Multiplex Real-Time PCR Genotyping From Crude Sample Lysates

USER GUIDE

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ThermoFisher SCIENTIFIC

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Products manufactured at this site:

- DNA Extract All Reagents Kit

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Products manufactured at this site:

- TaqPath™ ProAmp™ Master Mix

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Products manufactured at this site:

- Diomni™ Design and Analysis (RUO) Software 3

For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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1 Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

Product information

This user guide describes the workflow for multiplex real-time PCR genotyping with crude sample lysates.

The workflow includes the following components:

- qPCR Assay Design Hub (**https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/assay-design.html**)
- Multiplexing support request (**https://www.thermofisher.com/us/en/home/global/forms/life-science/assay-support.html**)
- TaqMan™ SNP Genotyping Assays and TaqMan™ Drug Metabolism Genotyping Assays
- DNA Extract All Reagents Kit (Cat. No. 4403319)
- TaqPath™ ProAmp™ Master Mix (Cat. No. A30865)
- Applied Biosystems™ QuantStudio™ real-time PCR instrument
- Diomni™ Design and Analysis (RUO) Software v3.0 and later

The workflow allows an end-to-end solution for multiplex SNP genotyping experiments.

Multiplex PCR is the simultaneous amplification of multiple targets in a single reaction tube. Different reporters with distinct fluorescence spectra are used in multiplex PCR to track each individual amplification reaction. The real-time PCR instrument detects a signal from each reporter dye and determines the amount of each target.

Multiplex support is a multiplex assay interaction check service that allows the user to confirm that individual assays are compatible with each other in a multiplex assay.

Overview of the recommended dyes

TaqPath™ ProAmp™ Master Mix allows for multiplex genotyping assays with 2 SNP assays when the recommended dyes are used.

Different combinations of dyes are possible for probe labeling. The following combinations are highly recommended for 2 SNP assay multiplexing using TaqPath™ ProAmp™ Master Mix.

- SNP assay 1: a FAM™ dye-labeled probe and a VIC™ dye-labeled probe
- SNP assay 2: an ABY™ dye-labeled probe and a Cyanine 5 dye-labeled probe

Your real-time PCR instrument must have the correct number of optical filters to support multiplexing. A multiplex genotyping assay with 2 SNP assays requires at least 4 optical filters. A multiplex genotyping assay with 3 SNP assays requires 6 optical filters.

Your real-time PCR instrument must be calibrated for the dyes that are used. The recommended dyes for 2 SNP assays are system dyes. Cyanine 5.5, recommended for a 3 SNP assay, is not a system dye. A custom calibration must be performed for this dye.

Assay design information

JUN™ dye uses the same optical channel as ROX™ dye. The TaqPath™ ProAmp™ Master Mix contains ROX™ dye to allow passive reference normalization in real-time PCR.

For genotyping assays with 2 SNP assays and the TaqPath™ ProAmp™ Master Mix that contains ROX™ dye, we do not recommend designing an assay with JUN™ dye.

JUN™ and Cyanine 5.5 dye can be used as the third dye combination for genotyping assays with 3 SNP assays. A custom TaqPath™ ProAmp™ Master Mix with no ROX™ dye must be used. A passive reference normalization cannot be included with three SNP assays. Contact **custom.solutions@lifetech.com**.

Table 1 Reporter and quencher dye options for predesigned TaqMan™ SNP Genotyping Assays

Reporter dye options	Quencher dye	Recommended SNP assay
FAM™ dye VIC™ dye	MGB-NFQ	SNP assay 1
ABY™ dye Cyanine 5 dye	MGB-NFQ	SNP assay 2
JUN™ dye	MGB-NFQ	SNP assay 3
Cyanine 5.5 dye	QSY2™ quencher	

A redesign of the probe sequence must be requested when converting an MGB-NFQ quencher dye to a QSY2™ quencher dye (**https://www.thermofisher.com/us/en/home/global/forms/life-science/assay-redesign.html**). The predesigned assay ID is used, then the probe is redesigned to be longer.

The quencher dye QSY2™ is used for the Cyanine 5.5 reporter dye in SNP genotyping assays.

Overview of the analysis software

Diomni™ Design and Analysis (RUO) Software 3 with the Genotyping Analysis Module or the Genotyping Analysis Module (project) can be used to analyze genotyping multiplex assays. The Genotyping Analysis Module (project) allows multi-plate analysis.

The following versions of the software are available:

- Diomni™ Design and Analysis (RUO) Software v3.0
- Diomni™ Design and Analysis (RUO) Software v3.1

Both available versions can be used to analyze genotyping data.

Two SNP assays or 3 SNP assays can be assigned to one well. Only automatic calling can be performed. Manual calls cannot be performed when more than 1 SNP assay is assigned to one well.

