LightMix® Universal Color Compensation Hexaplex Plus
Working instructions for use with LightMix® Modular kits

Cat.-No. 40-0320-12 Roche 06 296 971 001

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 or instruments must be verified by the user.

Index

1. Contents, Selection of Calibrator Dye and Storage

12 vials containing primer, dark quenched Hydrolysis Probe and DNA target for 15 reactions each. The vials labelled with an **S** are “special” reagents that can be substituted in place of an N reagent, where the assay or instrument shows residual crosstalk even though a cc file has been applied. See chapter 12 for examples.

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**	Rhodamin 6G	**N**ormal reagent for HEX, JOE, R6G (and VIC) assays.
Red	**580 S**	Rhodamin 6G	**S**pecial reagent. Use if crosstalk from channels 610/640 is seen.
Black	**580 Y**	Yakima Yellow	**S**pecial reagent for YAK assays.
Purple	**610 N**	LightCycler® Red 610	**N**ormal reagent for ROX, Texas Red and LC610 assays.
Purple	**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, or long term frozen (within expiry of the kit). Do not combine / mix the reagents.

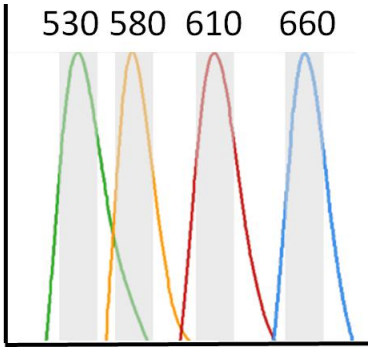
2. Additional Reagents and Materials Required:

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
or 1step RT Polymerase	TIB 60-9999-96
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 polymerase has not been tested but is expected to work in the same way. Mastermix must not contain fluorescent dyes (eg. 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 480 instruments use a combination of excitation and emission light filters to collect the fluorescence light mainly from one specific dye. Since the emission spectra overlap with the adjacent channels the instrument collects some signals from the neighbored dye.



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

To create a Color Compensation file the instrument is calibrated using wells containing pure dye Real-Time-PCR assays, reading the fluorescence in the affiliated and neighboring channels. The Roche Color Compensation is based on fluorescence data recorded in the melting step. A fresh made amplification plate may be used for the calibration of further 480 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	680-700
LightCycler® 2.0	•	530	560	•	•	•	•

The generated Color Compensation (CC) 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 might 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 (no significant) signals in the neighboring channels recorded.

The dye signals can be shifted dependent on a particular probe sequence and cause failure of the Color Compensation, generating very low signals in the neighbor channel which might be called by the instrument software as (false) positive result. The novel special reagents force the Color Compensation algorithm to make a subtle overcompensation visible as negative signals; negative curves will be not called positive. Negative signals will be subtracted from the positive signals in this same channel; so long the negative signals are less than approx 5% of the expected positive signals there is no change of calling of positive results to be expected, but there could be a shift of Cp values resulting in a change of quantity results. Use of the special reagent has to be tested before using for quantitative multiplex PCR assays.

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”. • Roche LightCycler® 2.0 settings cover two channels only. However, the 610 channel can be utilized for control reactions. Contact design@tib-molbiol.de for instructions.

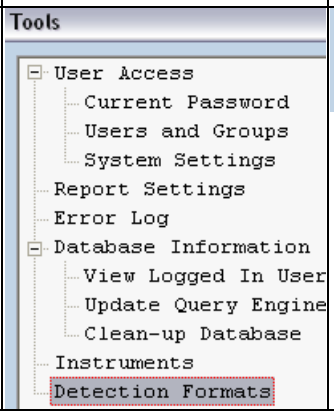
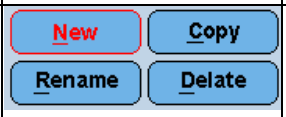
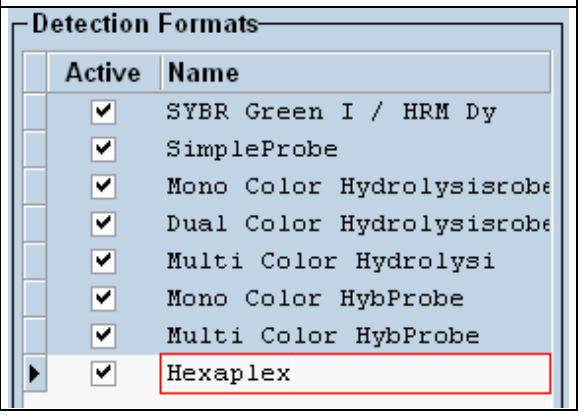
For dual color assays with LightCycler® 1.5 systems contact design@tib-molbiol.de for instructions

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 different from the default instrument settings.

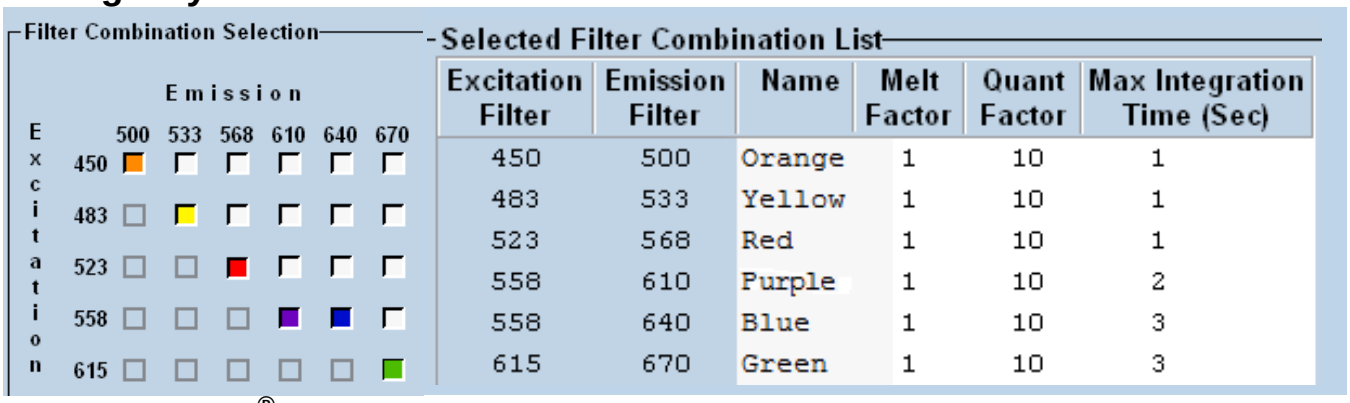
The CC can be included in a sample run (requires creation of a distinct subset for the CC).

