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I. Description :

Takara's Cycleave Human ALDH2 Typing Probe/Primer Set was designed to allow highly specific detection and typing of SNP of Aldehyde dehydrogenase-2 (ALDH2) through real time PCR, utilizing Cycling Probe Technology (CPT).
Aldehyde Dehydrogenase-2 (ALDH2) is the enzyme which degrades intermediately metabolized alcohol (acetoaldehyde). It is known that there is single nucleotide polymorphism (SNP) [ALDH2 type 1 (wild type), ALDH2 type 2 (mutant type)] within the exon 12 at ALDH2 in which 487 Glu (GAA) is replaced with Lys (AAA). This SNP is largely involved in difference in individual physical constitution which relates to alcohol drinking, and was also reported that it could be involved in carcinogenesis.
This set includes 2 kinds of cycling probe having different fluorescence; ROX labeled one for wild type detection and FAM labeled one for mutant type detection, and Postive Control. And it is used in combination with CycleavePCR® Core Kit (Cat.#CY501). For the performing the reaction, an instrument for real time DNA amplification is required, such as Smart Cycler® System ＊ or Smart Cycler® II System ＊ (Cepheid).

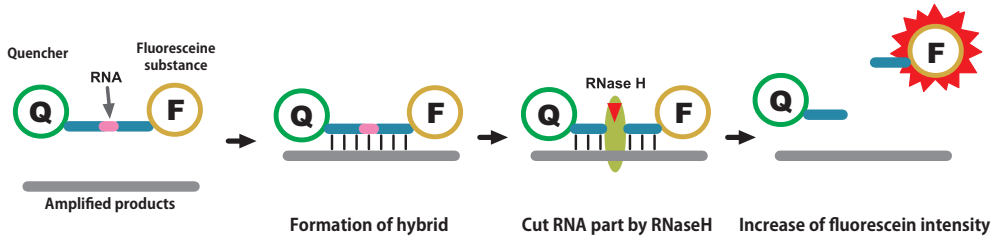
＊ : Smart Cycler® is a registered trademark of Cepheid.

II. Principle :

This set is used with CycleavePCR® Core Kit (Cat.#CY501). As the Core Kit employs Takara's proprietary PCR enzyme for hot start, *TaKaRa Ex Taq*® Hot Start Vertion, nonspecific amplification derived from mispriming or primer dimmer formation at the pre-cycling step, e.g. preparation of reaction mixture, can be minimized.

This set employs Cycling Probe Technology for detection, which is a high-sensitive detection method utilizing a combination of chimera probe, composed of RNA and DNA, and RNase H. The specific sequence of target gene to be amplified can be detected efficiently during or after amplification by this method. One end of the probe is labeled with a fluorescent substance and the another end is labeled with a quencher. As long as this probe remains intact, no strong fluorescence can be emitted because of the quenching function. When this probe forms a hybrid with the complementary sequence of amplified product, RNase H specifically cuts the RNA region of this probe, resulting in emission of strong fluorescence. By measuring the intensity of emitted fluorescence, the amount of amplified product can be monitored.

Principles of Cycling Probe Technology :



This set allows efficient detection and typing through amplifying a specific region of ALDH2 gene relating to SNP, and through simultaneous real time monitoring of fluorescence intensity of cycling probes which detect wild type and mutant type respectively. The probe for wild type detection forms hybrid with the wild type fragment in ALDH2 fragment which was amplified by PCR. Then RNA part of the probe is cut by RNase H, accordingly the fluorescence intensity of ROX labeled at the probe increases.

In this case, the fluorescence intensity of FAM labeled at the probe for mutant type detection would not increase, since they would not be subject to degradation of its RNA part by RNase H. The same way, for mutant type fragment in ALDH2 fragment, the probe for mutant type detection forms hybrid with the mutant type fragment in ALDH2 fragment which was amplified by PCR. Then RNA part of the probe is cut by RNase H, accordingly the fluorescence intensity of FAM labeled at the probe increases. In this case, the fluorescence intensity of ROX labeled at the probe for wild type detection would not increase, since they would not be subject to degradation of its RNA part by RNase H. In this way, Cycling Probe Technology cuts RNA part of the probes with RNase H by recognizing SNP. Monitoring of those two fluorescence intensity allows quick typing of SNP just in one tube, without performing electrophoresis.

III. Set Component (50 reactions×25 μl) :

1. Cycleave ALDH2 PCR Primer Mix	(10 μM each)	50 μl
2. Cycleave ALDH2 PCR Probe Mix ＊1	(25×)	50 μl
3. Cycleave ALDH2 Positive Control ＊2		50 μl (for 10 reactions)

＊1 : Make sure to store the fluorescent labeling probes in the light-shielding environment.

＊2 : Positive control of hetero type.