Contact Support for the option to perform manual calls when more than 1 SNP assay is assigned to one well.

Note: Your real-time PCR instrument must have the correct number of optical filters to support multiplexing. A multiplex genotyping assay with 2 SNP assays requires at least 4 optical filters. A multiplex genotyping assay with 3 SNP assays requires 6 optical filters.

Overview of the software documentation

Software settings and preferences, plate and project setup, and primary analysis are included in the following documents for Diomni™ Design and Analysis (RUO) Software 3:

- *Diomni™ Design and Analysis (RUO) Software 3 (Desktop) User Guide* (Pub. No. MAN0030162)
- *Diomni™ Design and Analysis (RUO) Software 3 (On-Premises) User Guide* (Pub. No. MAN1000091)
- *Diomni™ Design and Analysis (RUO) 3 User Guide*, for the Thermo Fisher™ Connect Platform (Pub. No. MAN0030163)

The following documents include genotyping analysis information:

- *Diomni™ Design and Analysis (RUO) Software 3 Genotyping Analysis Module User Guide* (Pub. No. MAN0030164)
- *Diomni™ Design and Analysis (RUO) Software 3 Genotyping Analysis Module (project) User Guide* (Pub. No. MAN1000138)

Workflow

Genotyping Multiplex Assay

Design and order assays

For more information, see “Product information” on page 5.

Prepare the sample (page 9)

Perform PCR amplification (page 12)

Analyze data

See Chapter 4, “Overview data analysis”.

2 Prepare the sample

Prepare the sample lysate from buccal swabs or blood with the DNA Extract All Reagents Kit.

For more information, see *DNA Extract All Reagents Kit Product Information Sheet* (Pub. No. MAN0030082).

Note: The instructions to prepare the sample lysate in this user guide are the same as the instructions in the *DNA Extract All Reagents Kit Product Information Sheet*. Instructions are provided in this user guide for a complete workflow with the sample types that are recommended for the assays in this user guide.

Stabilization with DNA Stabilizing Solution is required. We do not recommend proceeding to PCR immediately after lysis. DNA Stabilizing Solution stabilizes the DNA after the lysis reaction. DNA Stabilizing Solution also contains components that protect the PCR from inhibitors.

Overview sample types

The following sample types can be used with genotyping multiplex assays. The following table describes the quantity of the sample optimized for use with the DNA Extract All Reagents Kit.

Sample	Minimum quantity
Blood	2 µL
Buccal swab	1 swab

Buccal swabs can be air-dried, then stored at room temperature.

The reaction volume for blood can be scaled up, if needed. The volume of Lysis Solution and DNA Stabilizing Solution must be scaled up according to the volume of blood used.

Prepare blood samples

Lyse the samples

1. Thoroughly mix the Lysis Solution.
2. Add 20 µL of Lysis Solution to each 1.5-mL microcentrifuge tube or well of a 96-well plate containing the sample.

   The volume when preparing blood samples does not require a 96-well deep-well plate. A 96-well deep-well plate can be used if preferred.

3. Pipette up and down to mix the Lysis Solution and the sample in each tube or plate well.
4. Cap the tubes or seal the plate with an adhesive cover.

Incubate the samples

Incubate the samples at room temperature for 3 minutes.

Stabilize the DNA

1. Thoroughly mix the DNA Stabilizing Solution.
2. Open the tube or uncover the plate.
3. Add 20 µL of DNA Stabilizing Solution to each tube or well of the plate containing the sample.
4. Pipette up and down to mix the solution and the sample in each tube or plate well.
5. Cap the tubes or seal the plate with an adhesive cover.
6. *(Optional)* Centrifuge the tubes or plate briefly.

(Optional) Store the sample lysate at 4°C or store the sample lysate at –20°C for a longer storage time.

Proceed to performing the PCR (see Chapter 3, “Perform PCR amplification”).

Mix the sample lysate before performing PCR.

Prepare buccal swab samples

Lyse the samples

1. Thoroughly mix the Lysis Solution.
2. Add 400 µL of Lysis Solution to each 1.5-mL microcentrifuge tube or well of the 2-mL 96-well deep-well plate containing the swab sample.

   A 2-mL 96-well deep-well plate is required for lysis. The deep-well format accommodates the swab and the volume of Lysis Solution.
3. Immerse the swab in the tube containing the Lysis Solution.
4. Rotate the swab in the Lysis Solution 5 times.
5. Lift the swab above the Lysis Solution, then press the swab against the side of the tube to remove the liquid from the swab.
6. Dispose the swab after removing the liquid from the swab.