4. Define the 'Hexaplex' Detection Format

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

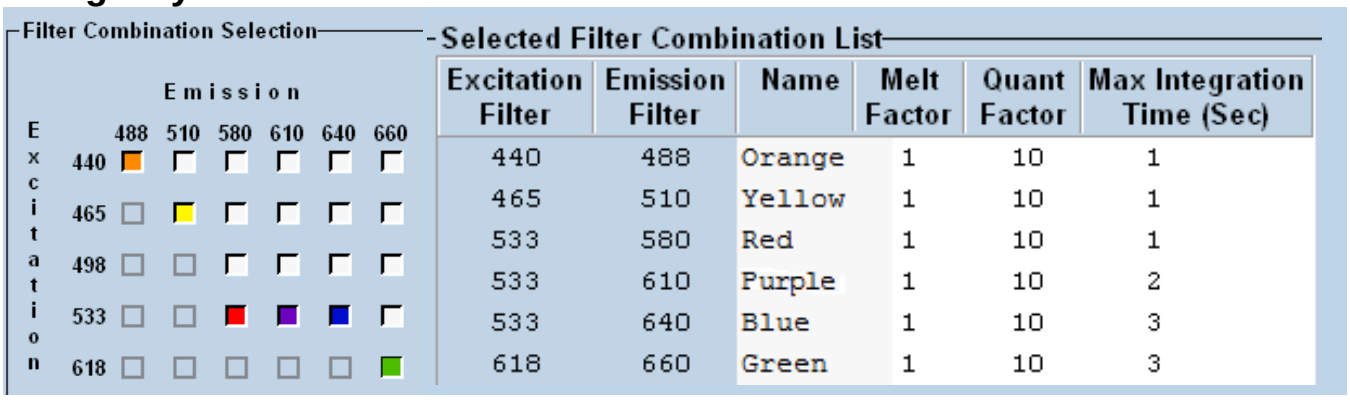
Select Filter Combination, edit Names, Melt Factor, Quant Factor and Max Integration Time :

LightCycler® 480



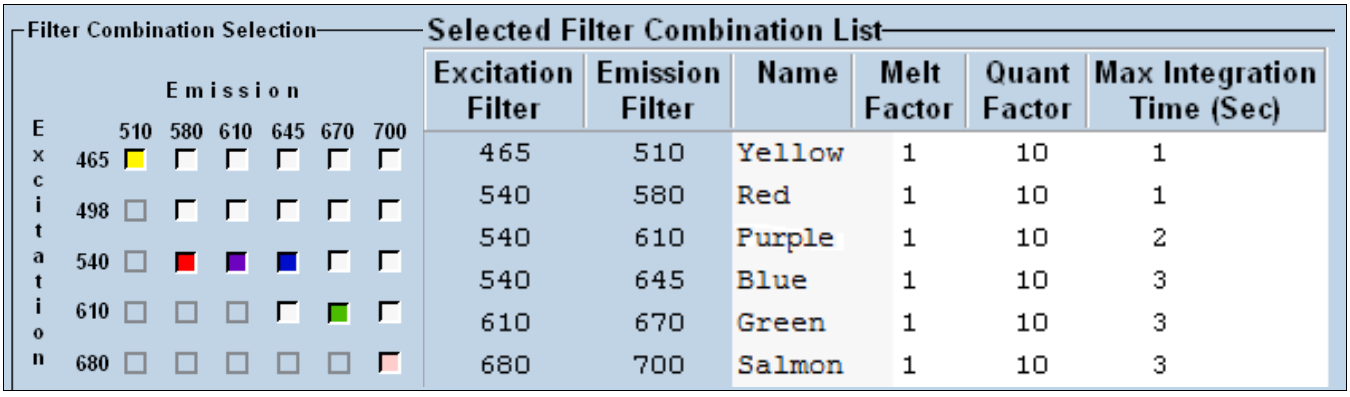
Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
450	500	Orange	1	10	1
483	533	Yellow	1	10	1
523	568	Red	1	10	1
558	610	Purple	1	10	2
558	640	Blue	1	10	3
615	670	Green	1	10	3

LightCycler® 480 II



Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
440	488	Orange	1	10	1
465	510	Yellow	1	10	1
533	580	Red	1	10	1
533	610	Purple	1	10	2
533	640	Blue	1	10	3
618	660	Green	1	10	3

cobas z 480



Excitation Filter	Emission Filter	Name	Melt Factor	Quant Factor	Max Integration Time (Sec)
465	510	Yellow	1	10	1
540	580	Red	1	10	1
540	610	Purple	1	10	2
540	645	Blue	1	10	3
610	670	Green	1	10	3
680	700	Salmon	1	10	3

