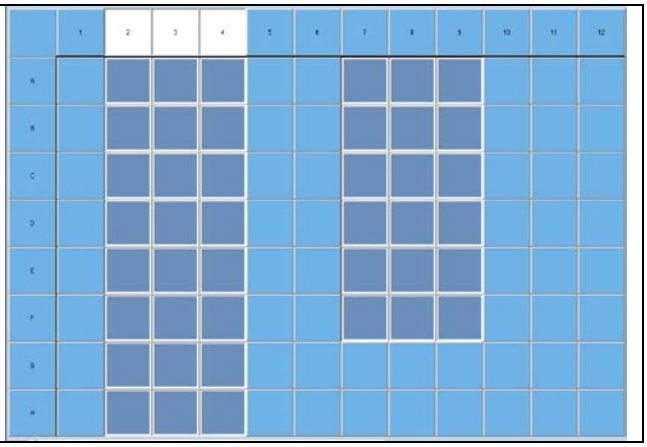
If using optional fluorophores, you need to create **one subset per dye combination**.
Go back into subsets and open additional subsets - examples:

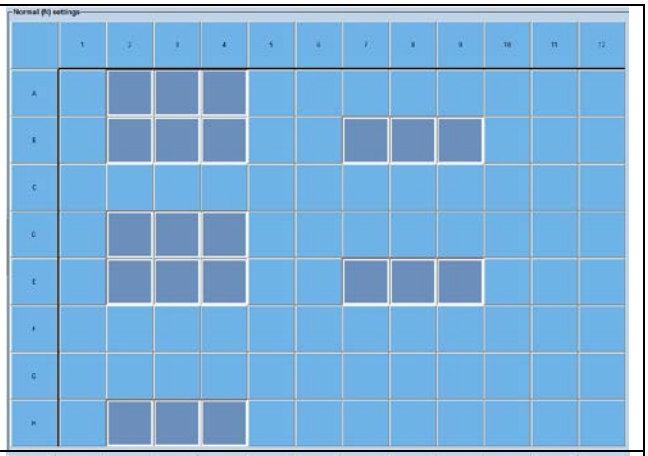
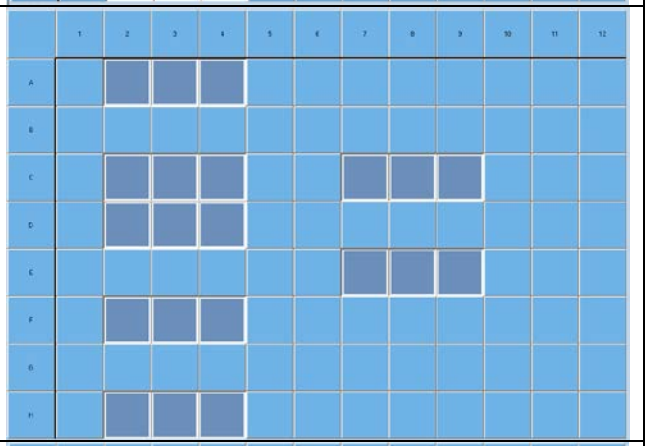
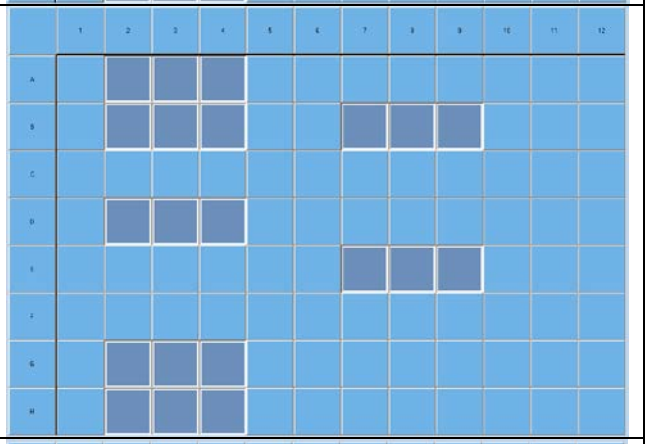
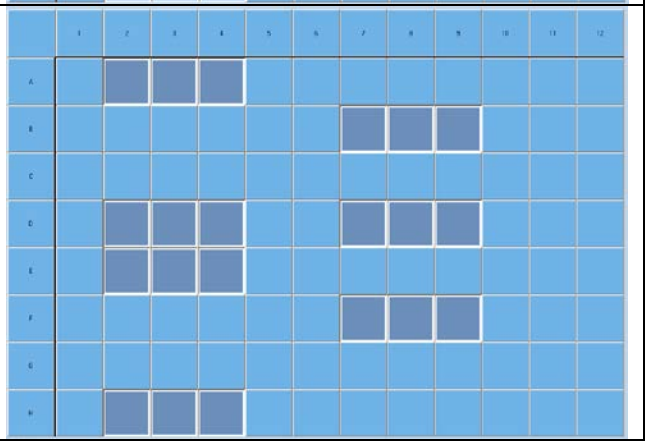
All wells selected (not useful for CC)

Use this subset for copying and remove dyes from the set and save by pressing button Apply:



See examples next page.