7. Pipette up and down to mix the Lysis Solution and the sample in each tube or plate well.
8. Cap the tubes or seal the plate with an adhesive cover.

Incubate the samples

1. Incubate the samples at 95°C for 3 minutes.
2. Cool the sample at room temperature for 30 seconds.

Stabilize the DNA

1. Thoroughly mix the DNA Stabilizing Solution.
2. Open the tube or uncover the plate.
3. Add 400 µL of DNA Stabilizing Solution to each tube or well of the plate containing the sample.
4. Pipette up and down to mix the solution and the sample in each tube or plate well.
5. Cap the tubes or seal the plate with an adhesive cover.
6. *(Optional)* Centrifuge the tubes or plate briefly.

(Optional) Store the sample lysate at 4ºC or store the sample lysate at –20°C for a longer storage time.

Proceed to performing the PCR (see Chapter 3, “Perform PCR amplification”).

Mix the sample lysate before performing PCR.

3 Perform PCR amplification

Procedural guidelines

Guidelines for sample lysate input

Sample lysate volume from the DNA Extract All Reagents Kit can be up to 25% of the total reaction volume.

Guidelines for PCR reactions

For reaction volumes that are different from those detailed, scale all components proportionally and include 10% overage.

IMPORTANT! Include at least 2 no-template control (NTC) reactions. NTC reactions are required for the software to accurately call the genotype.

It is recommended to include genomic DNA controls (PC) of known genotype to improve the accuracy of genotype calling.

Set up the PCR reactions

1. Prepare the required number of reactions according to the following table, plus 10% overage.

Table 2 PCR reactions for genotyping experiments

Component	Volume per reaction	
	10 µL (384-well and 96-well 0.1-mL)	20 µL (96-well 0.2-mL)
TaqPath™ ProAmp™ Master Mix	5.0 µL	10.0 µL
TaqMan™ SNP Genotyping Assay 1 or TaqMan™ Drug Metabolism Genotyping Assay 1 (20X)	0.5 µL	1.0 µL
TaqMan™ SNP Genotyping Assay 2 or TaqMan™ Drug Metabolism Genotyping Assay 2 (20X)	0.5 µL	1.0 µL
Sample lysate (up to 25% of the total reaction volume)	2.5 µL	5.0 µL
Nuclease-Free Water	1.5 µL	2.0 µL
Total volume	**10 µL**	**20 µL**

A third SNP assay can be added. Include 0.5 µL of a 20X concentration assay, then decrease the amount of water by 0.5 µL, for a total reaction volume of 10 µL. Include 1.0 µL of a 20X concentration assay, then decrease the amount of water by 1.0 µL, for a total reaction volume of 20 µL.

Note: For a genotyping assay with 3 SNPs, a custom TaqPath™ ProAmp™ Master Mix with no ROX™ dye must be used. For more information, see “Assay design information” on page 6.

2. Mix the components thoroughly, then centrifuge briefly to spin down the contents and eliminate air bubbles.
3. Add the appropriate volume of each reaction to each well of an optical plate.
4. Seal the plate with an optical adhesive cover, then centrifuge briefly to spin down the contents and eliminate air bubbles.

Set up and run the real-time PCR instrument

1. Load the reaction plate in the real-time PCR instrument.
2. Set the appropriate experiment settings and PCR thermal cycling conditions.
   - Select genotyping as the experiment.
   - Select ROX™ dye as the passive reference. If 3 SNP assays are used, select no passive reference.
   - Confirm that real-time data is collected.
   - Confirm that the following data collection points are selected:
     - Pre-read stage
     - Anneal/extend steps of the PCR stage
     - Post-read stage
   - Confirm that the filters are selected for each optical channel that is used for a reporter dye and the passive reference dye.

Note: Fast cycling is not recommended for TaqMan™ Drug Metabolism Genotyping Assays.

Table 3 Genotyping experiments: standard cycling

Step	Temperature	Time	Cycles
Pre-Read	60°C	30 seconds	Hold
Initial denature / Enzyme activation	95°C	5 minutes	
Denature	95°C	15 seconds	40
Anneal / Extend	60°C	60 seconds[1]	
Post-Read	60°C	30 seconds	Hold

[1] Use a 90-second anneal / extend step for TaqMan™ Drug Metabolism Genotyping Assays.

Table 4 Genotyping experiments: fast cycling

Step	Temperature	Time[1]	Cycles
Pre-Read	60°C	30 seconds	Hold
Initial denature / Enzyme activation	95°C	5 minutes	
Denature	95°C	5 seconds	40
Anneal / Extend	60°C	30 seconds	
Post-Read	60°C	30 seconds	Hold

[1] Optional cycling for Fast 96-well or 384-well plates only.