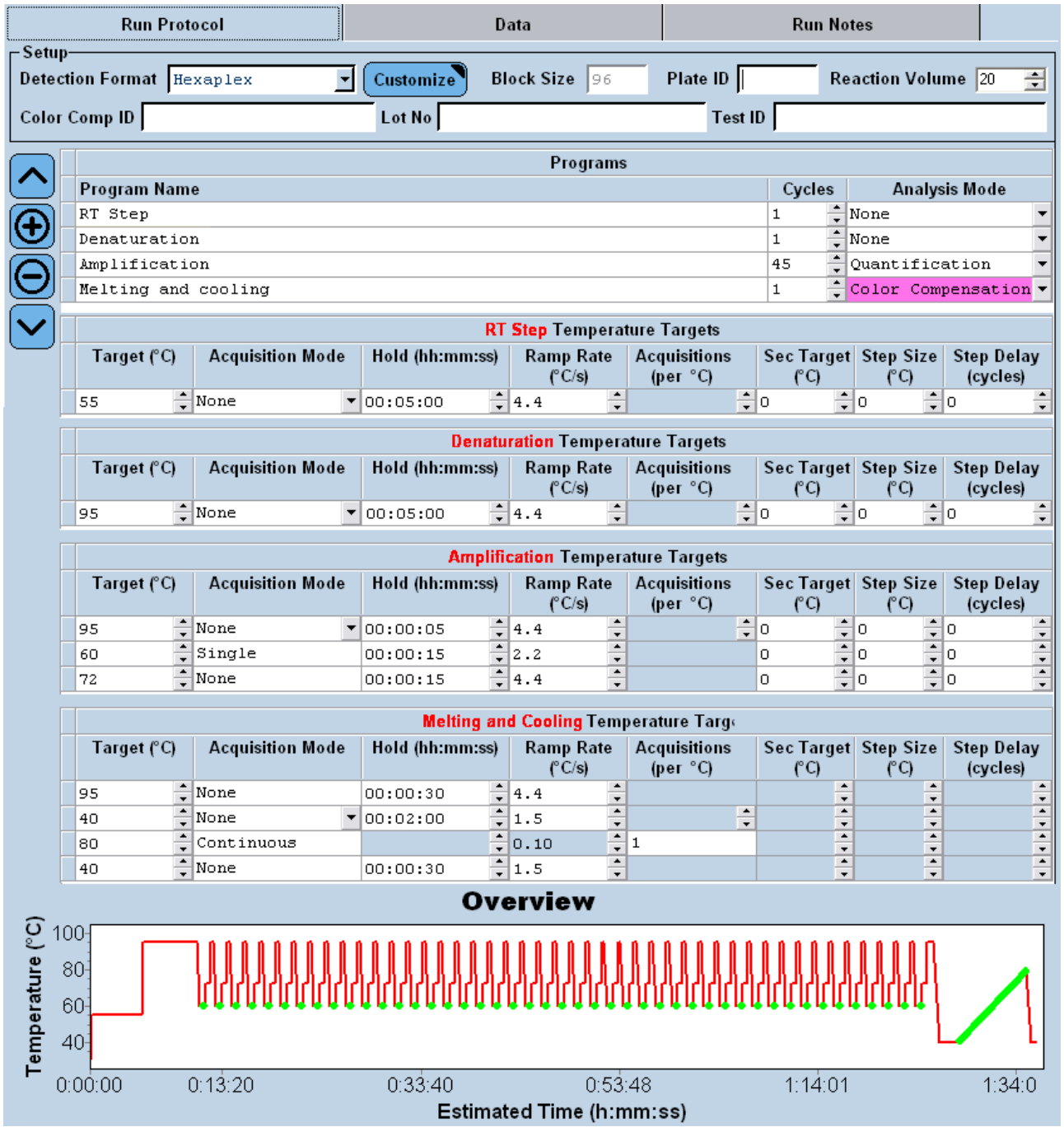
5. Instrument Programming

Color Compensation for Hydrolysis (TaqMan) Probes requires performance of a PCR experiment with single dye assays and water as 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.



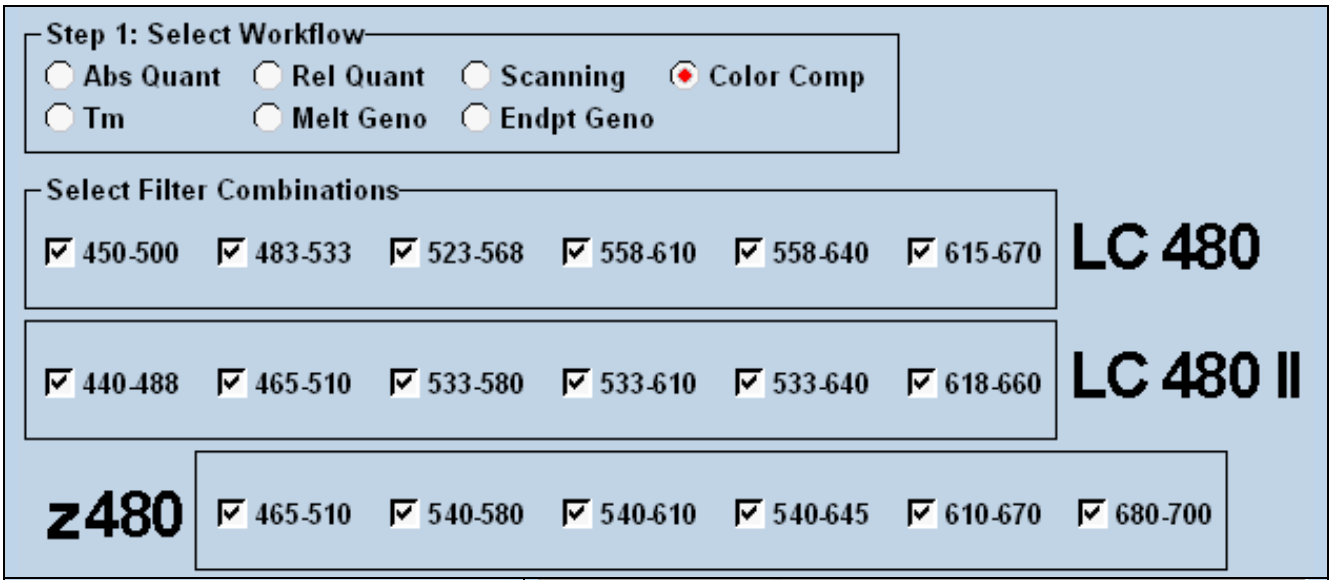
6. Sample Editor - Define the Dominant Channel

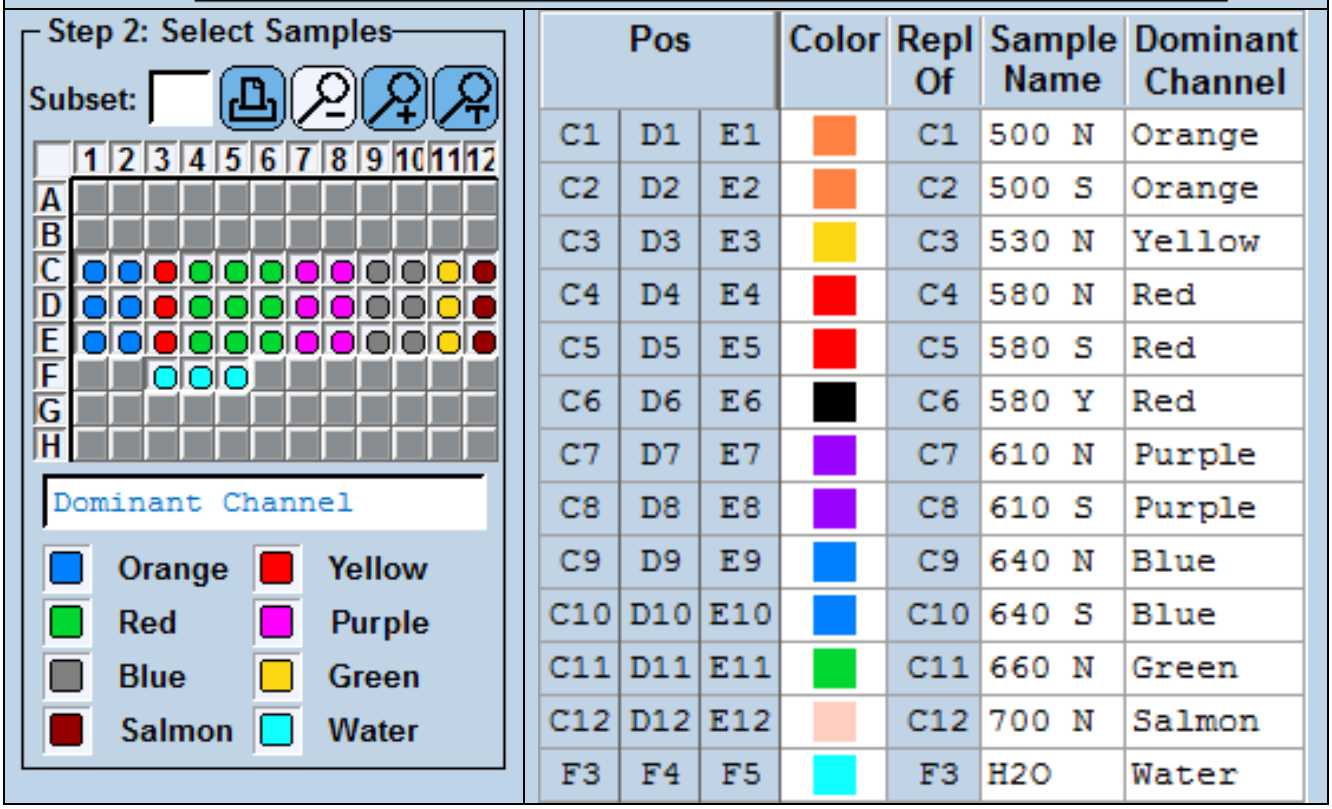
Select the Workflow **'Color Comp'**, then the filter combinations, and then the Dominant Channels.