IV. Reagents and Instruments Required but Not Supplied in the Kit :

[Reagents]

CycleavePCR® Core Kit (Cat.#CY501)

[Instrument]

1. Micropippets for 200 μl, 20 μl and 10 μl.
2. Micropippet tips (with hydrophobic filter)

[Equipment]

1. Thermal Cycler for real time PCR
   Smart Cycler® System, Smart Cycler® II System (Cepheid)
2. Special tubes for Smart Cycler®
3. Desk-op centrifuge for Smart Cycler®

V. Storage : − 20℃ (for shipping and storage)

VI. Precautions and warnings :

1. When handling Smart Cycler® System, be sure to follow the written instructions for the device.
2. If a chimera probe or a primer is decomposed by contamination with nuclease, such decomposition inhibits accurate detection. Sweat or saliva of an operator can cause contamination with nuclease. Extreme caution should be exercised during operation.
3. PCR reaction is of extremely high sensitivity. In order to prevent contamination, it is recommended to set the separate three areas described below in a flow from preparation of reaction solutions to detection, which are physically isolated from one another.

   Area 1 : Prepare reaction solutions and pipette into tubes. Do not open or close any tube which contains an amplified product or a sample.

   Area 2 : Prepare samples. Do not open or close any tube which contains an amplified product.

Area 3 : Add a reaction solution to a sample so as to allow reaction, followed by detection.
Do not open or close any tube which contains an amplified product.

This set simultaneously carries out both amplification and detection in real-time, which does not need to use amplified product obtained from the reaction to subsequent process, such as electrophoresis, etc. Do not take an amplified product out of tubes. It can cause contamination.

VII. Sample preparation (Perform in Area 2) :

Human genomic DNA samples are prepared from blood anticoagulated with EDTA or Sodium citric acid or buccal (cheek) cells.

[In case of Blood Sample]

Sample DNA can be prepared from Dr. GenTLE (from Whole Blood; Cat.#9081) or other methods that yield genomic DNA of a comparable purity and concentration. When using Dr. GenTLE (from Whole Blood), samples are prepared according to the protocol, and the DNA extracted from 100 μl blood sample is dissolved in sterilized distilled water of the final volume of 50 μl. Measure the concentration of the obtained DNA solution, and apply 10-200 ng of DNA per reaction with this set.

[In case of buccal (cheek) cells]

Prepare buccal cells using a commercial kit, e.g. Catch-All Sample Collection Swabs (EPICENTRE). And then extract DNA using a commercial kit for the use with buccal cells, e.g. QIAamp DNA Mini Kit (QIAGEN). When DNA is prepared with QIAamp DNA Mini Kit, extracted DNA should be eluted in sterilized distilled water of the final volume of 100 μl. The obtained DNA amount varied depending on the amount of original buccal cells. Apply at least 10 ng of genome DNA per reaction.

VIII. Protocol :

The protocol differs in several points between Smart Cycler® System and Smart Cycler® II System. When using Smart Cycler® System, Channel Ch#1 is used for the detection of mutant type through the fluorescence intensity of FAM labled probe, and Channel Ch#4 is used for the detection of wild type through the fluorescence intensity of ROX labled probe. And the cut off value should be set as 100. In case of using Smart Cycler® II System, Ch#1 is used for mutant type detection (FAM labeled probe) with the cut off value 100, and Ch#3 is used for wild type detection (ROX labeled probe) with the cut off value 200. The following protocol describes the protocol using Smart Cycler® II System. It is recommended to follow the instruction supplied with each system to be used, and perform analysis.

In case of using Smart Cycler® II System :

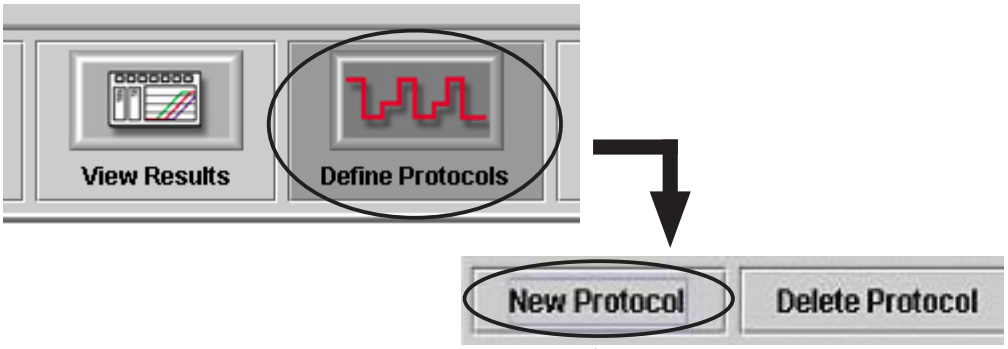
VIII-1. Setting of Smart Cycler® (Perform in Area 3)

(For more information on handling Smart Cycler® II System, see the instructions supplied with it.)

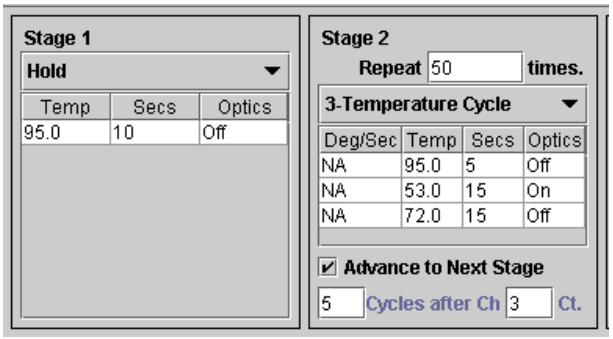
(1) Start the Smart Cycler® II System.

(2) Set the protocol.

