Sacace BIOTECHNOLOGIES

IVD For *in Vitro* Diagnostic Use

CE

IL28B rs17/rs60 Real-TM

Handbook

Real Time PCR kit for detection of polymorphisms rs8099917 and rs12979860 of Interleukin-28B gene region.

REF R-O5-100FRT

Σ 100

NAME

IL28B rs17/rs60 Real-TM

INTRODUCTION

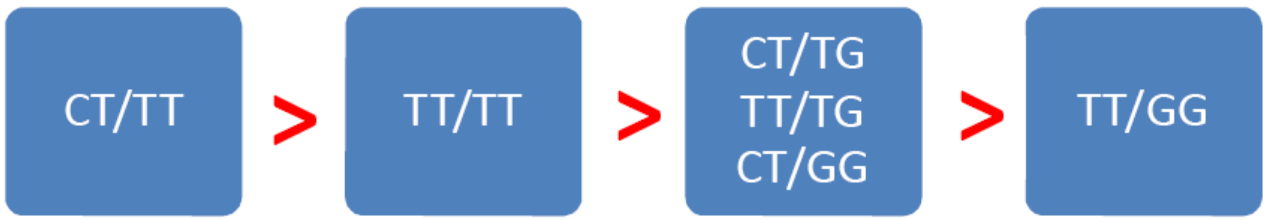
Interleukin-28 (IL28) is a cytokine that plays a role in immune defense against viruses. IL28B belongs to the type III interferon family of cytokines. Its classification as interferon is due to its ability to induce an antiviral state. Polymorphisms in the IL28B gene region are important in predicting outcome following therapy for chronic hepatitis C virus (HCV) infection.

Combined therapy INF pegylated (PEG-IFN) and ribavirin (RBV) is the current standard therapy against HCV infection and to know in detail the polymorphism in IL28B gene region of patients infected with HCV can be an important component of the decision to initiate treatment with PEG-IFN and RBV.

In particular, the rs12979860 polymorphism in the promoter of the IL28B human gene, is strongly associated to the SVR (Sustained Viral Response). It has been shown that patients who have the C/C genotype (genotype associated with favorable response to standard therapy) have a higher SVR rate compared to patients with genotype C/T and T/T (genotypes associated with less favorable response).

The rs8099917 polymorphism in the promoter of the IL28B human gene is associated with the success or failure of treatment in patients with viral genotype HCV1. It has been shown that patients who have the genotype T/T (favorable genotype) are associated with a greater likelihood of successful treatment than patients with genotype G/T and G/G. The rs8099917 polymorphism is also a valid predictor of successful treatment for HCV / HIV positive.

In summary the CC genotype at rs12979860 (regardless of the genotype of rs8099917) is the most significant predictor of SVR. With genotype other than CC at rs12979860 likelihood of achieving SVR, depending on the combination of variants analyzed of the polymorphisms rs12979860/rs8099917 decreases in the following order:



INTENDED USE

Kit **IL28B rs17/rs60 Real-TM** is a test for the qualitative detection of rs8099917 and rs12979860 polymorphisms of Interleukin 28B gene region in genomic DNA.

PRINCIPLE OF ASSAY

Kit **IL28B rs17/rs60 Real-TM** is based on two major processes: isolation of DNA from specimens and Real Time amplification. The test is performed by the detection of the genetic variants within the IL28B gene using competitive allele-specific amplification in Real Time PCR. Kit contains one primer PCR mix tube for the detection of rs8099917 polymorphism and one primer PCR mix tube for the detection of rs12979860 polymorphism. Internal Control (IC) serves as an amplification control for each individually processed specimen and to identify possible inhibition reactions. IC is detected in a channel other than the polymorphisms.

Reaction Mix	**<<rs17>>**	**<<rs60>>**
Fluorophore channel	**Detected nucleotide**	
FAM	T	T
JOE	G	C
ROX	IC	IC

MATERIALS PROVIDED

"IL28B rs17/rs60 Real-TM": Real Time amplification kit

- **PCR-mix-1-FRT IL28B rs8099917**, 2 x 0,6 ml;
- **PCR-mix-1-FRT IL28B rs12979860**, 2 x 0,6 ml;
- **PCR-mix-2-FRT**, 2 x 0,6 ml;
- **TaqF Hot Start DNA Polymerase**, 2 x 0,06 ml;
- **Pos C+ IL28B rs8099917**, 0,2 ml;
- **Pos C+ IL28B rs12979860**, 0,2 ml;
- **Negative Control (C-)***, 1,2 ml
- **TE-buffer****, 3 x 0,07 ml