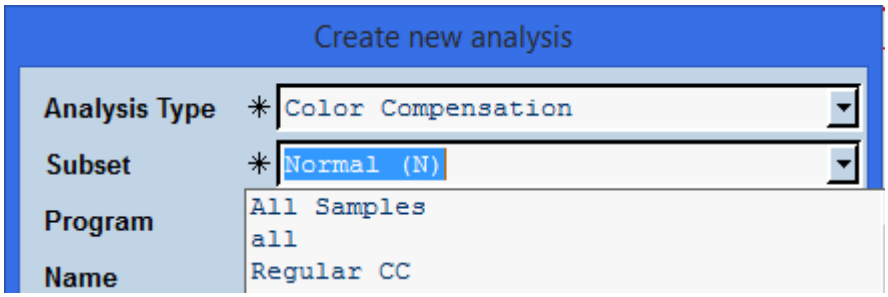


Normal dyes (positions see table 8.2): Water 500 N 530 N 580 N 610 N 640 N 660 N (500S, 580S, 580Y, 610S, 640S, 700N removed)	
Special dyes: Water 500 S 530 S 580 S 610 S 640 S 660 S (500N, 580N, 580Y, 610N, 640N, 700N removed)	
Normal dyes but channel 580 Yakima: Water 500 N 530 N 580 Y 610 N 640 N 660 N (500S, 580S, 580N, 610S, 640S, 700N removed)	
cobas z 480 Water 530 N 580 N 610 N 640 N 660 N 700 N (500N, 500S, 580S, 580Y, 610S, 640S, removed)	