Click the icon "Define Protocols" and then "New Protocol" button to create the protocol by following the steps shown below. Save the protocol by clicking "Save Protocol" (Since the created protocol is stored, no entry is required in subsequent reactions).



Define the Protocol name and click the OK button.



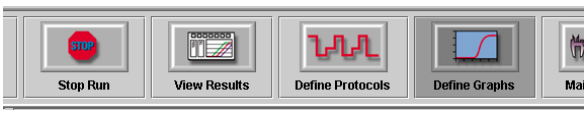
Create this Protocol

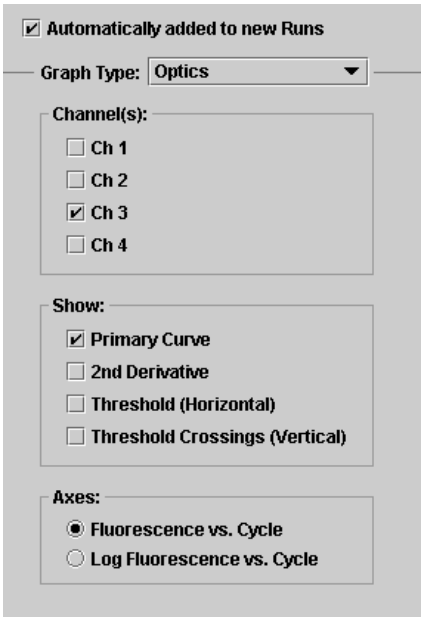
Click the Save Protocol.

NOTE : Function of "Advance to Next Stage"
This function allows quicker analysis by finishing the reaction at the time when amplified products are detected. This function is available with Smart Cycler® Software Verion 2.0. Although it cannot be used with Smart Cycler® Software Version 1.2, it can be used by upgrading the software using Smart Cycler® Software Version 2.0 Upgrade Kit. This is upgrade of the software alone, and the upgrade of an instrument Is not necessary.

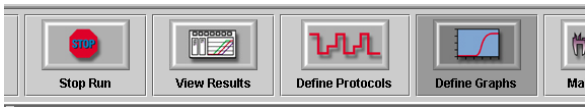
(3) Graph setting.
(Since the created graphs are stored, no entry is required in subsequent reactions).

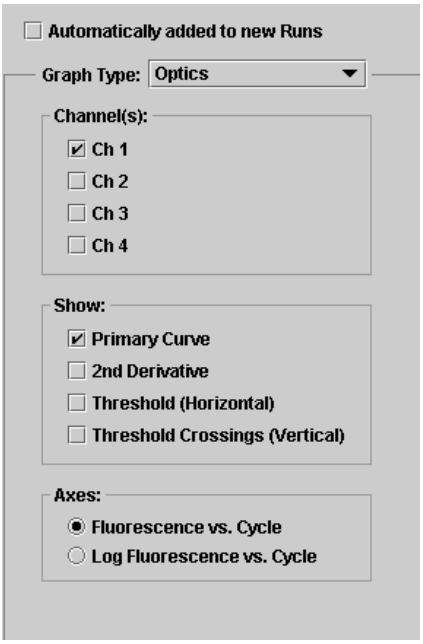
(3)-1. Setting of signal curve <channel 3 (TxRed)> for wild type detection (ROX).
Analyze ROX in channel (Ch#3) the same as Texas Red.
Click the icon "Define Graphs" and create the graphs by following the steps shown below.





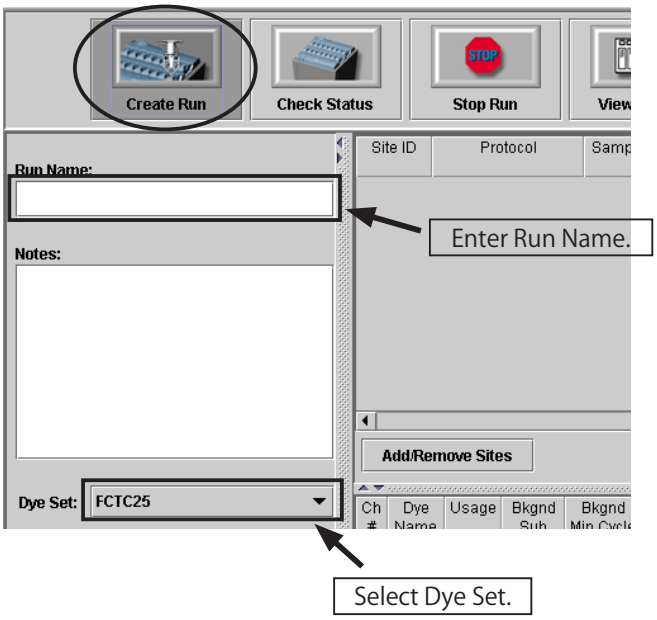
(3)-2. Setting of signal curve <channel 1 (FAM)> for mutant type detection (FAM).
Click the icon "Define Graphs" and create the graphs by following the steps shown below.
(Since the graphs have been set under a name "FAM" at initialization, no entry is required here).