Step 1 Channel setting for LightCycler® 480, LightCycler® 480 II or cobas z 480 analyzer.

Step 2 **Left:** Reference dyes have been placed on row C, D, E and H2O in row F.
Note: Colors in the subset are set automatically and can be not chosen !

Step 3 **Right:** Set “Repl of”, “Sample Name” and “Dominant Channel”.
Note: Colors have been modified to increase readability, but are not relevant for the function of the software ! See the LightCycler® operator’s manual of the specific LightCycler® Instrument for details.





Pos			Color	Repl Of	Sample Name	Dominant Channel
C1	D1	E1		C1	500 N	Orange
C2	D2	E2		C2	500 S	Orange
C3	D3	E3		C3	530 N	Yellow
C4	D4	E4		C4	580 N	Red
C5	D5	E5		C5	580 S	Red
C6	D6	E6		C6	580 Y	Red
C7	D7	E7		C7	610 N	Purple
C8	D8	E8		C8	610 S	Purple
C9	D9	E9		C9	640 N	Blue
C10	D10	E10		C10	640 S	Blue
C11	D11	E11		C11	660 N	Green
C12	D12	E12		C12	700 N	Salmon
F3	F4	F5		F3	H2O	Water

Left:
The default color code for the 'Dominant Channel' is blue for 500, red for 530, green for 580, pink for 610, grey for 640, green for 660, brown for 700 and light blue for water.

Right:
Color code inserted manually according to the cap color of the reagents.

7. Reagent Preparation

We recommend to include all dyes to allow to generate different color compensation files defined by different subsets (see chapter 9). The '500' reagent can be omitted if using only z 480 analyzer; the '700' reagent can be omitted for use with LightCycler® 480 instruments.

Spin down the reagent tubes and check to contain a colored pellet. Reconstitute the pellets in each 75 µl water. Vortex and allow to dissolve for 5 minutes. Vortex again and spin down.

Do not mix dye reagents. Do not transfer into self-labeled tubes. Store frozen.

Note: Intended Use is single use. No in-use / on board stability studies performed. However, from exisiting data frozen product it is expected to be stable until end of the stated expiry (printed on the outer label).

Plate based instruments require a melting curve. Each measurement is done in triplicate (3 wells). Read the instrument manual for details on the generation of a CC file.

7.1 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	5.3 µl	5.0 µl	2.5 µl	10.9 µl	5.45 µl	11.0 µl	5.5 µl
Amplification Master	2.0 µl	1.0 µl	10.0 µl	5.0 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	1.2 µl	----	----	----	----	----	----
Fluorophore (contains DNA)	5.0 µl	2.5 µl	5.0 µl	2.5 µl	5.0 µl	2.5 µl	5.0 µl	2.5 µl
Final volume	20.0 µl	10.0 µl	20.0 µl	10.0 µl	20.0 µl	10.0 µl	20.0 µl	10.0 µl

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

7.2 Reagents needed for one experiment (96well plate) :

Prepare for 37 [(11 tubes+ H_2O) x 3 +1 excess] reactions using LightCycler® 480 instruments
Prepare for 34 [(10 tubes+ H_2O) x 3 +1 excess] reactions using cobas z 480 analyzer

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	**37 rxns**	**34 rxn**	**37 rxns**	**34 rxn**	**37 rxns**	**34 rxn**	**37 rxns**	**34 rxn**
Water, PCR-grade	392.2 µl	360.4 µl	185.0 µl	170.0 µl	403.3 µl	370.6 µl	407.0 µl	374.0 µl
Amplification Master	74.0 µl	68.0 µl	370.0 µl	340.0 µl	148.0 µl	136.0 µl	148.0 µl	136.0 µl
RT Enzyme solution	----	----	----	----	3.7 µl	3.4 µl	----	----
$MgCl_2$ solution 25 mM	88.8 µl	81.6 µl	----	----	----	----	----	----
Final volume	555.0 µl	510.0 µl	555.0 µl	510.0 µl	555.0 µl	510.0 µl	555.0 µl	510.0 µl

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

7.3 Reagents needed for one 10 µl experiment (384well plate) :

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 10 µl reactions	**37 rxns**	**34 rxn**	**37 rxns**	**34 rxn**	**37 rxns**	**34 rxn**	**37 rxns**	**34 rxn**
Water, PCR-grade	196.1 µl	180.2 µl	92.5 µl	85.0 µl	201.7 µl	185.3 µl	203.5 µl	187.0 µl
Amplification Master	37.0 µl	34.0 µl	185.0 µl	170.0 µl	74.0 µl	68.0 µl	74.0 µl	68.0 µl
RT Enzyme solution	----	----	----	----	1.9 µl	1.7 µl	----	----
$MgCl_2$ solution 25 mM	44.4 µl	40.8 µl	----	----	----	----	----	----
Final volume	277.5 µl	255.0 µl	277.5 µl	255.0 µl	277.5 µl	255.0 µl	277.5 µl	255.0 µl

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

8. Pipetting the Plate

Use eleven different dyes plus water for LightCycler® 480 systems and ten dyes plus water for cobas z 480 analyzer. Define subsets to define specific dye combinations - for example 'Hexaplex Normal' including the dyes 500N + 530N + 580N + 610N + 640N + 660N + water (see chapter 10.).

One plate can be applied to different instruments: If using one plate for LightCycler® 480 and cobas systems then use 12 dyes and water and define subsets for the different instrument types.

Well position	Tube Name	Cap color	LC480	LC480II	z 480
C1; D1; E1	**500 N**	**Orange**	**5.0 µl**	**5.0 µl**	**----**
C2; D2; E2	**500 S**	**Orange**	**5.0 µl**	**5.0 µl**	**----**
C3; D3; E3	**530 N**	**Yellow**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C4; D4; E4	**580 N**	**Red**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C5; D5; E5	**580 S**	**Red**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C6; D6; E6	**580 Y**	**Black**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C7; D7; E7	**610 N**	**Purple**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C8; D8; E8	**610 S**	**Purple**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C9; D9; E9	**640 N**	**Blue**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C10; D10; E10	**640 S**	**Blue**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C11; D11; E11	**660 N**	**Green**	**5.0 µl**	**5.0 µl**	**5.0 µl**
C12; D12; E12	**700 N**	**Salmon**	**----**	**----**	**5.0 µl**
F3; F4; F5	**H_2O**		**5.0 µl**	**5.0 µl**	**5.0 µl**

9. Start Run.

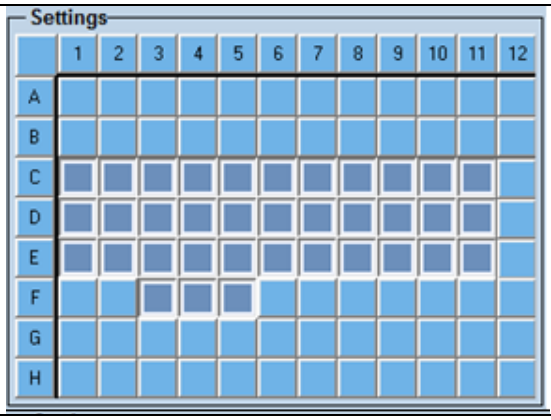
Seal the plate.
Centrifuge the plate.
Start run.