Contains reagents for 100 samples

* *must be used in the isolation procedure as Negative Control of Extraction.*
** *must be used in the amplification procedure as Negative Control of Amplification*

MATERIALS REQUIRED BUT NOT PROVIDED

- DNA extraction kit
- Real Time Thermal cycler
- Reaction tubes
- Workstation
- Pipettes (adjustable)
- Sterile pipette tips with filters
- Vortex mixer
- Freezer, refrigerator

STORAGE INSTRUCTIONS

Store kit at 2-8°C. TaqF Hot Start DNA Polymerase must be stored at -20°C. The kit can be shipped at 2-8°C but should be stored at 2-8°C and -20°C immediately on receipt.

STABILITY

IL28B rs17/rs60 Real-TM is stable up to the expiration date indicated on the kit label. The product will maintain performance through the control date printed on the label. Exposure to light, heat or humidity may affect the shelf life of some of the kit components and should be avoided. Repeated thawing and freezing of these reagents should be avoided, as this may reduce the sensitivity. Components stored under conditions other than those stated on the labels may not perform properly and may adversely affect the assay results.

QUALITY CONTROL

In accordance with Sacace's ISO 13485-Certified Quality Management System, each lot is tested against predetermined specifications to ensure consistent product quality.

WARNINGS AND PRECAUTIONS

IVD

***In Vitro* Diagnostic Medical Device**

For *In Vitro* Diagnostic Use Only

The user should always pay attention to the following:

- Use sterile pipette tips with aerosol barriers and use new tip for every procedure.
- Store extracted positive material (samples, controls and amplicons) away from all other reagents and add it to the reaction mix in a separate area.
- Thaw all components thoroughly at room temperature before starting an assay.
- When thawed, mix the components and centrifuge briefly.
- Use disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Thoroughly wash hands afterwards.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in laboratory work areas.
- Do not use a kit after its expiration date.
- Dispose of all specimens and unused reagents in accordance with local authorities' regulations.
- Specimens should be considered potentially infectious and handled in a biological cabinet in accordance with appropriate biosafety practices.
- Clean and disinfect all sample or reagent spills using a disinfectant such as 0.5% sodium hypochlorite, or other suitable disinfectant.
- Avoid sample or reagent contact with the skin, eyes, and mucous membranes. If skin, eyes, or mucous membranes come into contact, rinse immediately with water and seek medical advice immediately.
- Material Safety Data Sheets (MSDS) are available on request.
- Use of this product should be limited to personnel trained in the techniques of DNA amplification.
- The laboratory process must be one-directional, it should begin in the Extraction Area and then move to the Amplification and Detection Areas. Do not return samples, equipment and reagents to the area in which the previous step was performed.

Some components of this kit contain sodium azide as a preservative. Do not use metal tubing for reagent transfer.

PRODUCT USE LIMITATIONS

All reagents may exclusively be used in in vitro diagnostics. Use of this product should be limited to personnel trained in the techniques of DNA amplification (UNI EN ISO 18113-2:2012). Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use a kit after its expiration date.

SAMPLE COLLECTION, STORAGE AND TRANSPORT

IL28B rs17/rs60 Real-TM can analyze genomic DNA extracted from:

- *Whole blood* collected in either ACD or EDTA tubes
- *Buccal swab*: insert the swab into the nuclease-free 1,5 ml tube and add 0,2 ml of Transport medium. Vigorously agitate swabs in medium for 15-20 sec.

Specimens can be stored at +2-8°C for no longer than 12 hours, or frozen at -20°C to -80°C.

Transportation of clinical specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.

DNA ISOLATION

Any commercial RNA/DNA isolation kit, if IVD-CE validated for the specimen types indicated herein at the “SAMPLE COLLECTION, STORAGE AND TRANSPORT” paragraph, could be used.

The following kit is recommended:

⇨ **Genomic column DNA Express** (Sacace, REF K-1-1/E);

⇨ **SaMag Blood Extraction kit** (Sacace, REF SM001).

Please carry out DNA extraction according to the manufacture’s instruction.

PROTOCOL

Reaction volume = 25 µl

Prepare required quantity of reaction tubes for samples and controls.