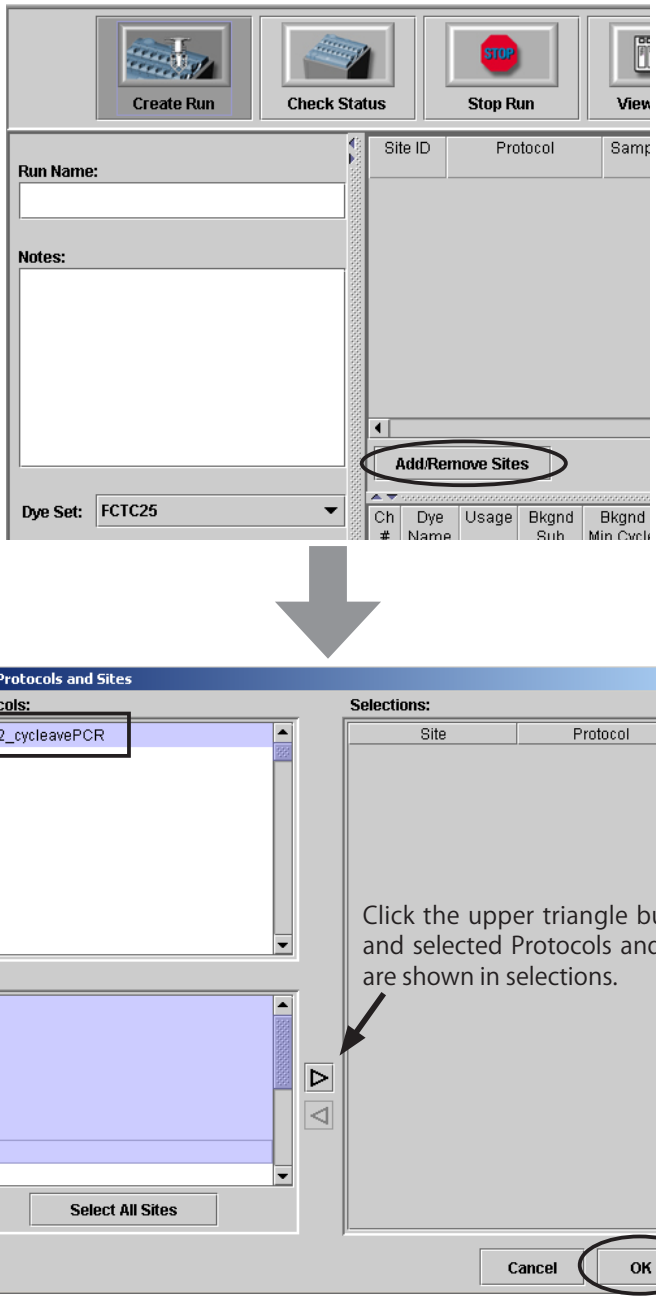


(4) Setting of reaction

(4)-1. Click the icon "Create Run" , enter Run Name, and select Dye-Set (FTTR25).



(4)-2. Click the "Add/Remove" button and the "Select Protocols and Sites" screen appears.
From the menu, select the Site and Protocol to be used, and check "OK" button.



VIII-2. Preparation of PCR reaction mixture (Perform in Area 1) :

It is recommended to duplicate the reaction per sample to secure the ALDH2 phosphorism in samples. In addition, the negative control reaction should be carried out simultaneously using sterilized distilled water instead of sample. This is to verify that there is not contamination in the reaction mixture. Also the positive control reaction using the supplied ALDH2 Positive Control should be done to verify that the ICAN ™ reaction and detection process proceed correctly.

The following reaction mixture is prepared in a requined quantity on ice.

<Per reaction>

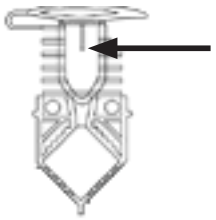
10 × CycleavePCR Buffer ＊1	2.5 μl
Mg^{2+} solution (25 mM) ＊1	3 μl
dNTP Mixture (2.5 mM each) ＊1	3 μl
ALDH2 PCR Primer Mix ＊2	1 μl
ALDH2 Probe Mix ＊2	1 μl
TaKaRa Ex Taq® HS (5 units/μl) ＊1	0.25 μl
Tli RNase H II (200 units/μl) ＊1	0.5 μl
dH_2O ＊1	X μl ＊3

＊1 : Supplied in CycleavePCR™ Core Kit (Cat.#CY501)

＊2 : Supplied in Cycleave Human ALDH2 Typing Probe/Primer Set (Cat.#CY403)

＊3 : Prepared sample solution (template) is added in 1-13 μl. So dH_2O should be added to have the final volume of 25 μl.

Add the components of the above reaction mixture without sample (template DNA) into a fresh tube in 12-24 μl, by pouring on the tube wall. Please refer to the following figure.



Place the reaction mixture into this reserver part.

Close gently the lid of the tubes, not so tightly, and move to the Area 3.

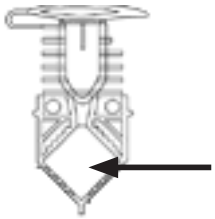
VIII-3. Addition of sample (template) (Perform in Area 3) :

Prepare one tube of negative control by adding sterilized distilled water instead of sample.

For the rest tubes, add the prepared samples into the reaction mixture, and close the lid of the tubes tightly. When performing Positive Control reaction, add 5 μl of ALDH2 Positive Control solution.