3. Set the reaction volume appropriate for the reaction plate.
4. Start the run.

4 Overview data analysis

Perform data analysis with Diomni™ Design and Analysis (RUO) Software 3 with the Genotyping Analysis Module or the Genotyping Analysis Module (project).

For more information about the software, see “Overview of the analysis software” on page 6.

For more information about using the software, see “Overview of the software documentation” on page 7 and “Related documentation” on page 19.

Confirm that no template control (NTC) wells and positive control wells are assigned.

When analyzing data where crude samples and purified samples are run on the same plate, it is recommended to analyze the crude samples and purified samples independently of each other due to differences in the matrix background.

Samples are reviewed for the following assignments:

- Assay 1 allele 1 homozygote
- Assay 1 allele 2 homozygote
- Assay 1 allele 1/2 heterozygote
- Assay 2 allele 1 homozygote
- Assay 2 allele 2 homozygote
- Assay 2 allele 1/2 heterozygote

A third assay can be included and samples can be reviewed for homozygote and heterozygote assignments.

The software performs automatic calls.

IMPORTANT! Manual calls cannot be performed when more than 1 SNP assay is assigned to one well. A manual call can be performed after the data are exported. Alternatively, contact Support for the option to perform manual calls when more than 1 SNP assay is assigned to one well.

A Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

AVERTISSEMENT ! PRÉCAUTIONS GÉNÉRALES EN CAS DE MANIPULATION DE PRODUITS CHIMIQUES. Pour minimiser les risques, veiller à ce que le personnel du laboratoire lise attentivement et mette en œuvre les consignes de sécurité générales relatives à l'utilisation et au stockage des produits chimiques et à la gestion des déchets qui en découlent, décrites ci-dessous. Consulter également la FDS appropriée pour connaître les précautions et instructions particulières à respecter :

- Lire et comprendre les fiches de données de sécurité (FDS) fournies par le fabricant avant de stocker, de manipuler ou d'utiliser les matériaux dangereux ou les produits chimiques. Pour obtenir les FDS, se reporter à la section « Documentation et support » du présent document.
- Limiter les contacts avec les produits chimiques. Porter des équipements de protection appropriés lors de la manipulation des produits chimiques (par exemple : lunettes de sûreté, gants ou vêtements de protection).
- Limiter l'inhalation des produits chimiques. Ne pas laisser les récipients de produits chimiques ouverts. Ils ne doivent être utilisés qu'avec une ventilation adéquate (par exemple, sorbonne).
- Vérifier régulièrement l'absence de fuite ou d'écoulement des produits chimiques. En cas de fuite ou d'écoulement d'un produit, respecter les directives de nettoyage du fabricant recommandées dans la FDS.
- Manipuler les déchets chimiques dans une sorbonne.

- Veiller à utiliser des récipients à déchets primaire et secondaire. (Le récipient primaire contient les déchets immédiats, le récipient secondaire contient les fuites et les écoulements du récipient primaire. Les deux récipients doivent être compatibles avec les matériaux mis au rebut et conformes aux exigences locales, nationales et communautaires en matière de confinement des récipients.)
- Une fois le récipient à déchets vidé, il doit être refermé hermétiquement avec le couvercle fourni.
- Caractériser (par une analyse si nécessaire) les déchets générés par les applications, les réactifs et les substrats particuliers utilisés dans le laboratoire.
- Vérifier que les déchets sont convenablement stockés, transférés, transportés et éliminés en respectant toutes les réglementations locales, nationales et/ou communautaires en vigueur.
- **IMPORTANT !** Les matériaux représentant un danger biologique ou radioactif exigent parfois une manipulation spéciale, et des limitations peuvent s'appliquer à leur élimination.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  cdc.gov/labs/bmbl
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  who.int/publications/i/item/9789240011311

B

Documentation and support

Related documentation

Document	Publication number
DNA Extract All Reagents Kit Product Information Sheet	MAN0030082
TaqPath™ ProAmp™ Master Mixes User Guide	MAN0015758
Diomni™ Design and Analysis (RUO) Software 3 (Desktop) User Guide	MAN0030162
Diomni™ Design and Analysis (RUO) Software 3 (On-Premises) User Guide	MAN1000091
Diomni™ Design and Analysis (RUO) 3 User Guide (Thermo Fisher™ Connect Platform)	MAN0030163
Diomni™ Design and Analysis (RUO) Software 3 Genotyping Analysis Module User Guide	MAN0030164
Diomni™ Design and Analysis (RUO) Software 3 Genotyping Analysis Module (project) User Guide	MAN1000138

Customer and technical support

Visit **thermofisher.com/support** for the latest service and support information.

- Worldwide contact telephone numbers
- Product support information
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.