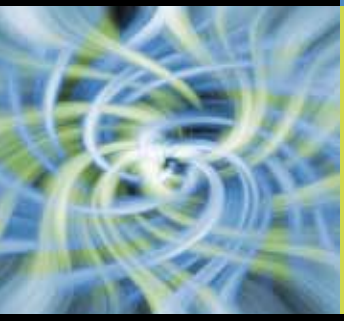

Thermo Scientific
DyNAmo SNP Genotyping Master Mix

Technical Manual

F-480S 200 reactions (25 µl each)
F-480L 1000 reactions (25 µl each)
F-480XL 4000 reactions (25 µl each)

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1. Description

Thermo Scientific DyNAmo SNP Genotyping Master Mix is designed for allelic discrimination (such as single nucleotide polymorphism genotyping) using detection methodology based on hydrolysis probes. Single nucleotide polymorphisms (SNPs) have a significant role as markers for various genetic properties. SNP genotypes can also be used for identification of individuals or characterization of populations by SNP genotype frequencies.

A typical SNP has two alleles. Therefore two labeled probes are designed – one for each sequence variant. When using hydrolysis probes (such as TaqMan® probes), signal is generated when the 5'→3' nuclease activity of the DNA polymerase cleaves the hybridized probe yielding an unquenched free fluorophore. Primers and probes for the analysis can either be designed by the user or predesigned by commercial companies.

DyNAmo™ SNP Genotyping Master Mix is a ready-to-use master mix containing a uniquely engineered hot start DNA polymerase optimized to provide maximal resolution between different genotypes. The master mix also contains a blue dye that helps to visualize reaction mixes during setup. Additionally, a sample buffer with a yellow dye is provided to keep track of pipetting. Using the yellow sample buffer is optional, but it significantly helps to visualize the wells where sample has already been added. The PCR premix without the sample is blue, and adding the sample turns the reaction mix green. DyNAmo SNP Genotyping Master Mix is compatible with all major real-time PCR instruments, including Thermo Scientific PikoReal Real-Time PCR System.

2. Kit components

DyNAmo SNP Genotyping Master Mix	F-480S	F-480L	F-480XL
2x DyNAmo SNP Genotyping Master Mix (contains a hot start Tbr DNA polymerase, optimized PCR buffer, $MgCl_2$, dNTP mix including dUTP)	2 × 1.25 ml (200 reactions of 25 µl)	10 × 1.25 ml (1000 reactions of 25 µl)	4 × 12.5 ml (4000 reactions of 25 µl)
50x ROX passive reference dye	1 × 250 µl	1 × 1.25 ml	2 × 1.25 ml
40x Sample Buffer with yellow dye	1 ml	2 × 1 ml	1 × 10 ml

Material safety data sheet (MSDS) is available at www.thermoscientific.com/fzmsds.

3. Shipping and storage

DyNAmo SNP Genotyping Master Mix is shipped on gel ice. Upon arrival, store all the components at -20°C. When using the 2x master mix, the leftover thawed mix can be refrozen and stored at -20°C without affecting the performance of the product.

4. Notes about reaction components

Table 1. General recommendations.

Categories	Comments
Kit storage	Store at -20°C.
Consumables	Follow the recommendations of the PCR instrument manufacturer.
Reaction volume	Follow the recommendations of the PCR instrument manufacturer. See section 4.8 for more detailed information.
Template amount	Recommended template amount is 1-10 ng per reaction.
Primer and probe design	Use primers with matched Tm. Avoid inter-primer and intra-primer complementary sequences. We recommend calculating Tm by the nearest-neighbor method as described by Breslauer *et al.*[1] Instructions for Tm calculation and a link to a calculator using the nearest-neighbor method can be found on the Thermo Scientific website (www.thermoscientific.com/pcrwebtools).

4.1 2x DyNAmo SNP Genotyping Master Mix

The 2x DyNAmo SNP Genotyping Master Mix contains a uniquely engineered hot start Tbr DNA polymerase, optimized PCR buffer, $MgCl_2$ and dNTP mix including dUTP. Only the template, primers and probes need to be added by the user. The 2x master mix also contains a blue dye that helps track pipetting of the master mix into the reaction wells. The absorption maximum of the blue dye is at 615 nm.