1. **Setup two Reaction mix respectively for each polymorphisms "rs17" and "rs60"**
2. Reaction Mix:

   - **Polymorphism "rs17":** prepare in the new sterile tube for each sample:
     - **5 µl of PCR-mix-2-FRT,**
     - **0,5 µl of TaqF Hot Start DNA Polymerase,**
     - **10 µl of PCR-mix-1-FRT IL28B rs8099917.**

     Vortex and centrifuge briefly.

   - **Polymorphism "rs60":** prepare in the new sterile tube for each sample:
     - **5 µl of PCR-mix-2-FRT,**
     - **0,5 µl of TaqF Hot Start DNA Polymerase,**
     - **10 µl of PCR-mix-1-FRT IL28B rs12979860.**

     Vortex and centrifuge briefly.
3. Add to each sample tube **15 µl** of the appropriate **Reaction Mix** and **10 µl** of **extracted DNA** sample. Mix by pipetting.
4. Prepare for each polymorphism the controls as follow:
   - Add **10 µl** of **Pos C+ IL28B rs8099917** to the tube labeled **PCR Positive Control "rs17";**
   - Add **10 µl** of **Pos C+ IL28B rs12979860** to the tube labeled **PCR Positive Control "rs60";**
   - Add **10 µl** of **TE-buffer** to the tube labeled **Amplification Negative Control.**
5. Insert the tubes in the thermalcycler.

Amplification

1. Create a temperature profile on your instrument as follows:

Step	**Rotor type instruments[1]**			**Plate or modular type instruments[2]**		
	Temperature, °C	***Time***	***Cycles***	***Temperature, °C***	***Time***	***Cycles***
Hold	**95**	15 min	1	**95**	15 min	1
Cycling	**95**	5 s	5	**95**	5 s	5
	60	20 s		**60**	20 s	
Cycling 2	**95**	5 s	40	**95**	5 s	40
	60	**40 s fluorescence detection**		**60**	**50 s fluorescence detection**	

[1] *For example Rotor-Gene™ 6000/Q (Corbett Research, Qiagen)*
[2] *For example, SaCycler-96™ (Sacace), CFX/iQ5™ (BioRad)*

Fluorescence is detected at the 2nd step of Cycling 2 stage (60 °C) in FAM (Green), JOE (Yellow)/Hex and ROX (Orange) fluorescence channels.

INSTRUMENT SETTINGS

Rotor-type instruments

Channel	Calibrate/Gain Optimisation…	Threshold	More Settings/ Outlier Removal	Slope Correct
FAM/Green	*from 5 Fl to 10 Fl*	*0.03*	*30 %*	*On*
JOE/Yellow	*from 5 Fl to 10 Fl*	*0.03*	*30 %*	*On*
ROX/Orange	*from 5 Fl to 10 Fl*	*0.03*	*30 %*	*On*

Plate-type instruments

The threshold line should cross only sigmoid curves of signal accumulation of positive samples and should not cross the baseline; otherwise, the threshold level should be raised. Set manually the threshold: its level must be put at 30% of the maximum fluorescence level reached by the positive control in the last amplification cycle.

DATA ANALYSIS

The fluorescent signal intensity is detected in three channels.

- The signal from T_{rs17} and T_{rs60} DNA amplification product is detected in the FAM/Green channel;
- The signal from G_{rs17} and C_{rs60} DNA amplification product is detected in the JOE (Yellow)/ HEX channel;
- The signal from the Internal Control (IC) amplification product is detected in the ROX (Orange) channel.

Interpretation of results

The results are interpreted with the software of instrument through the presence of crossing of fluorescence curve with the threshold line.

Depending on the type of instrument make the evaluation of the results as described below:

Rotor –type instruments:

If ROX (Orange) Ct is present and <25 evaluate the results as follow:

- Only FAM (Green) Ct present = TT_{rs17} / TT_{rs60} polymorphisms;
- Only JOE (Yellow)/HEX Ct present = GG_{rs17} / CC_{rs60} polymorphisms;
- Both FAM (Green) and JOE (Yellow)/HEX Cts present:
  - FAM (Green) Ct < JOE (Yellow)/HEX Ct = TT_{rs17} / TT_{rs60} polymorphisms;
  - FAM (Green) Ct > JOE (Yellow)/HEX Ct = TG_{rs17} / CT_{rs60} polymorphisms;

If ROX (Orange) Ct is not present or > 25 repeat the test.