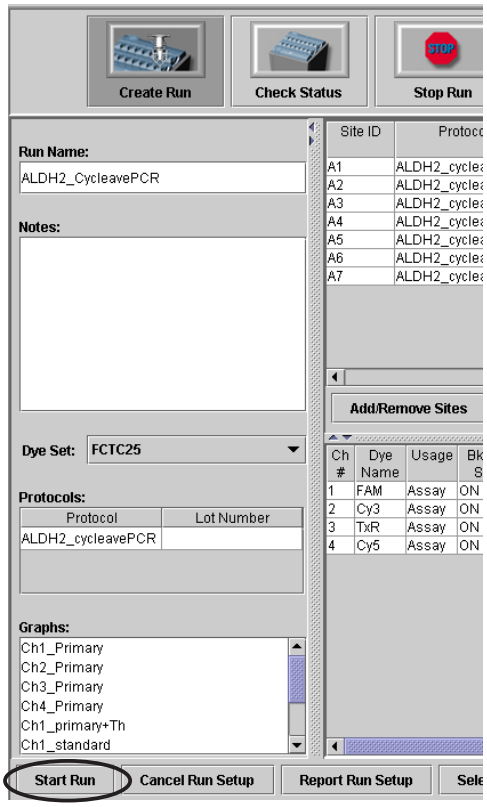
VIII-4. PCR Reaction and detection (Perform in Area 3) :

Centrifuge using the centrifuge exclusive use for Smart Cycler®. Drop the reaction mixture in the reserver into the lower rhombic room.



Reaction solution should be loaded down in this part of the tube.

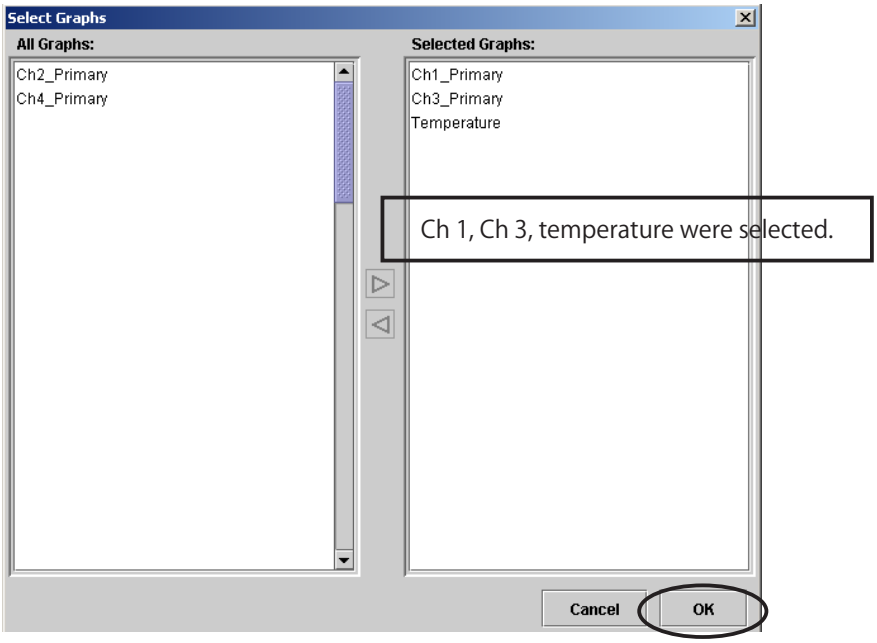
Load the reaction tubes on Smart Cycler® System and click the "Start Run" button to start the reaction process.



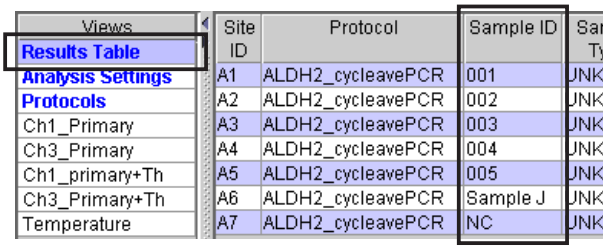
VIII-5. Display of results :

(1) View the View Results screen. (The same time the reaction process is started, the View Results screen automatically appears. If another screen is open, click the icon "View Results").

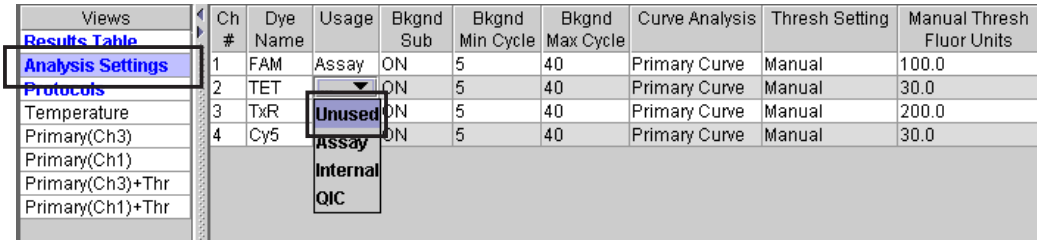
(2) Click the "Select Graphs" button, and the Select Graphs screen appears. From the menu, select Channel (Ch#3) for wild type detection, Ch#1 (for mutant type detection) and temperature (graph to display temperature chart). When the graphs of Ch#3, Ch#1, and temperature are already selected in the initial setting, no need to set again.