4.2 Hot Start *Tbr* DNA polymerase

The uniquely engineered hot start *Tbr* DNA polymerase present in DyNAmo SNP Genotyping Master Mix is a chemically reversibly inactivated enzyme. The hot start characteristic of the polymerase facilitates reaction setup at room temperature, as reactivation of the polymerase does not occur until the initial denaturation step in the PCR protocol. Prior to this, the polymerase is inactivated, preventing extension of non-specifically bound primers during reaction setup and the first heating cycle, maintaining PCR specificity. The hot start *Tbr* DNA polymerase also displays the required 5'→3' exonuclease activity necessary for hydrolysis probe chemistries, such as for TaqMan chemistry.

4.3 40x Sample Buffer with yellow dye

The 40x Sample Buffer with yellow dye is used to visualize pipetting of samples into the reaction mix. After adding the sample to the blue reaction mix, the reaction turns green, making it easy to track pipetting of samples. The yellow sample buffer can be added to existing samples, and the samples can then be stored at -20°C if not used immediately. The buffer is provided as a 40x concentrate and used in 1x concentration in the final reaction. Using the yellow sample buffer is optional. The absorption maximum of the yellow dye is at 413 nm.

4.4 PCR primers and probes

Careful primer and probe design is important to minimize nonspecific primer annealing and primer-dimer formation. Standard precautions must be taken to avoid primer-dimer or hairpin loop formation. Most primer design software tools will yield well-designed primers for use in real-time PCR.

The optimal final concentration for the primers is usually 0.5 µM and 0.25 µM for the probes. Primers and probes for genotyping analysis can either be designed by the user or predesigned by commercial companies

4.4.1 Hydrolysis probes

Allelic discrimination with the DyNAmo SNP Genotyping Master Mix is based on two allele-specific hydrolysis probes, each being a perfect match for one allele and labeled with a specific fluorescent dye. Binding to a matching sequence is more efficient than binding to a sequence with one mismatch. Therefore, a perfect match generates a stronger signal.

A hydrolysis probe consists of a target-specific sequence, which is usually around 20 bp long. The probe has a fluorescent reporter molecule (fluorophore) at one end and a quencher at the other end of the probe. The quencher receives the energy from the fluorophore and quenches the fluorescence. During the PCR protocol the probe hybridizes to its complementary sequence in the target and one of the PCR primers anneals in the same strand upstream from the probe. When the polymerase extends the primer, it encounters the probe, hydrolyses it from the 5′ end, and thus cleaves the reporter from the probe. When the reporter is cleaved, it is no longer quenched and the increase in the fluorescence can be detected with the real-time PCR instrument.

4.5 Template preparation and quality

Purity of nucleic acid templates is particularly important in real-time PCR applications, as contaminants may interfere with fluorescence detection and/or DNA amplification. Most commercial DNA purification kits give satisfactory results for real-time PCR.

4.6 ROX passive reference dye

For most real-time instruments ROX passive reference dye is not required, but on some instruments it is used to normalize for non-PCR-related fluorescence signal variation. Passive reference dye does not take part in the PCR and its fluorescence remains constant during the reaction. The amount of ROX passive reference dye needed can vary depending on the type of excitation. The amount of ROX dye needed with real-time cyclers which use argon laser as the excitation light source or which have excitation filters that are not optimal for ROX dye may be greater than with instruments that excite efficiently near 585 nm.

The ROX dye is provided as a 50x solution dissolved in a buffer that is compatible with the reaction buffer. Usually the ROX concentrations recommended in Table 2 are sufficient for passive reference dye normalization with all instruments, but it is important to make sure that the intensity is strong enough to produce stable signal for normalization. Note that the use of ROX passive reference dye may not be possible with some fluorescent dyes.

Table 2. ROX concentration.

