TIB MOLBIOL

LightCycler® *Scrapie Susceptibility* Mutation Detection Kit

Dual Color LightCycler® Kit for the detection of ovine prion protein (PRNP) gene mutations in the codons 136, 154, 171 in a single reaction for the use with LightCycler® 1.x / 2.0 instruments.

Kit for 6 x 16 (96) reactions with a final volume of 20 µl each - **store at room temperature (18-25°C)**

Additional reagents required: (Roche Diagnostics)	FastStart DNA Master Hybridization Probes	Cat. 03 003 248 001
	LightCycler® – Color Compensation Set	Cat. 12 158 850 001
	HighPure PCR Template Preparation Kit	Cat. 11 796 828 001
Other reagents needed	Ethanol, Isopropanol	
Roche Diagnostics instrument	LightCycler® 1.0 (grey instrument, version II)	
	LightCycler® capillaries, 8 x 96	Cat. 11 909 339 001
This product	if ordered through Roche Diagnostics	Cat. 04 374 100 001

1. Introduction

Scrapie is a fatal neurodegenerative disease of sheep that belongs to the group of prion diseases. The bovine spongiform encephalopathy (BSE) and the new Creutzfeldt-Jakob (nCJD) disease in humans are closely related. The host encoded prion protein (PRNP) plays a central role in the disease process.

PRNP gene polymorphisms have been found to be associated with disease susceptibility and pathogenesis. Based on the triplet sequences present at codons 136, 154 and 171, the most important allelic variants reported are PRNP VRQ, ARR, ARQ, ARH and AHQ.

Literature: Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. Belt PB, Muileman IH, Schreuder BE, Bos-de Ruijter J, Gielkens AL, Smits MA. J Gen Virol. 1995 Mar;76 (Pt 3):509-17.

2. Kit Description

The ovine PRNP gene is amplifed by PCR. Hybridization probes are designed to cover the inidicated codons. Base changes cause a lowered melting temperature, which can be read out in genotypes. A high melting point in channel F3 (65°C) indicates the presence of the scrapie-resistant variant.

PRNP	Susceptibility	Channel F2	Channel F2	Channel F3
ARQ (wildtype)	highly susceptible	62.3°C	56.1°C	60.2°C
VRQ	highly susceptible	**67.0°C**	56.1°C	60.2°C
ARH	heterologously susceptible	62.3°C	56.1°C	**55.6°C**
AHQ	susceptible	62.3°C	**51.3°C**	60.2°C
ARR	**resistant**	62.3°C	56.1°C	**65.3°C**

Table 1. Genotypes and melting temperatures (differences are indicated in bold)

3. Kit Contents and Storage

6 x 1 vial containing premixed primers and hybridization probe for 16 reactions

Store the reagents in a dark place at room temperature (18-25°C). **Do not freeze** lyophilized reagents. Reagents are stable for at least 3 months after purchase. Store dissolved reagents refrigerated (4°C).

4. Experimental – Extraction Protocol (total time approx. 40 min)

Preparation of solutions (HighPure PCR Template Preparation Kit)

- Dissolve proteinase K in 4.5 mL water, store in aliquots at 4°C (daily use) or at –20°C
- Inhibitor Removal Buffer (black): add 20 mL ethanol, store buffer at room temperature
- Wash Buffer (blue) : add 80 mL ethanol, store buffer at room temperature

Extraction protocol (optimized protocol, Roche Diagnostics protocol see ref. below)

- Pipette 200 µl ovine whole blood into 1.5 mL microreaction tube,
  add 40 µl ProtK and 200 µl Lysis buffer (green), vortex, and keep for 30 min at 70°C

- Add 100 µl isopropanol, vortex, add solution to the extraction column
- Centrifuge column for 1 min at 8,000 rpm, discard flowthrough
- Add 450 µl Inhibitor Removal Buffer (black) and repeat centrifugation at 8,000 rpm, discard
- Add 450 µl Wash Buffer (blue) and repeat centrifugation at 13,000 rpm, discard flowthrough
  (optional repeat this step – for reference see Roche protocol, internet address below)
- Put column into new tube and add 100 µl preheated (70°C) Eluation Buffer (t ransparent)
- Centrifuge column for 1 min at 8,000 rpm, keep flowthrough = purified DNA

http://www.roche-applied-science.com/prod_inf/manuals/napi_man/pdf/chapter2/page_15-24.pdf

Nucleic acid extraction can also be performed using the "MagNA Pure LC DNA Isolation Kit I" (Cat. 300 3990) using the "MagNA Pure Instrument" as described in the manufacturer's instructions.

5. Experimental – Amplification Protocol

The following procedure was developed for use with the LightCycler® Instrument.
Start programming before preparing the solutions. See the LightCycler® Operator's Manual for details.

Sample material: Use aqueous nucleic acid extracts (e.g. High Pure PCR Template Preparation kit).

Negative control: Always run one negative control - replace the template DNA with water.

Using the kit for the first time, run pretyped reference samples for all genotypes. Repeat this control from time to time. Run controls in case of dubious results.

(A) Preparation of parameter-specific Reagents (one vial = 16 reactions)

One reagent vial (labeled transparent vial with a violet lid containing a blue pellet) contains all primers and probes to run 16 LightCycler® reactions.

Add 65 µl PCR-grade water (e.g. tube 3 from the Roche FastStart kit) to reagent vial containing the blue pellet, mix the solution (vortex) and spin down.

►Use each 4 µl **reagent** for a 20 µl PCR reaction

| This solution is stable three days or longer if stored refrigerated at 4°C. Avoid prolonged exposure t o light.