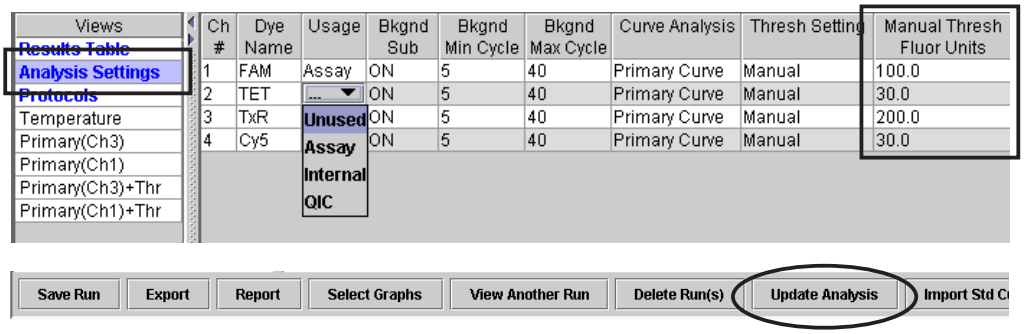
(3) From the Views list, select "Results Table" and enter Sample ID.



(4) Click "Analysis Settings" for opening. Click "Usage" next to Ch#2 and Ch#4 and select "Unused" from the pull-down menu. (After this setting is activated, data on the Ch#3 in Results Table is invisible.)



(5) In the Analysis Settings screen, set "Manual Thresh Fluor. Units for Ch#1" to 100, and for Ch#3 to 200. After entering data in the cells, click the "Update Analysis" button. Then the settings are activated. This value is used as a cut-off value.

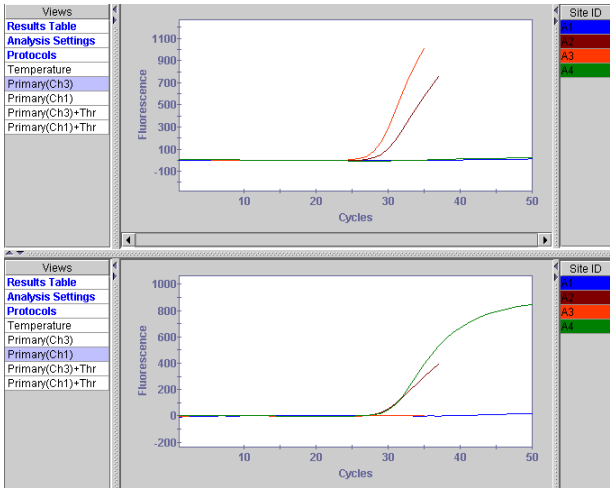


(6) Select Ch#3 at the upper "Views", and monitor the amplification curve of signal intensity for wild type detection in real time. (After the reaction starts, graphs appear on the screen soon.)

(7) Select Ch#1 at the lower "Views" and monitor the amplification curve of signal intensity for mutant type detection. (After the reaction starts, glaphs appear on the screen soon.)

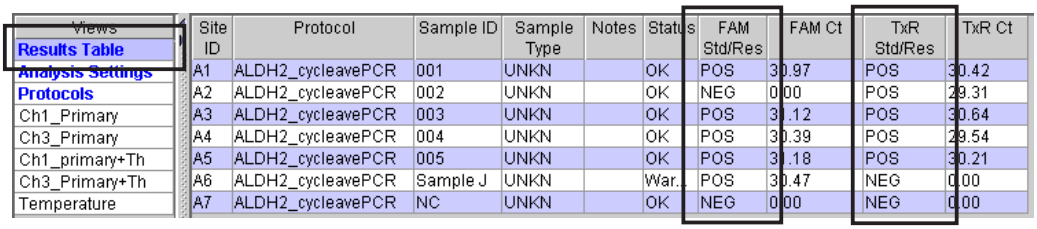
Amplified curve for ROX (detection for wild type)

Amplified curve for FAM (detection for mutant type)



When base line of graphs is not shown (constant, adjust the value of "Bkgnd Max Cycle" around at 15-20 in "Advanced to Next Stage" was selected to be "5 cycles after Ch#3 ct.". Therefore, the reaction terminates at 5 cycles after when the signal intensity of Ch#3 reaches 200 of "Manual Thresh Flour." which was set for Ch#3 at "Analysis Settings".

(8) After the reaction process terminates, click "Results Table" for viewing. Look at data in column of the TxR (Ch#3) Std/Res (Standard/Results), and FAM (Ch#1) Std/Res (Standard/Results) for verifying the results. If the fluorescent signal value for amplified product in TxR Std/Res is 200 or larger, "POS" is displayed in the cell, while if it is smaller than 200, "NEG" is displayed.
In FAM Std/Res, "POS" is displayed in the cell when the fluorescent signal value is 100 or longer, while "NEG" is displayed when it is smaller than 100.