Real-time PCR instrument	Recommended ROX concentration
Applied Biosystems StepOne™ Real-Time PCR System	1x
Applied Biosystems 7300 Real-Time PCR System	1x
Applied Biosystems 7900HT Fast Real-Time PCR System	1x
Applied Biosystems ViiA 7 Real-Time PCR System	0.3x
Applied Biosystems 7500 Real-Time PCR System	0.3x
Agilent Mx3000P® QPCR System	0.3x (optional)
Agilent Mx3005P® QPCR System	0.3x (optional)
Agilent Mx4000® QPCR System	0.3x (optional)

4.7 UNG (UDG) treatment

Due to the high sensitivity of real-time PCR, even minute amounts of contaminating DNA can lead to false positive results. If dUTP is used in real-time PCR, the carry-over contamination from previous PCR runs can be prevented by treating the reaction samples with UNG before PCR. UNG (uracil-N-glycosylase) digests dU-containing DNA, and the digested DNA cannot act as a template in PCR[2]. UNG is inactivated during the first denaturation step in PCR. The UNG treatment step (50°C for 2 min) has no negative effect on real-time PCR performance because the hot-start DNA polymerase is not reactivated at 50°C. DyNAmo SNP Genotyping Master Mix contains dUTP and therefore UNG treatment can be used.

To minimize contamination risk in general, tubes or plates containing reaction products should not be opened or analyzed by gel electrophoresis in the same laboratory area that is used to setup reactions.

4.8 Reaction volume

Recommended reaction volume depends on the real-time PCR instrument. A reaction volume of 20-50 µl is usually recommended for 96-well real-time PCR instruments. Typical reaction volume range for 384-well systems is 5–20 µl. Follow the recommendations of the instrument and consumables manufacturer.

5. Reaction setup and cycling protocols

- Perform the reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- As the hot-start DNA polymerase is inactive during PCR setup, it is not necessary to do the setup on ice.
- Make sure all the reaction components are properly mixed.
- Pipette with sterile filter tips.
- Minimize the exposure to light after adding ROX passive reference dye and/or probes to the 2x master mix.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes to avoid pipetting very small volumes.
- Use optically clear caps or sealers to achieve maximum signal.
- Use a cap sealing tool or firm finger pressure to close caps properly, or use a film sealer.
- Avoid touching the optical surface of the cap or sealing film without gloves, as fingerprints may interfere with fluorescence measurements.
- Use powder-free gloves.
- Plates or strips should be centrifuged before starting the cycling program to force the solution to the bottom of the tubes and to remove any bubbles.
- Use molecular biology grade H_2O.

5.1 Reaction setup for instruments not using ROX normalization

(If you are using an Applied Biosystems real-time PCR instrument, see section 5.2 in this chapter.)

1. Program the real-time PCR instrument for allelic discrimination analysis (see cycling protocol on section 5.3 in this chapter).
2. Perform reaction setup as instructed below.
3. Run PCR protocol (For analyzing results, see chapter 6).

Reaction setup

1. Thaw the template DNA, primers, probes and 2x master mix (and the ROX passive reference dye, if needed. Refer to the section 5.2 in this chapter for more guidelines). Mix the individual solutions to ensure homogeneity (e.g. by vortexing). This is especially important for the master mix. Note that vortexing typically introduces less air bubbles than other methods.
2. If using the yellow sample buffer (optional), add buffer to the samples to a concentration that will yield 1x in the final reaction volume. For example, if 5 µl of sample is to be used in a 25 µl reaction volume, 5x buffer concentration in the sample results in 1x buffer concentration in the final reaction (for example 0,63 µl/5 µl sample).
3. Assemble the reactions into strip tubes or plate wells following the guidelines in Table 3. Reverse pipetting technique can be used to avoid air bubble formation.
4. Seal the strips or plate with appropriate sealer, place them in the thermal cycler and start the cycling program.

   Note: It is also possible to store the prepared reactions before cycling up to 3 days at +4°C.

Table 3. Reaction setup for instruments not using ROX normalization.