Figure 1: Schematic interpretation results on Rotor-type instruments

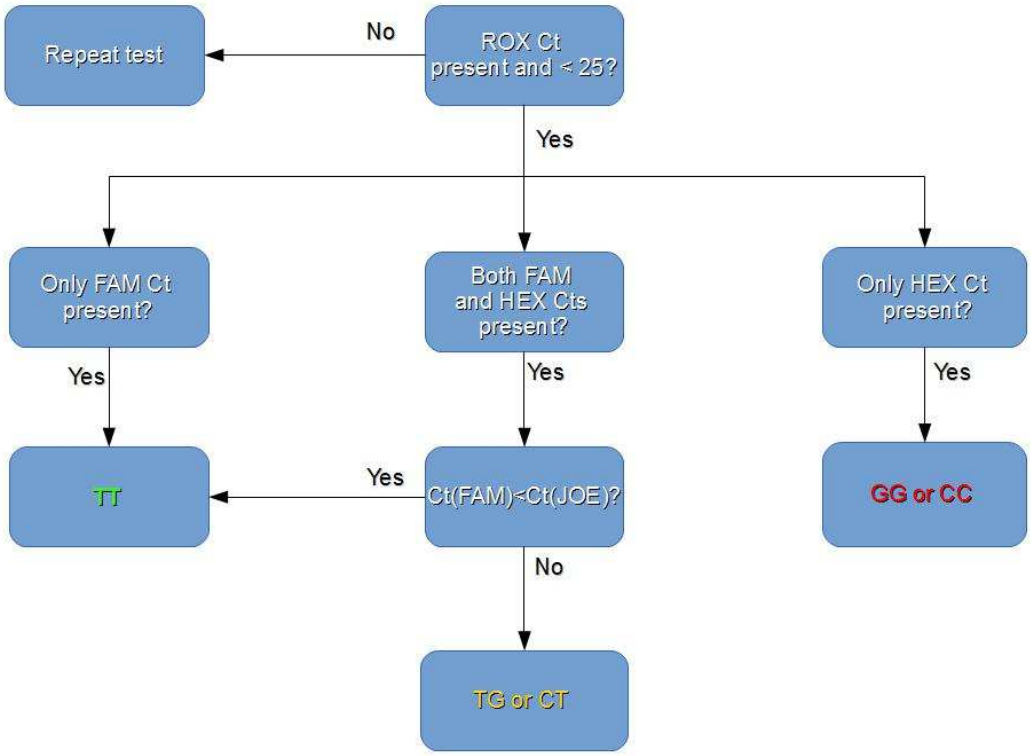


Plate-type instruments:

If ROX (Orange) Ct is present and <28 evaluate the results as follow:

- Only FAM (Green) Ct present = TT_{rs17} / TT_{rs60} polymorphisms;
- Only JOE (Yellow)/HEX Ct present = GG_{rs17} / CC_{rs60} polymorphisms;
- Both FAM (Green) and JOE (Yellow)/HEX Cts present:
    - FAM (Green) Ct - ROX (Orange) Ct > N* = GG_{rs17} / CC_{rs60} polymorphisms
    - JOE (Yellow)/HEX Ct - ROX (Orange) Ct > N* = TT_{rs17} / TT_{rs60} polymorphisms;
    - JOE (Yellow)/HEX Ct - ROX (Orange) Ct < N* = TG_{rs17} / CT_{rs60} polymorphisms;

* *N = 10 for iQ5, CFX / N = 15 for SaCycler-96*

If ROX (Orange) Ct is not present or >28 repeat the test.