Views
Results Table
Analysis Settings
Protocols
Ch1_Primary
Ch3_Primary
Ch1_primary+Th
Ch3_Primary+Th
Temperature

Site ID	Protocol	Sample ID	Sample Type	Notes	Status	FAM Std/Res	FAM Ct	TxR Std/Res	TxR Ct
A1	ALDH2_cycleavePCR	001	UNKN		OK	POS	30.97	POS	30.42
A2	ALDH2_cycleavePCR	002	UNKN		OK	NEG	0.00	POS	29.31
A3	ALDH2_cycleavePCR	003	UNKN		OK	POS	31.12	POS	30.64
A4	ALDH2_cycleavePCR	004	UNKN		OK	POS	30.39	POS	29.54
A5	ALDH2_cycleavePCR	005	UNKN		OK	POS	31.18	POS	30.21
A6	ALDH2_cycleavePCR	Sample J	UNKN		War...	POS	30.47	NEG	0.00
A7	ALDH2_cycleavePCR	NC	UNKN		OK	NEG	0.00	NEG	0.00

IX. Judgement :

If the fluorescent signal value of ROX detection is larger than the cut off value (100 at Smart Cycler® System, 200 at Smart Cycler® II System) when the reaction is completed, "POS " is displayed in the cell of the ROX Std/Res at Smart Cycler® System or "TxR Std/Res" at Smart Cycler® II System (the results of detection of the fluorescent signal derived from probe for wild type detection). If the value is below the cut off value, "NEG" is displayed. The same pattern of display is shown about FAM Std/Res (the results of detection of the fluorescent signal derived from probe for mutant type detection).

Using these results, judge the sample by referring to the following "Quick Reference Table for Judgment".

Quick reference table for judgement (reactions with a sample)

		FAM Std/Res (Probe for mutant type detection)	
		POS	NEG
Std/Res or TxR Std/Res (Probe for wild type detection)	POS	Hetero (w/m) ＊1	Wild homo (w/w)
	NEG	Mutant homo (m/m)	Judgement impossible ＊2 or the same result that is obtained with Negative control ＊3

＊1 : When performing positive control reaction using the supplied ALDH2 Positive Control, the result should show Hetero. If other result is obtained in positive control reaction, either of PCR reaction or detection by cycling probe method were not performed correctly.

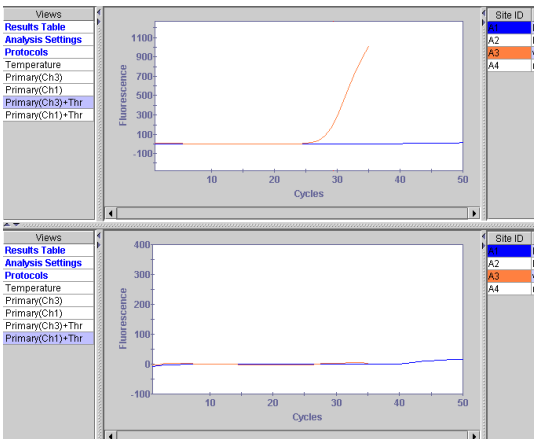
＊2 : For some reasons, PCR reaction or detection by cycling probe method were not performed correctly. Repeat the PCR reaction again. It is suspected that the DNA amount might be below detection limit or that any substance inhibiting reaction might be contaminated.
(Refer to "XI. Trouble Shooting")

＊3 : Negative control added with sterilized distilled water instead of sample DNA solution should be judged "NEG/NEG". If "POS" appears in the column of ROX Std/Res or FAM Std/Res in Negative control reaction, contamination is suspected. (Refer to "X. Trouble Shooting")

When judgment differs among two results obtained from the duplicated reactions per sample, repeat the reaction again.

○ **Application (Smart Cycler® II System was used.)**

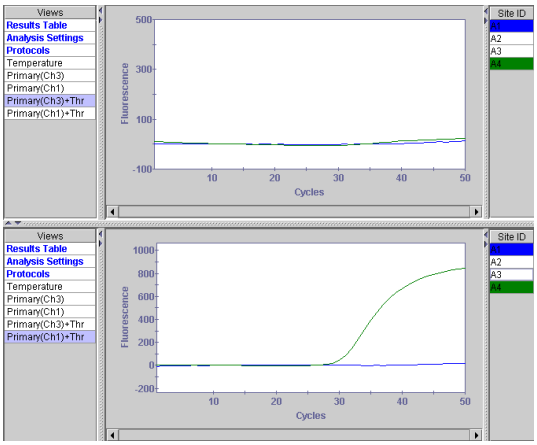
(1) Amplified curve of fluorescent intensity when using 20 ng of DNA as template which was proved to be wild homo (w/w).



Signal of Ch#3
(for wild type detection)

Signal of Ch#1
(for mutant type detection)

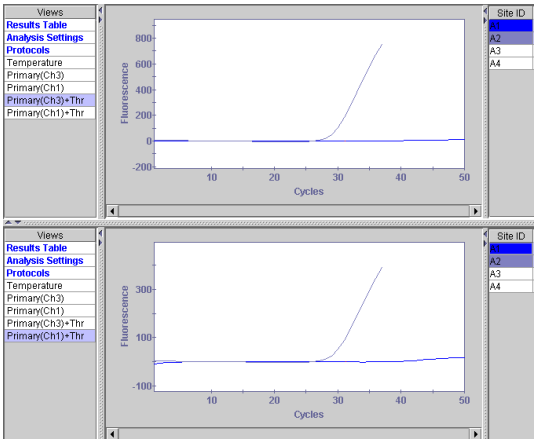
(2) Amplified curve of fluorescent intensity when using 20 ng of DNA as template which was proved to be mutant homo (m/m)