Components (In order of addition)	25 µl reaction	5 µl reaction	Final concentration	Comments
2x DyNAmo SNP Genotyping Master Mix	12.5 µl	2.5 µl	1x	Mix thoroughly. Avoid air bubble formation.
Forward primer	X µl	X µl	0.5 µM	
Reverse primer	X µl	X µl	0.5 µM	
Probe for allele A	X µl	X µl	0.25 µM	
Probe for allele B	X µl	X µl	0.25 µM	
Template DNA	X µl	X µl		Optimal amount 1-10 ng
H_2O	add to 25 µl	add to 5 µl		

For different volumes, adjust all components proportionally. To include ROX passive reference dye, follow the guidelines described in the next chapter (Applied Biosystems instruments).

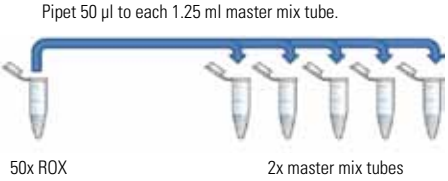
5.2 Reaction setup for Applied Biosystems real-time PCR instruments

1. Program the real-time PCR instrument for allelic discrimination analysis (see cycling protocol on section 5.3 in this chapter).
2. Add ROX passive reference dye to the 2x DyNAmo SNP Genotyping Master Mix (optional, see below).
3. Perform reaction setup as instructed on page 10.
4. Run PCR protocol (For analyzing results, see chapter 6).

Addition of ROX passive reference dye

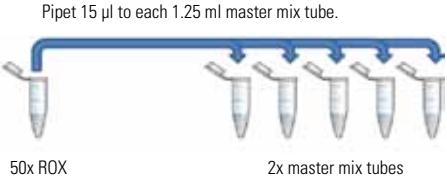
ABI 7300, 7900, StepOne™: 1x ROX final concentration

1. Thaw and carefully mix 50x ROX and 2x master mix tubes.
2. Add 50 µl of 50x ROX to each 1.25 ml 2x master mix tube.
3. Mix carefully again.
4. Store at -20°C.



ABI 7500, Viia7: 0.3x ROX final concentration

1. Thaw and carefully mix 50x ROX and 2x master mix tubes.
2. Add 15 µl of 50x ROX to each 1.25 ml 2x master mix tube.
3. Mix again carefully.
4. Store at -20°C.



1. Thaw the template DNA, primers, probes and 2x master mix (where ROX passive reference dye has been added). Mix the individual solutions to ensure homogeneity (e.g. by vortexing). This is especially important for the master mix. Note that vortexing typically introduces less air bubbles than other methods.
2. If using the yellow sample buffer (optional), add buffer to the samples to a concentration that will yield 1x in the final reaction volume. For example, if 5 µl of sample is to be used in a 25 µl reaction volume, 5x buffer concentration in the sample results in 1x buffer concentration in the final reaction (for example 0,63 µl/5 µl sample).
3. Assemble the reactions into strip tubes or plate wells as instructed in Table 4. Reverse pipetting technique can be used to avoid air bubble formation.
4. Seal the strips or plate with appropriate sealer, place them in the thermal cycler and start the cycling program.

Note: It is also possible to store the prepared reactions before cycling up to 3 days at +4°C.

Table 4. Reaction setup for Applied Biosystems real-time PCR instruments.

Components (In order of addition)	25 µl reaction	5 µl reaction	Final concentration	Comments
2x DyNAmo SNP Genotyping Master Mix with ROX added (see instructions above)	12.5 µl	2.5 µl	1x	Mix thoroughly. Avoid air bubble formation.
Forward primer	X µl	X µl	0.5 µM	
Reverse primer	X µl	X µl	0.5 µM	
Probe for allele A	X µl	X µl	0.25 µM	
Probe for allele B	X µl	X µl	0.25 µM	
Template DNA	X µl	X µl		Optimal amount 1-10 ng
H_2O	add to 25 µl	add to 5 µl		

For different volumes, adjust all components proportionally.