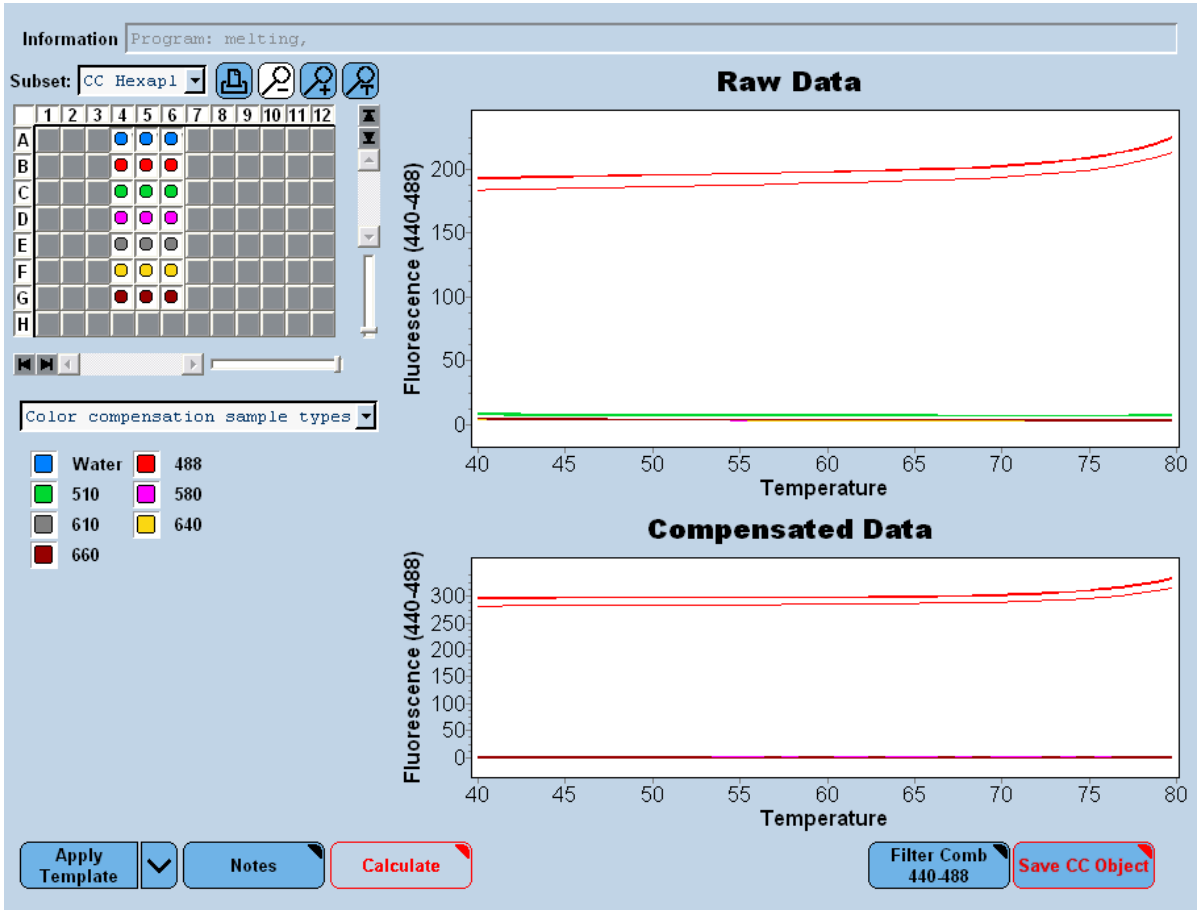
11. Create the Color Compensation File

Left side of the screen Open **Analysis** and press the **+** button:

Chose Color Compensation and the appropriate subset (chapter 10) :



Press the button **Calculate**

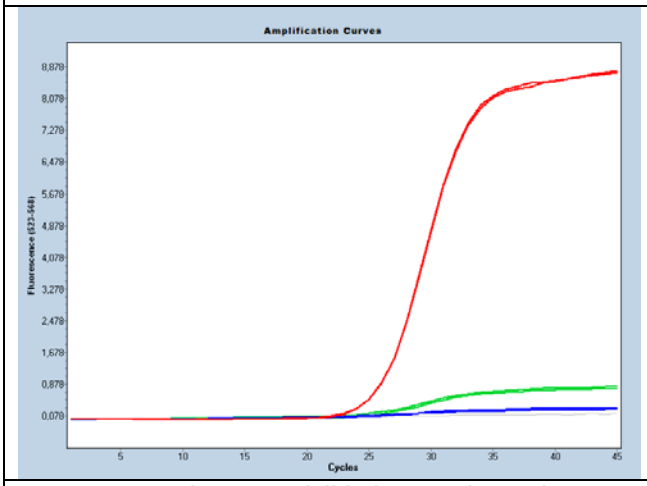
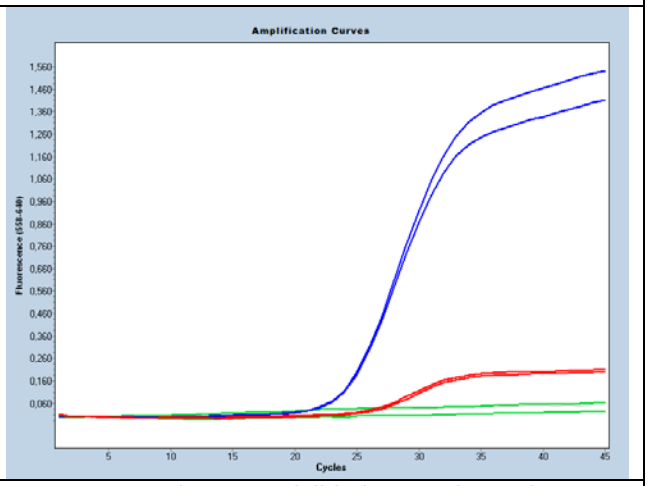
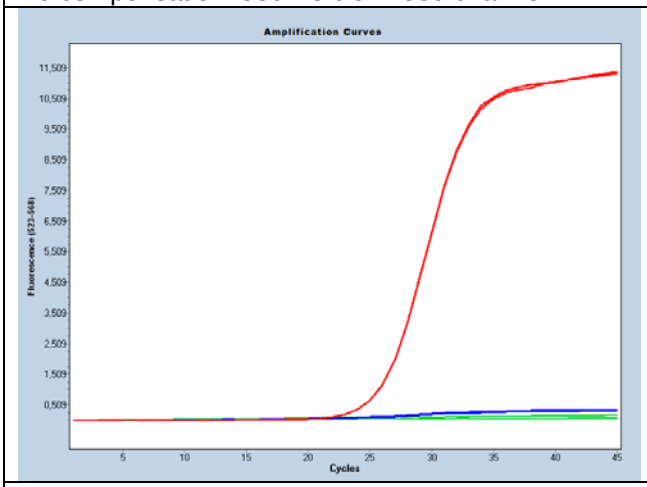
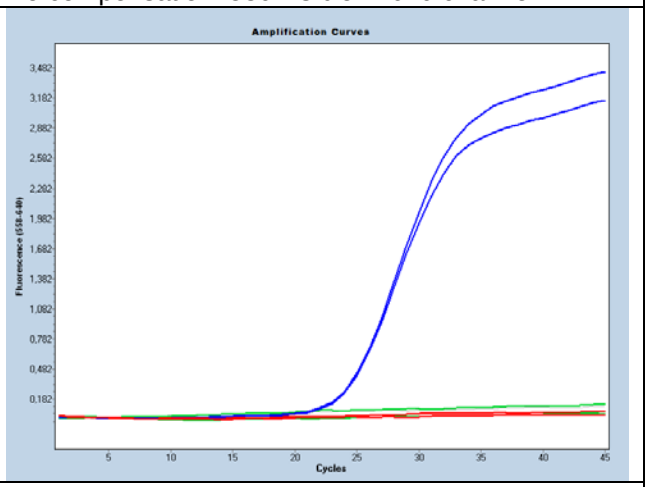
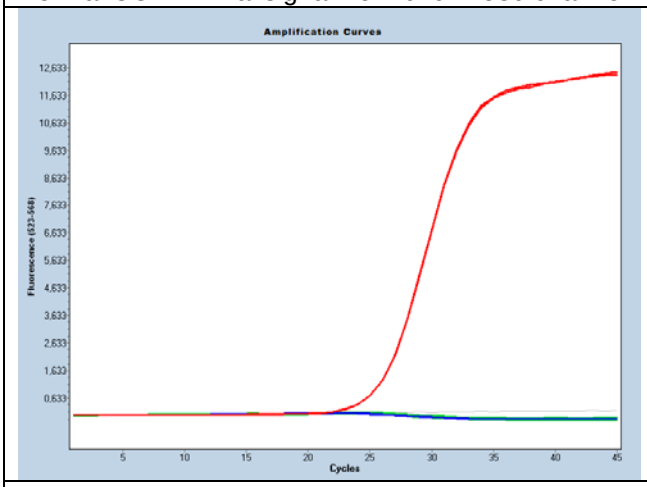
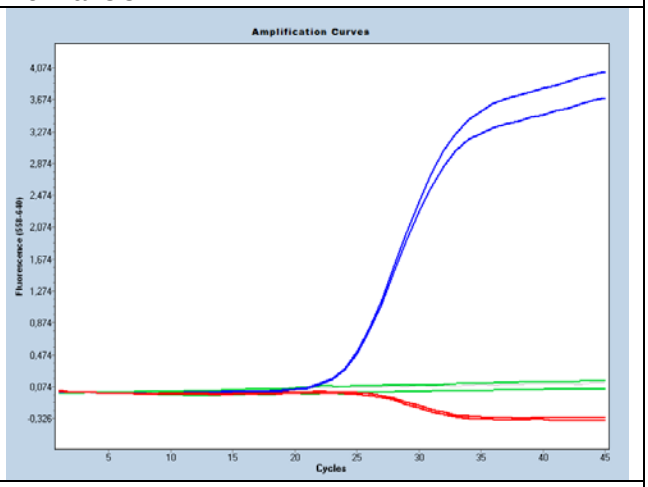