Figure 2: Schematic interpretation results on Plate-type instruments

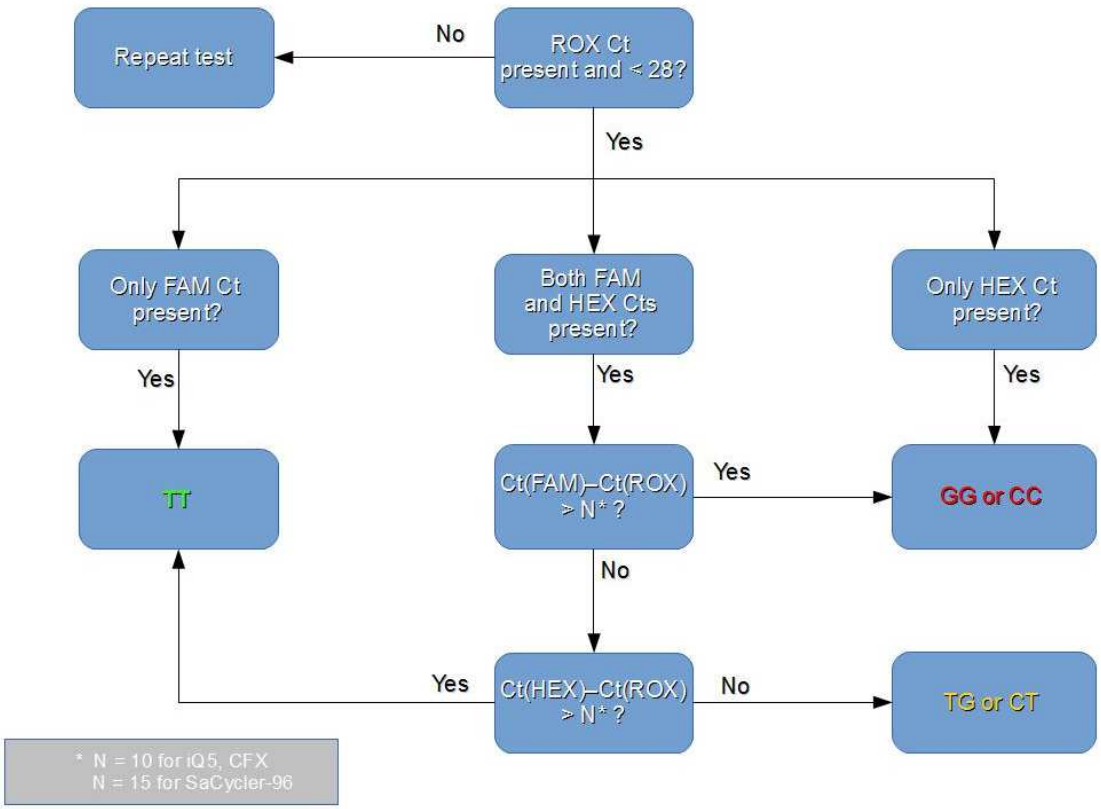


Table 1. Results for controls

Reaction Mix		<<rs17>>			<<rs60>>		
Control	**Stage for control**	**Ct FAM**	**Ct JOE**	**Ct ROX**	**Ct FAM**	**Ct JOE**	**Ct ROX**
NCE	DNA isolation	NEG	NEG	NEG	NEG	NEG	NEG
NCA	PCR	NEG	NEG	NEG	NEG	NEG	NEG
C+ $_{rs17}$	PCR	POS	POS	POS	-	-	-
C+ $_{rs60}$	PCR	-	-	-	POS	POS	POS

QUALITY CONTROL PROCEDURE

A negative control of extraction (NCE), negative amplification control (NCA), positive amplification control (C+) are required for every run to verify that the specimen preparation, the amplification and the detection steps are performed correctly.

If the controls are out of their expected range (see table Results for Controls), all of the specimens and controls from that run must be processed beginning from the sample preparation step.

TROUBLESHOOTING

1. Weak or no signal of the Positive Control.
    - The PCR conditions didn't comply with the instructions.
        - ⇒ Check the amplification protocol and select the fluorescence channel reported in the manual.
2. Fam (Green), Joe/Yellow/Hex and ROX (Orange) signal with Negative Control of extraction.
    - Contamination during DNA extraction procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol.
        - ⇒ Use only filter tips during the extraction procedure. Change tips between tubes.
        - ⇒ Repeat the DNA extraction with the new set of reagents.
3. Any signal with Negative Control of PCR (DNA-buffer).
    - Contamination during PCR preparation procedure. All samples results are invalid.
        - ⇒ Decontaminate all surfaces and instruments with sodium hypochlorite and ethanol or special DNA decontamination reagents.
        - ⇒ Pipette the Positive control at last.
        - ⇒ Repeat the PCR preparation with the new set of reagents.

KEY TO SYMBOLS USED

REF	List Number		Caution!
LOT	Lot Number		Contains sufficient for <n> tests
	Expiration Date	VER	Version
	Store at	**NCA**	Negative Control of Amplification
	Manufacturer	**NCE**	Negative control of Extraction
	Consult instructions for use	**C+**	Positive Control of Amplification
IC	Internal Control	IVD	For *in Vitro* Diagnostic Use

* SaCycler™ is a registered trademark of Sacace Biotechnologies
* CFX™ and iQ5™ are registered trademarks of Bio-Rad Laboratories
* Rotor-Gene™ is a registered trademark of Qiagen

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