5.3 Cycling protocol for all instruments

Program the real-time PCR instrument to perform an allelic discrimination run. Depending on the instrument, data collection for allelic discrimination is setup either by reading the fluorescence from the first and last PCR cycle, or adding pre-PCR and post-PCR data collection steps. Follow the instrument manufacturer's recommendations.

Table 5. Cycling protocol for all instruments.

Step	Purpose	Temp	Time	Cycles	Comments
Pre-read (Baseline RFU)	Pre-PCR data collection	25°C*	30 s	1	The time required depends on the instrument used; 30 seconds should be enough for all instruments. If this step is not possible, perform the pre-PCR data collection in the first PCR cycle.
	UNG incubation			1	Optional, see section 4.7 and instructions below this table.
1	Initial denaturation	95°C	7 min	1	This step is needed to activate the hot start DNA polymerase and to denature the template DNA.
2	Denaturation	95°C	5 s	40 cycles	
3**	Annealing / extension	60°C	30 s		
4	Fluorescence data collection				
5	Cooling	30°C	10 s	1	Cooling down the plate for safe handling.
Post-read (Endpoint RFU)	Post-PCR data collection	25°C*	30 s	1	The time required depends on the instrument used; 30 seconds should be enough for all. If this step is not possible, perform the post-PCR data collection in the last PCR cycle.

* Use 25°C if possible, or follow the instrument manufacturer's recommendations.

** Use the Tm calculator at www.thermoscientific.com/pcrwebtools to determine Tm of the primers. Use 50 mM KCl and 0.5 µM primer concentration when calculating Tm (or the primer concentration in your reaction if optimized to other than 0.5 µM). Design primers to anneal efficiently at 60°C (Tm should be about 65°C).

UNG incubation (optional)

If UNG enzyme is used, incubate 2 minutes at 50°C. This step does not negatively affect real-time PCR performance because the hot-start DNA polymerase is not active at 50°C. If heat-labile UNG is used, decrease the incubation temperature and increase time in accordance with the manufacturers' instructions.

Initial denaturation/reactivation at 95°C for 7 minutes is needed to ensure a complete reactivation of the hot start DNA polymerase and denaturation of the template.

Denaturation at 95°C for 5 seconds is sufficient in most cases.

Annealing and extension

For most amplicons, a combined annealing and extension for 30 seconds at 60°C works well if the primers are designed to anneal efficiently at 60°C (Tm should be about 65°C). An

annealing temperature of 60°C has proven to be successful for a wide range of primer pairs. These guidelines are based on Tm values (50 mM salt and 0.5 µM primer) calculated by the nearest-neighbor method as described by Breslauer *et al.*[1] Instructions for Tm calculation and a link to a calculator using the nearest-neighbor method can be found on www.thermoscientific.com/pcrwebtool.

Different software may give different Tm values. If primer-dimers are observed, the easiest solution is often to redesign primers. Another alternative is to optimize the annealing temperature by performing additional runs, varying the annealing temperature in each cycle by 2°C. A temperature gradient feature on the thermocycler can also be used, if available.

Number of cycles

For most applications, 40 cycles of amplification should be sufficient even when the template is present at a very low copy number.

6. Analysis

In hydrolysis probe-based genotyping, results are typically based on end-point fluorescence intensities. Two TaqMan probes, each being a perfect match for one allele, are labeled with different fluorescent dyes. The two probe sequences only differ at the SNP site. Binding to a matching sequence is more efficient than binding to a sequence with one mismatch. Therefore a perfect match generates a stronger signal.

With homozygous samples the fluorescence from a matching probe is significantly higher than that from the mismatching probe. In heterozygous samples a complementary sequence is present for both probes, which results in relatively high signal from each probe (Figure 1,2).