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

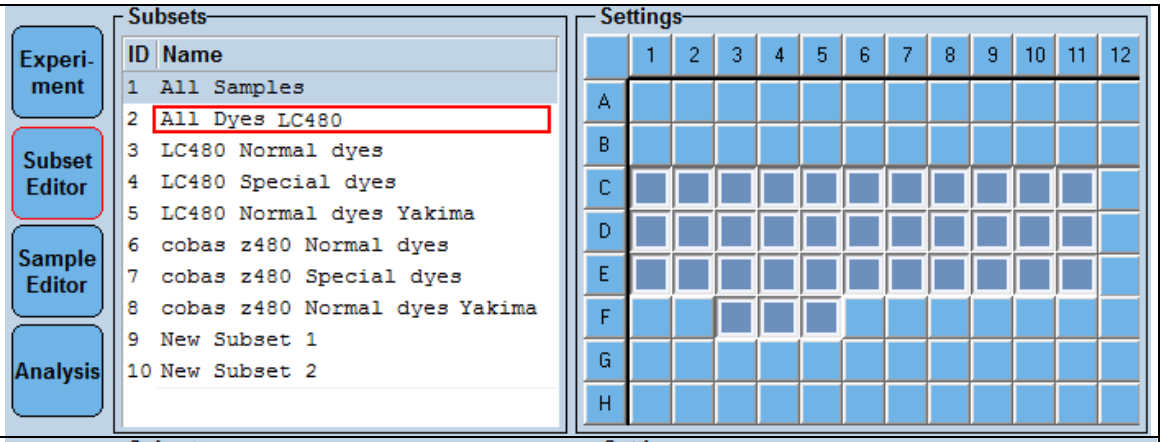
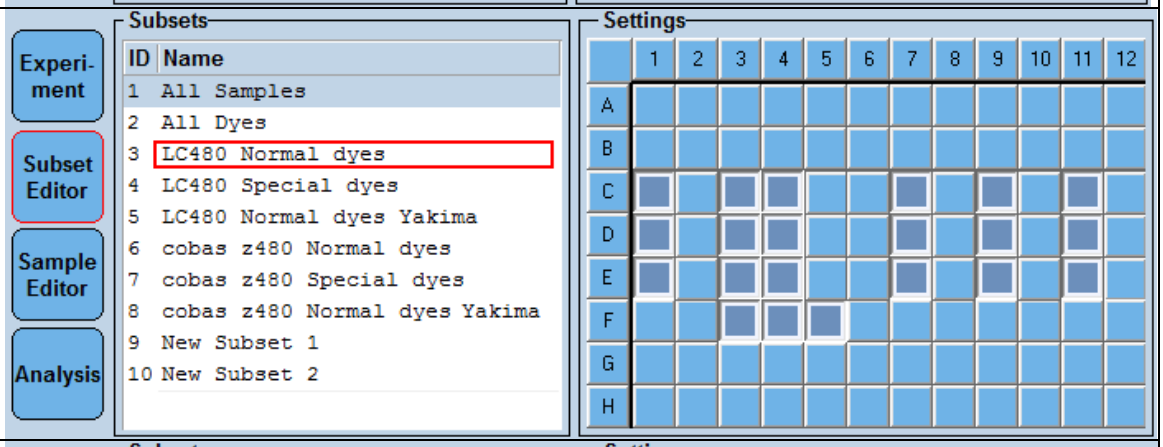
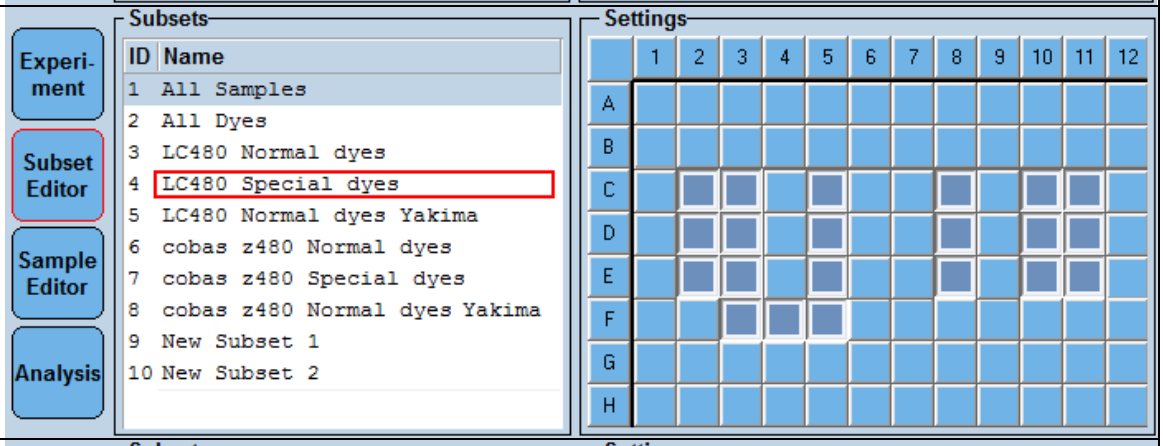
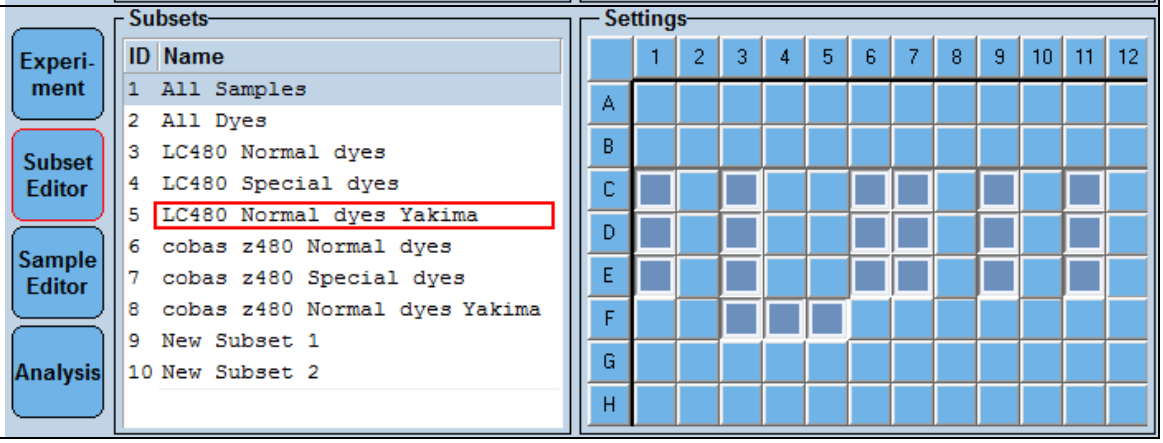
10. Define Subsets for the Analysis

