Preface

Potassium channels are important regulators of membrane excitability, which in turn determines cellular function. They form a super-family of ion channels that are regulated by a diverse range of chemical and physical stimuli. In excess of 80 genes in the human genome encode pore-forming potassium channel subunits. Diversity is further enhanced by alternative splicing of subunit mRNA, heteromeric assembly between pore-forming subunits, or by association with accessory proteins. The basis of several inherited diseases lies in potassium channel gene mutations, and the pharmacological manipulation of potassium channel function is increasingly important as a strategy in the treatment of disease. This provides researchers with the challenge of developing tools and experimental procedures to probe potassium channel function and identify chemicals that modulate their behaviour.

We have progressed since the initial mid-twentieth century studies of delayed rectifier, anomalous rectifier, and leak potassium currents, and now ask questions such as "What does a potassium channel look like?", "How do changes in membrane potential gate a potassium channel?", or "Which proteins assemble to form a potassium channel complex in this particular cell?". Many of these questions cannot be answered by electrophysiological techniques alone, thus scientists have adopted techniques from other disciplines to assist their studies.

This volume describes a range of experimental approaches that have been developed to investigate potassium channel structure, function, pharmacology, cell biology, gene expression, and their role in disease. Many of these techniques study potassium channels as cellular proteins as well as the resultant membrane biophysics. They are proteins that are synthesised according to mRNA sequence, trafficked to the correct location in the cell at the appropriate time, interact with other protein components, change conformation following stimulation, all in addition to providing a conduit for potassium ions to cross membranes. Naturally, content covering the topic of ion channels would not be without electrophysiological techniques, but here they are focused on those that enable the study of intracellular modulation, which is of particular importance to many potassium channels.

All investigators, including new researchers and discipline-hopping scientists, will benefit from this volume. Within the various chapters you will also find protocols for several standard laboratory techniques, such as cell culture, transfection, *Xenopus* oocyte preparation, Western blotting, and whole-cell patch clamp recording. Indeed, the techniques found in this book can be applied to the study of many other types of ion channel. There is an increasing trend to answer scientific questions using a wide range of approaches and it is hoped that the techniques outlined in this volume will help provide finishing touches to research projects or provide new avenues of investigation.

Leeds, UK — Jonathan D. Lippiat

Chapter 2

Quantitative RT-PCR Methods for Investigation of Low Copy Potassium Channel Gene Expression in Native Murine Arteries

Alex Cheong, Samuel J. Fountain, and David J. Beech

Summary

Voltage-gated K^+ channels (K_V channels) are encoded by the *KCNx* gene family and have such a wide range of properties that it is necessary to identify the precise expression profile that is instrumental in governing the electrical phenotype of a cell and its response to extrinsic factors. Real-time quantitative RT-PCR methodology has been developed and validated for specific RNA species in vascular smooth muscle cells. We have shown that most of the *KCNA* gene family, encoding the major $K_V\alpha1$ subunits, was markedly up-regulated in the resistance artery compared to the thoracic aorta, in line with reported patch-clamp recordings. Thus quantitative real-time RT-PCR data can be translated into physiological response.

Keywords: Potassium channel, Real-time RT-PCR, Smooth muscle.

2.1. Introduction

KCNA genes are mammalian homologues of the *Drosophila* melanogaster *Shaker* K^+ channel gene. Each of the *KCNA* gene products are K^+ pore-forming subunits that can be functional homotetramers, or heterotetramers consisting of any $K_V\alpha1$ but not α- or β-subunits of other K_V channels *(1)*. $K_V\alpha1$ subunits are known to be expressed throughout the vasculature including the cerebral arteries *(2–4)*, the mesenteric artery *(5, 6)* the pulmonary arteries *(7, 8)*, the coronary arteries *(9)*, the renal artery *(10)*, the portal vein *(11)* and the aorta *(5, 6, 12)*. Such differential phenotypes may be a mechanism for physiological control of a blood vessel and local tissue perfusion.

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Several challenges present themselves when using small tissues such as murine mesenteric arteries: the isolation of RNA from a specific type of cell – in our case the smooth muscle layer, the small amount of RNA which can be isolated, and the low copy number of genes encoding the potassium channels. Nonetheless, the advent of real-time quantitative RT-PCR has provided a reliable and straightforward methodology which we have used to describe the distinctive cell-specific expression patterns of *KCNA* genes between a conduit artery and a resistance artery *(6)*.