Signal of Ch#3
(for wild type detection)

Signal of Ch#1
(for mutant type detection)

(3) Amplified curve of fluorescent intensity when using 20 ng of DNA as template which was proved to be hetero (w/m)



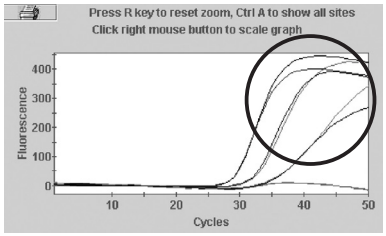
Signal of Ch#3
(for wild type detection)

Signal of Ch#1
(for mutant type detection)

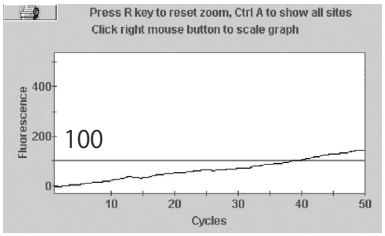
XI. Trouble Shooting :

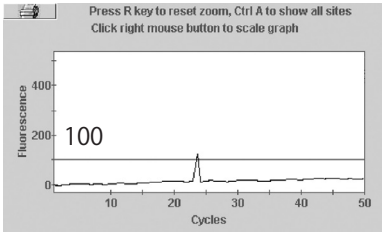
TROUBLE	REMEDY
(1) ・No signal was obtained. ・Signal intensity was weak.	・Perform control experiment using ALDH2 Positive Control supplied in the kit to see if signal is obtained. If signal is detected in the control experiment, there is no problem about the reagents for the reaction. ・Check the purity of sample DNA. Sample DNA is suspected to be contaminated with substances which may inhibit the reaction. Prepare sample DNA again, or repurify the sample again. ・Check the concentration of sample DNA. Reaction would not proceed with lower concentration, or the signal intensity would be weak when the concentration is low. ・Check a real time DNA amplification instrument to see if the temperature condition was set correctly. ・Read through the instruction of a real time DNA amplification instrument to confirm if all the procedures were followed correctly. ・It is recommended to perform control reaction using Positive Control supplied in the kit, along with a sample DNA.
(2) POS appears in both ROX and FAM Std/Res columns about all samples.	・All samples are suspected to be hetero. If there are any samples which had already been verified as wild homo or mutant homo, perform the reaction using those known samples. ・Perform negative control reaction using 5 μl of sterilized distilled water instead of sample. If POS still appears in both ROX and FAM Std/Res columns, the reaction reagents are suspected to be contaminated with amplified products. Careful attention should be paid not to cause contamination during operations. ・In order to prevent cross contamination among tubes, caution should be paid when opening and closing tubes including sample DNA to avoid the generation of aerosol.
(3) FAM (both in Smart Cycler® and in Smart Cycler® II) or ROX (TxR in Smart Cycler® II) Std/Ros shows values, not show POS or NEG.	If "Sample Type" in Results table is set STD, values will be shown. All columns in "Sample Type" should be set UNKN.

(4) POS is shown incorrecty due to signal noise derived from any other reason than an amplified product.	Judge from the amplified curve. When it is difficult to judge the result, perform the reaction again.



Increased signal due to any other reason than an amplified product





Amplified curve derived from an amplified product (Sigmoid curve is drawn)

(5) The background appears to be high when the fluorescence intensity curve is shown.	The reason is assumed that the Y-axis scale on the graph has been automatically adjusted to the value for the detected signal, since the weak fluorescent signal was detected Adjust the Y-axis scale manually. [In case of using Smart Cycler® System] 1. Click any point near the Y-axis scale with a right mouse button to open the Axes Graph Scale screen. 2. Enter an appropriate value in the Max filed in the y.Axes (Fl.) (for example, the value of Y-axis at the Positive Control reaction). Click the [Apply] button. 3. Click the [Exit] button to close the Axes Graph Scale screen. During the reaction processes, no scale can be adjusted. Scale adjustment must be done after all the reactions terminated. [In case of using Smart Cycler® II System] 1. Click any point near the Y-axis scale with a right mouse button. Select "Scale Graph" from pop up menu, and Axes Graph Scale screen appears. 2. Enter an appropriate value in the Max filed in the y.Axes (Fl.) (for example, the value of Y-axis at the Positive Control reaction). Click the [Apply] button. 3. Click the [Exit] button to close the Axes Graph Scale screen.

XII. Reference :

1) Yamamoto K, *et al*. (1993) *Jpn. J. Alchol & Drug Dependence*, **28** (1), 13-25
2) Yokoyama A, *et al.* (1998) *Carcinogenesis*, **19** (8), 1383-1387

XIII. Related products :

CycleavePCR® Core Kit (Cat.#CY501)
Smart Cycler® System (Cepheid)
Smart Cycler® II System (Cepheid)

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[L4] Quencher

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NANOGEN®

[M45] RNase-resistant Cycleave probe

This product is the subject of the pending Japanese patent application.

[M46] ICAN®-Cycleave and PCR-Cycleave

This product is the subject of the pending Japanese patent application.

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