and Save CC Object as: **Hexaplex (N) CC YYYY-MM-DD** (e.g., “Hexaplex (N) CC 2016-05-08”).

Create one Color Compensation File per dye combination (per subset).

NB: If the same plate will be used to generate colour compensation files for multiple instruments, ensure the plate seal is intact prior to re-use.

12. Color Compensation Function and Sample Data

580 Channel	640 Channel
	
No compensation. 530 visible in 580 channel	No compensation. 580 visible in 640 channel
	
Normal CC: Minimal signal from 640 in 580 channel	Normal CC
	
Special CC: Negative signals for neighboring dyes	Special CC: Negative signals for neighboring dyes
Red VZV (580), Green HSV-1 (530), Blue HSV-2 (640)	

13. 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 Material Safety Data Sheet (MSDS).

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

14. Version History

Notes in red mark events require to change procedures

V120218	Release Version
V121221	Editorial changes
V130220	Revised version / new editorial format
V130409	Corrected annealing time for LC480
V130724	Procedure for LC480 Probes Master included
V140212	Correction filter settings table / detection format for 480II Procedure for LC Multiplex RNA Virus Master included
V141206	Section 7 Experimental Protocol PCR programming changed Color coding adapted to ModularDx kit colors
V151215	Section 6: “Max Integration Time” revised Amplification protocol harmonized with modular kits
V160606	SAP N° corrected, Editorial changes; Storage condition
V170404	Note: No reagent with 705 (Cy5.5) label contained.
V190123	Primer/probe/target systems changed from parasite genome targets to a plasmid target to reduce the risk of laboratory contaminations. Color Compensation reagent for channel 700 (cobas z 480) added. Alternative Color Compensation reagents for reduced crosstalk included. Order no. 40-0320-12
V190808	Renaming of reference dyes to N (normal) and S (special)

Roche SAP No. 06296971001

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Notice to Purchaser

The purchase of this product does not convey any right for its use in clinical diagnostic applications.
These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.