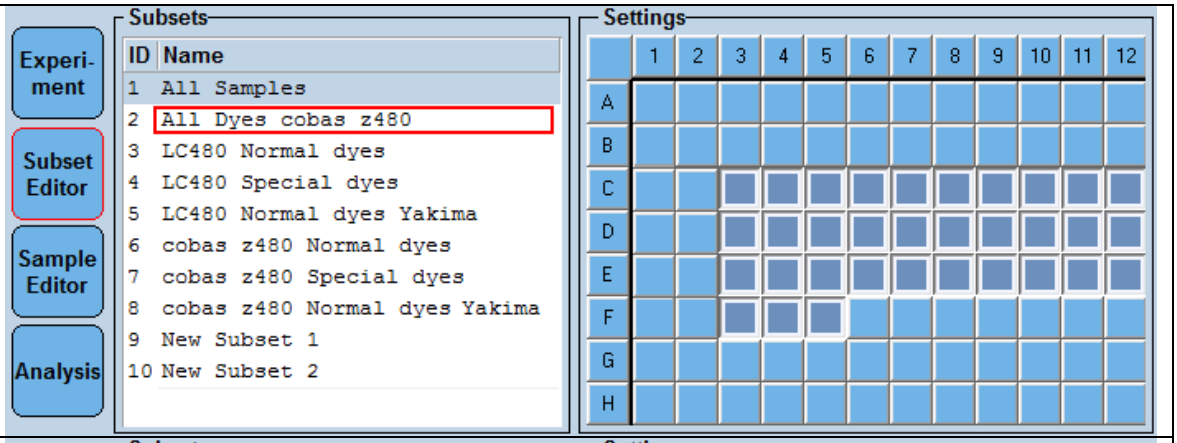
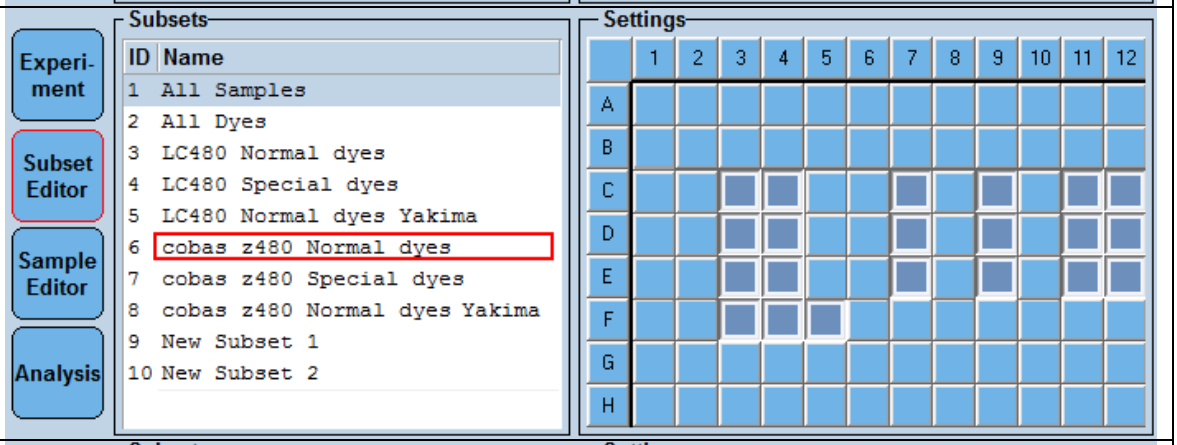
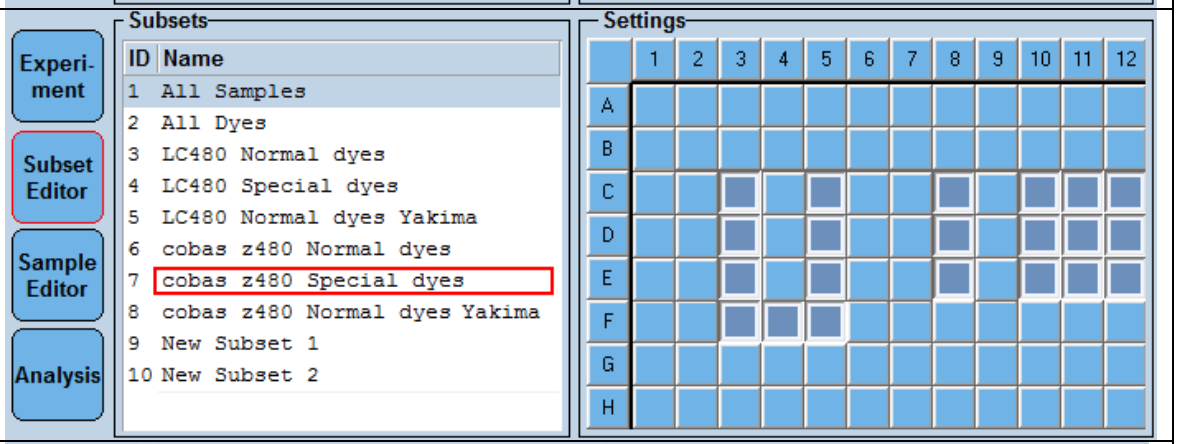
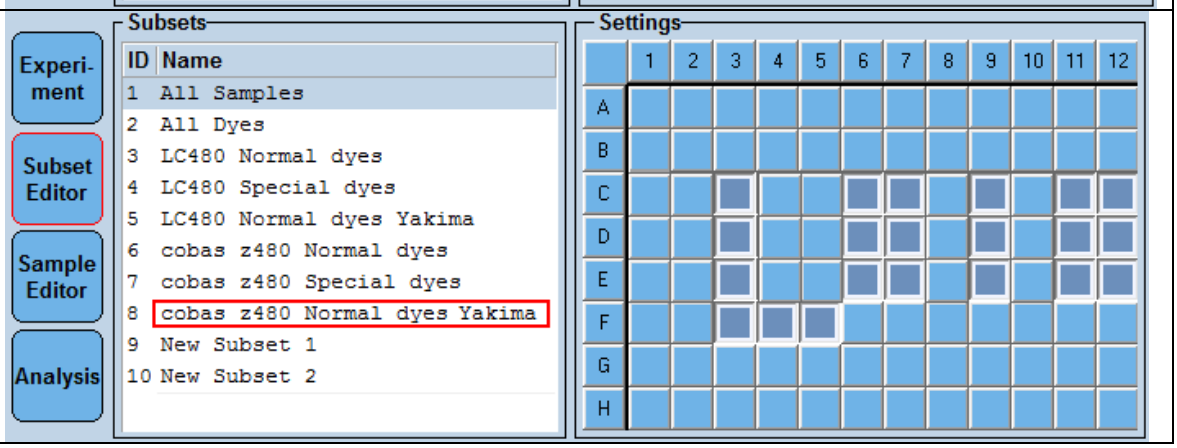
Copy the selected cc subset and remove replicate dyes that shall not be included in the custom cc, save by pressing button Apply and rename:

NB do not include both S and N reactions for the same fluorophore in a single subset.