Coupled with other techniques such as immunocytochemistry, western blot and electrophysiology, quantitative real-time RT-PCR can provide solutions to translate the expression profile of potassium channel genes and proteins into functional signals.

2.2. Materials

2.2.1. Tissue Dissection

1. Hank's solution for dissection: 137-mM NaCl, 5.4-mM KCl, 0.34-mM NaH_2PO_4, 0.44-mM K_2HPO_4, 8-mM glucose, 5-mM HEPES, 0.01-mM $CaCl_2$, pH 7.4 with NaOH. Filtered through 0.2-μm membrane filter (NUNC), and kept on ice.
2. Dumonstar 55 dissection forceps.
3. Scalpel, size 10.
4. Sylgard 184 silicone elastomer (Dow Corning).
5. Entomological pins (local Hobby shop).
6. Endothelial removal solution: 0.1% (v/v) Triton X-100 in water.
7. Perfusion syringe for endothelial removal (1-ml Terumo syringe with Microlance 3, BD).

2.2.2. RNA Isolation and Quantification

1. Liquid nitrogen for snap-freezing tissue immediately after dissection.
2. Tissue homogeniser (Kinematica).
3. Tri-Reagent (Sigma; *see* **Note 1**).
4. Bromophenol chloroform.
5. Glycogen to act as charge carrier for RNA (Sigma).
6. Isopropanol.
7. 75% ethanol in water.
8. DNAse I (Ambion) for removal of DNA contamination.

9. DNAse inactivating reagent (Ambion).
10. RiboGreen and ribosomal RNA standards (Molecular Probes) for quantification of RNA (*see* **Note 2**).
11. PCR grade water.
12. Tris–EDTA (TE).
13. Bench-top centrifuge for quick-spin.
14. Cooled centrifuge for 13,000 rpm at 4°C.
15. LightCycler instrument (v1.5, Roche).

2.2.3. cDNA Preparation

1. Transcriptor reverse transcriptase (Roche).
2. dNTPs (Roche).
3. Oligo-dT and random primers (Roche) or gene-specific primers (Sigma).
4. DNase inhibitor (Roche).
5. PCR grade water (Roche).
6. Water bath (thermal cycler preferable).

2.2.4. RT-PCR

1. LightCycler FastStart DNA Master SYBR Green I kit (*see* **Note 3**).
2. LightCycler glass capillaries (20 µl).
3. LightCycler capillary cooling box.
4. LightCycler instrument (v1.5).

2.2.5. Gel Electrophoresis

1. Agarose.
2. Tris–borate–EDTA electrophoresis buffer (*see* **Note 4**).
3. Ethidium bromide (*see* **Note 5**).
4. 100-bp DNA ladder (New England Biolabs).
5. Electrophoresis chamber and power supply.
6. Transilluminator.

2.3. Methods

2.3.1. Dissection of Tissues

1. Sacrifice 8- week-old male C57/BL5 mice by CO_2 asphyxiation and cervical dislocation, in accordance with the local legislature and code of practice.
2. Surgically remove the thoracic aorta (approximately 0.75 mm in diameter) and place on ice-cold Hank's solution.

3. Pin out the blood vessel using entomological pins on a Sylgard-coated dissection plate (*see* **Note 6**) and submerge it in ice-cold Hank's solution.
4. Remove the fat completely by dissection.
5. Flush out the blood cells from the lumen with Hank's solution.
6. Perfuse the lumen briefly with the endothelial removal solution (*see* **Note 7**).
7. Using fine forceps, completely remove the adventitial layer (*see* **Note 8**).

2.3.2. RNA Isolation

1. The medial layer of the individual aorta is placed in a sterile 1.5 ml Eppendorf tube and snap-frozen in liquid nitrogen immediately after dissection. This sample can be stored at –80°C until required.
2. Pipet 0.5 ml of Tri-Reagent into the tube and homogenise the tissue at maximum speed for 2 min.
3. Make sure there is no carryover for the next sample by thoroughly cleaning the aggregate in molecular biology grade water.
4. Add an extra 0.5 ml of Tri-Reagent to the tube, giving a final volume of 1 ml.
5. Add 100 µl of bromophenol chloroform, mix and quick-spin.
6. Leave for 15 min at room temperature to allow separation.
7. You should be able to see separation of the protein, DNA, and RNA phases.
8. Centrifuge at 16,000×*g* for 15 min at 4°C.
9. The protein, DNA and RNA phases should be clearly defined now.
10. Carefully pipet the clear RNA solution above the white DNA ring into a sterile 1.5 ml Eppendorf tube. You should get approximately 600 µl (*see* **Note 9**).
11. Add 12.5 µl of glycogen, mix and quick-spin.
12. Add 600 µl of ice-cold isopropanol to the clear solution.
13. Mix thoroughly and leave on ice for 30 min.
14. Centrifuge at 16,000×*g* for 15 min at 4°C.
15. There should be a white pellet at the bottom of the Eppendorf tube.
16. Carefully pipet out the solution and pipet in 1 ml of ice-cold 75% ethanol.