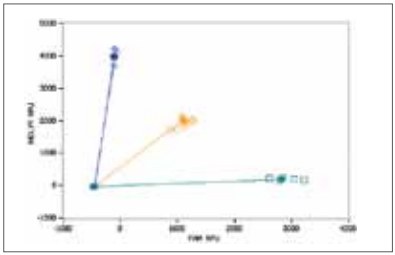


Figure 1. Allelic discrimination analysis performed in PikoReal Real-Time PCR System. Different genotypes are discriminated based on the change of fluorescence before and after amplification. The fluorescence intensity of probe A is plotted against the fluorescence intensity of probe B, which creates a scatter plot. The software uses the plot to automatically call the genotypes.

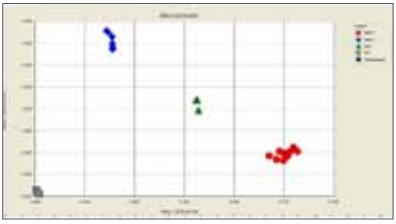


Figure 2. Allelic discrimination analysis performed in Applied Biosystems 7500 Fast Real-Time PCR System.

7. Troubleshooting

Possible causes	Comments and suggestions
No or late increase in fluorescence signal	
Error in cycler setup	• Make sure that the instrument settings are correct for the experiment.
Missing components (e.g. primers, probes or template) or pipetting error	• Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
Missing essential step in PCR protocol/ protocol not optimal	• Make sure the recommended PCR protocol is used. If necessary, optimize using the recommended protocol as a starting point.
Insufficient activation of the hot start *Tbr* DNA polymerase	• Make sure 95°C 7 min was used for the initial denaturation step. • Make sure cycler block temperature is accurate.
Sample not configured properly	• Check the plate configuration.
qPCR primer and/or probe design or concentration not optimal	• Re-check primer and probe design. See section 4.4. • Use primer concentration of 0.5 µM and probe concentration of 0.25 µM if not otherwise optimized.
Improperly stored or expired reagents	• Check storage conditions and expire dates of the reagents.
Insufficient starting template	• Check the calculation of the template stock concentration; increase the template amount if possible.
No clear clusters	
No amplification	• See instructions above.
Plate read analysis not defined correctly	• Make sure the recommended PCR protocol is used.
Too many clusters	
Sample components affect fluorescence end point signal Inhibitors in the sample	• Use less sample if possible to introduce less interfering factors in to the reaction. • Check the purity of the sample. Re-purify if necessary.
Sequence variation in primer or probe binding regions (other than the SNP that probes are designed for)	• Check sequence region from databases for known variation. • Sequence the product if necessary.
Clusters too close to each other	
Suboptimal probe design	• Check the assay design and redesign if necessary.
Data acquisition in too high temperature	• Use 25°C for pre and post read. See protocol in section 5.3.
Increased fluorescence or C_q value for "No Template Control" (NTC)	
Suboptimal assay design	• Check the assay design for primer/probe dimers and redesign if necessary.
Contamination	• Clean the lab environment and equipment. Consider UNG treatment if PCR product contamination is suspected.

8. References

1. Breslauer *et al.* (1986) *Proc. Nat. Acad. Sci.* 83: 3746-50.
2. Longo M.C. *et al.* (1990) *Gene* 93: 125-28.

Appendix I: General molecular biology data

Table 6. Spectrophotometric conversions for nucleic acid templates.

1 A_{260} unit*	Concentration (µg/ml)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

* Absorbance at 260 nm = 1 (1 cm detection path).

Table 7. Molar conversions for nucleic acid templates.

Nucleic acid	Size	pmol/µg	Copies/µg**
1 kb DNA	1 000 bp	1.52	9.1×10^{11}
pUC19DNA	2 686 bp	0.57	3.4×10^{11}
Lambda DNA	48 502 bp	0.03	1.8×10^{10}
Escherichia coli	4.7×10^{6} bp	3.2×10^{-4}	1.9×10^{8}
Human	3.2×10^{9} bp	4.7×10^{-7}	2.8×10^{5}

** For single-copy genes.

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DyNAmo™ SNP Genotyping Master Mix contains patent-pending technology.

The quality system of Finnzymes Oy is certified according to standard SFS-EN ISO9001:2008.

Thermo Scientific DyNAmo SNP Genotyping Master Mix Technical Manual

v2_12.2011

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