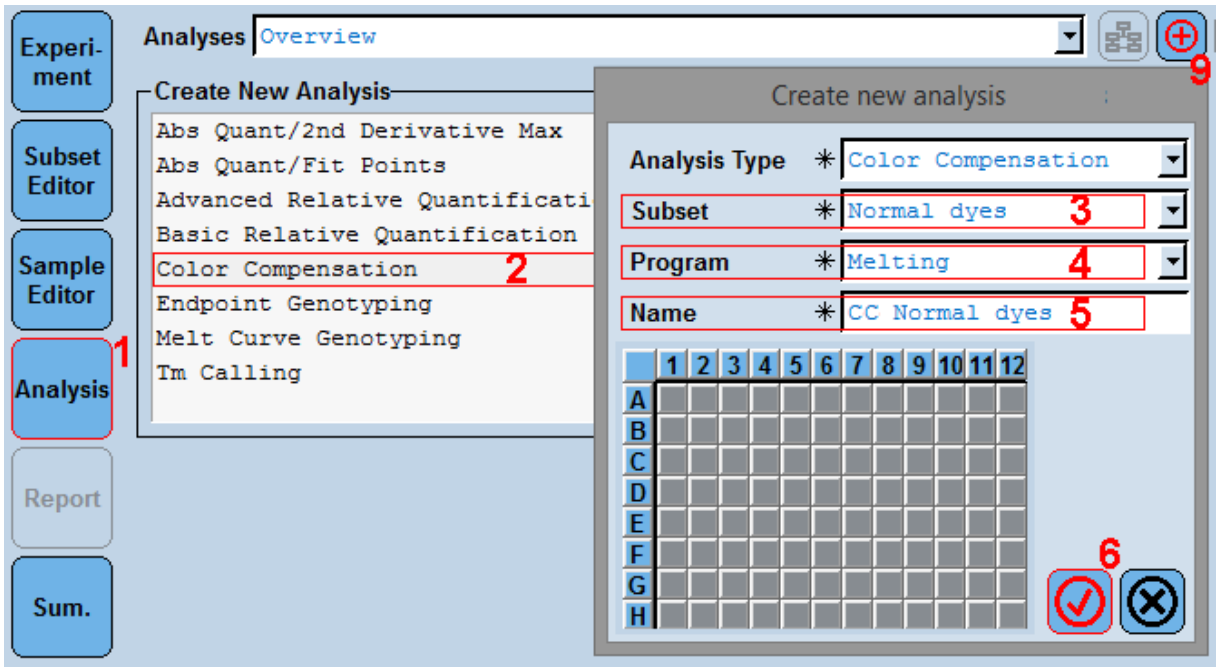
Generate the subset appropriate for the instrument utilized as described:

LC 480 **All Dyes**	
Normal Dyes	
Special Dyes	
Normal Dyes with Yakima	

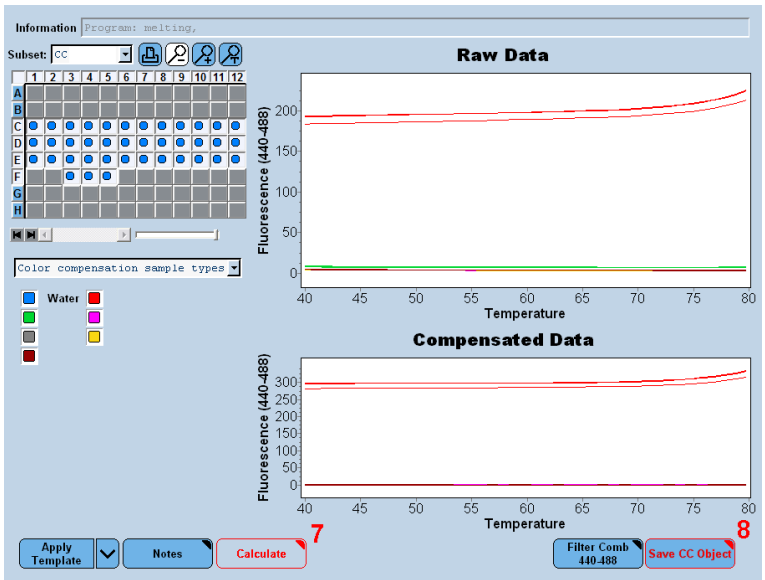
z 480 **All Dyes**	
Normal Dyes	
Special Dyes	
Normal Dyes with Yakima	

11. Create the Color Compensation File - step by step :

1. Open Analysis screen
2. Select Color Compensation
3. Select Subset eg. "**Normal dyes**"
4. Select Program "Melting
5. Name "**CC Normal dyes**"
6. Select **OK**