(B) Preparation of the LightCycler® Reaction Mix (total time approx. 10 min)

In a 1.5 mL reaction tube placed on ice, add the following components in the order mentioned below:

Volume		Component	Note
11.2	µl	water, PCR-grade	(eg. vial 3, colorless cap, from the Roche Diagnostics FastStart kit)
0.8	µl	Mg solution 25 mM	(vial 2, blue cap, provided in the Roche Diagnostics FastStart kit)
4.0	µl	**reagent** mix	(parameter specific reagents containing primers and probes, see **A**)
2.0	µl	FastStart mix	(vial 1, combined from vials 1a and 1b, red cap, see manual)
2.0	µl	sample or **reference**	
20	µl	final volume	

Mix gently, spin down and transfer to a LightCycler® capillary.

(C) Run the LightCycler® (total run time approx. 50 min)

Start run. Results are obtained within 1 hour (less than 2 hours including extraction)

6. Experimental – Programming

The protocol consists of four program steps :

- Program 1: Denaturation of sample and activation of the reagents (FastStart)
- Program 2: PCR-amplification of the target DNA (see step 'Quantification' in table 2)
- Program 3: Melting curve for genotype analysis (see step 'Melting Curve' in table 2)
- Program 4: Cooling the instrument

Fluorescence parameters / gain settings (LightCycler software older than version 3.5): Channel F1:1, F2:10, F3:30

For use with the Roche FastStart reagents, run an initial heating for 8:00 min at 95°C.

Composition of the reaction mix			Parameter	Value			Parameter	Value		
H_2O (tube 3)	11.2	µl	Cycles	45			Cycles	1		
$MgCl_2$ (tube 2)	0.8	µl	Analysis Mode	**Quantification**			Analysis Mode	**Melting Curve**		
Reagent mix	4.0	µl		Segment				Segment		
FastStart mix	2.0	µl		1	2	3		1	2	3
Sample	2.0	µl	Target Temp [°C]	95	60	72	Target Temp [°C]	95	45	75
Final volume:	20	µl	Incubation Time [sec]	10	10	15	Incubation Time [sec]	120	60	0
Final $MgCl_2$ concentration:			Transition Rate [°C/s]	20	20	2	Transition Rate [°C/s]	20	20	0.2
$MgCl_2$	2.0	mM	Acquisition Mode	none	**single**	none	Acquisition Mode	none	none	**cont**

Finally the Rotor should be cooled down to 40°C for 30 s. Table 2

7. Data Analysis

Switch the color compensation mode on. If this mode is not enabled, run the color compensation program. Use the melting curve analysis section. Follow the instructions in the manual.

Select F2/back F1 for the analysis for mutations in the codons 136 and 154
Select F3/back F1 for the analysis for mutation in the codon 171

Compare data with samples of known genotypes (controls). Repeat samples with unclear results. Check these PCR products by agarose gel electrophoresis; the product must be one band. Different melting curves are an indication of the presence of other (rare) genotype variants. We recommend analyzing these samples by DNA sequencing or sending these samples to a reference center.

Check the amount of the samples - the quantification plot gives you information about the amount of the samples; high concentrated samples could yield problems for the melting curve. Signals should rise after 25-35 cycles. Dilute samples if the curves start much earlier. Perform data analysis, as described in the LightCycler Operator's Manual. We recommend using the Second Derivative Maximum method. The cycle number of the Crossing Point of each sample is calculated automatically.

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These reagents were developed and manufactured by TIB MOLBIOL®, Berlin, Germany. LightCycler® hybridization probes produced under license from Roche Diagnostics. LightCycler® is a registered trademark of a member of the Roche group.
The protocol was optimized by Dr. Jean-Marc Costa, Paris. Publication submitted 2003, Dr. Bertrand Le Tallec, Chambéry.
Reverse primer was changed november 2006 based on the report of a deviation due to a SNP at codon 176 (version 1.3).

8. Sample Data - Typical Results

Sample data
Channel F2

Sample data
Channel F3

Data supplied by B. Le Tallec

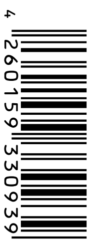
Fig.1. Sample data for PRNP typing **Upper panels**: LC640
Lower panels: LC705

Samples 1, 2, 3 genotype ARR / ARR (homozygous, both alleles position 171 mutated, 171R)
Samples 4, 5, 6 genotype VRQ / VRQ (homozygous, both alleles position 136 mutated, 136V)
Sample 7 genotype ARH / ARQ (heterozygous, one allele position 171 mutated, 171H)
Samples 8, 9 genotype ARQ / ARQ (homozygous, both alleles wildtype, ARQ)
Sample 10 genotype AHQ / AHQ (homozygous, both alleles position 154 mutated, 154H)

Position	Amino Acid	Genotype	Melting temperature	LC Channel
136	**A**	Wildtype	**62,3°C**	F2 (LC640)
	V	Mutation	**67,0°C**	F2 (LC640)
154	**R**	Wildtype	**56,1°C**	F2 (LC640)
	H	Mutation	**51,3°C**	F2 (LC640)
171	**Q**	Wildtype	**60,2°C**	F3 (LC705)
	H	Mutation	**55,6°C**	F3 (LC705)
	R	Mutation	**65,3°C**	F3 (LC705)

Table 3

Note: Observed temperatures can differ due to buffer components (salt) coming from the extraction process and possibly due to different instrument settings. However, the temperature differences between the individual types are rather constant. Use known (pretyped) samples for calibration.



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