7. Calculate
8. Save CC Object "**CC Normal dyes YYYY-MM-DD**"

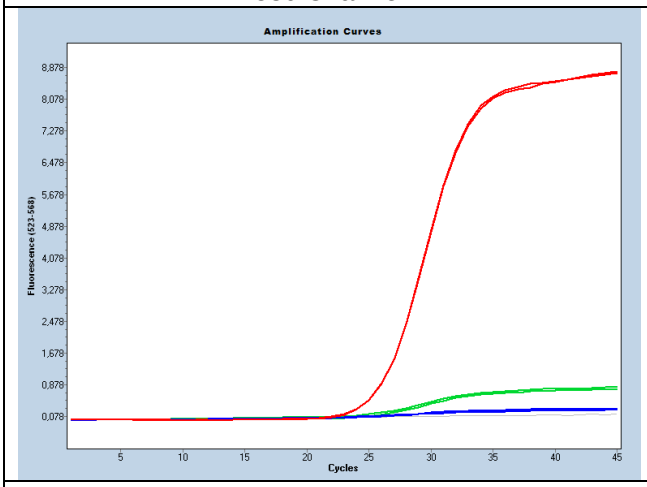
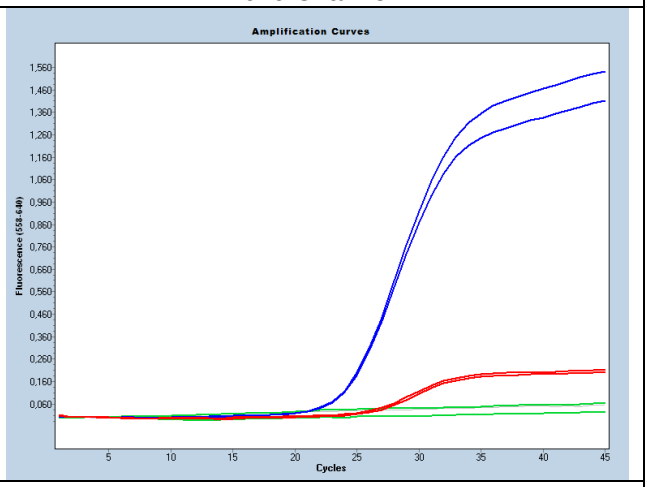
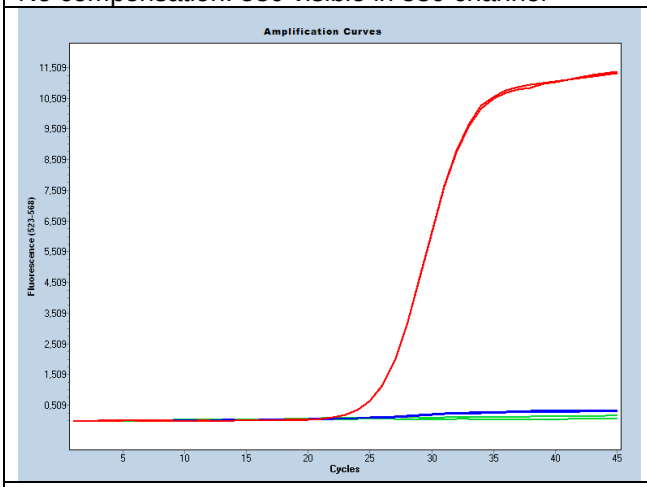
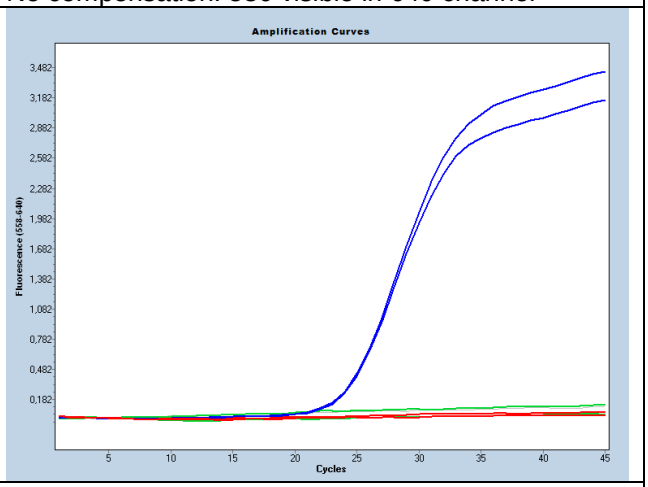
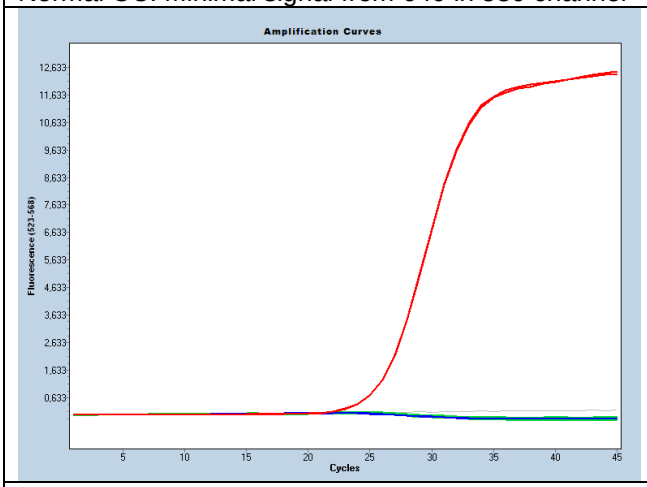
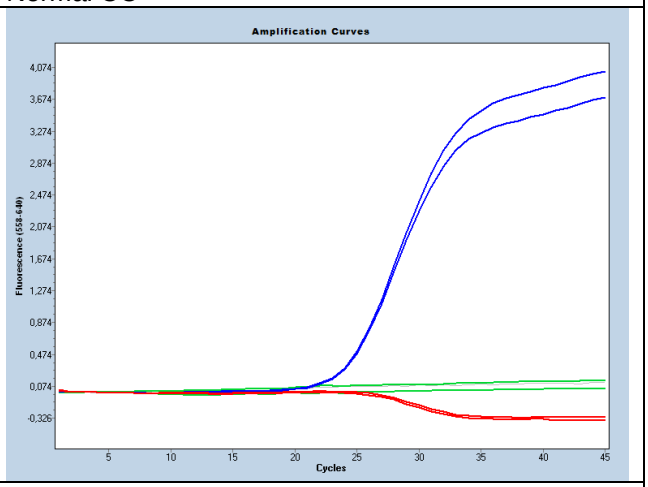


9. For every next CC file, e.g. for "**Special Dyes**" or for "**Normal Dyes with Yakima**" repeat from step 3.

Procedure for re-use of the colour compensation plate.

1. Remove plate; ensure seal is intact. If the seal has lifted discard plate
2. Place the plate in the next instrument, program melt curve only (chapter 5).
3. Follow procedure in this document for detection format and sample definition, start run.
4. Perform analysis.

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 neighbor dyes	Special CC: Negative signals for neighbor dyes

13. Contents and Material Safety Data (MSDS)

Typical product amount is ~ 30 µg (0,00003 gram). Product may contain :

> 99% Synthetic oligonucleotides. Synonym CAS 93384-16-8
< 0,5% CAS 77-86-1 Tris (hydroxymethyl) aminomethane
< 0,5% CAS 60-00-4 Ethylenediamine tetraacetic acid (EDTA)
< 0,5% CAS 99-20-7 Trehalose
< 0,5% CAS 9004-54-0 Dextran

This product is not hazardous (according to regulation (EC) No 1272/2008), not toxic, not IATA-restricted. Not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and EU Directives (EC) No 1907/2006 and (EC) No 2015/830 any products which do not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a MSDS.

14. Version History

Notes in red mark events require to change procedures

V120218	Release Version
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)
V210505	New manual structure and description of the selection of subsets

Certificate of Analysis / Certificate of Product

Panels tested ▼			**Lot no. 4680**		**Expiry: YYYY-MM-DD**	
Carba-penemase	**Gastro Bacteria**	**Gastro Parasites**	**LC480**	**LC480 II**	**cobas z 480**	**Passed**
50-0633	50-0601	50-0611	450 - 500	440 - 488	-	✓
53-0627	53-0602	53-0612	483 - 533	465 - 510	465 - 510	✓
58-0628	58-0603	58-0613	523 - 568	533 - 580	540 - 580	✓
61-0626	61-0604	61-0614	558 - 610	533 - 610	540 - 610	✓
64-0631	64-0605	64-0615	558 - 640	533 - 640	540 - 645	✓
66-0625	66-0625	66-0625	615 - 670	618 - 660	610 - 670	✓
-	70-0605	70-0611	-	618 - 660	610 - 700	✓

DOM (manufactured): YYYY-MM-DD | QC Acceptance: YYYY-MM-DD

We, the undersigned, certify that the product designated above has been obtained in accordance with the rules of production and quality control.

Name1 Name2

4260159330991

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.