17. Vortex the Eppendorf tube to wash the pellet and quick-spin.
18. Carefully pipet out the 75% ethanol and repeat the wash step.
19. Remove as much ethanol as possible and leave the pellet to air-dry.
20. Reconstitute the pellet in 10 µl of TE buffer.
21. Add 1.3 µl of 10× DNase buffer and mix.
22. Add 3 µl of DNase I and mix.
23. Incubate at 37°C for 45 min for DNA digestion.
24. Add 5 µl of DNase inactivation reagent. Leave for 5 min and quick-spin. The smooth muscle RNA is ready for quantification.

2.3.3. RNA Quantification Using Ribogreen

1. Prepare sufficient volume (10 µl per sample) of a working concentration of RiboGreen reagent at 1:200 dilution in TE buffer.
2. Protect the working solutions from light by covering them with foil or placing them in the dark, as RiboGreen is susceptible to photo-degradation.
3. Prepare a 10 µg/ml solution of ribosomal RNA standards in TE. This is provided in the RiboGreen Quantification kit.
4. Dilute the 10 µg/ml RNA solution to give final RNA concentrations in the TE buffer in the range of 0.1, 0.2, 0.4, 0.6, 0.8 and 1 µg/ml.
5. Transfer 10 µl of the RNA standards into individual LightCycler glass capillaries.
6. Dilute the unknown RNA 100-fold in the TE buffer and transfer 10 µl into a LightCycler glass capillary.
7. Add 10 µl of RiboGreen reagent per capillary.
8. Quick-spin in the LightCycler centrifuge.
9. Load into the LightCycler and leave for 5 min to allow the reaction to occur.
10. Start the LightCycler software and select the real-time Fluorimeter.
11. Record the Fluorescence F1 values for each sample.
12. Subtract the background F1 value of the reagent blank from that of each of the samples.
13. Use the subtracted data to construct a standard curve of fluorescence vs. RNA concentration.
14. The unknown RNA concentration can be determined from the equation of the standard curve (**Fig. 2.1**, *see* **Note 10**).

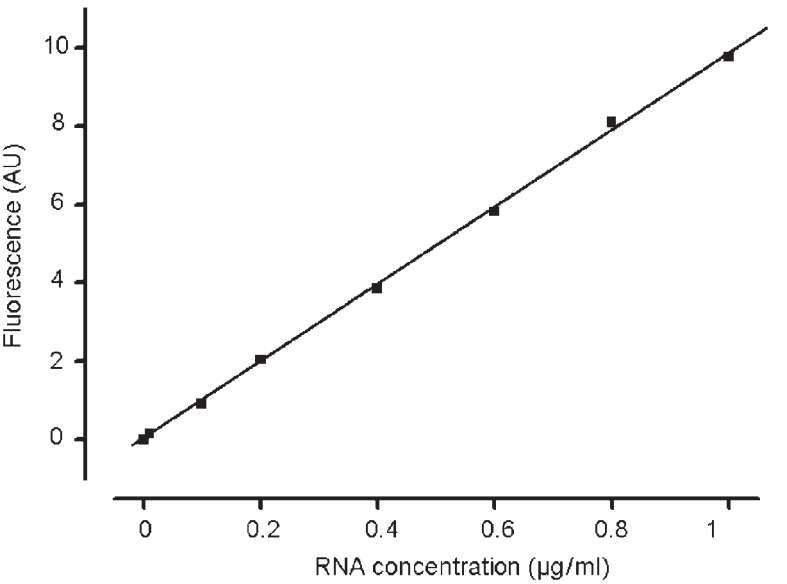

Fig. 2.1. RNA quantification curve.

2.3.4. cDNA Preparation

1. Calculate the volume of RNA required for cDNA synthesis. We usually use 0.5–1 µg.
2. Total reaction volume is 20 µl.
3. Add either gene-specific primers (final concentration 1 µM) or random hexamers (final concentration 60 µM) and oligo-dT primers (final concentration 2.5 µM) (*see* **Note 11**).
4. Add PCR grade water up to 13 µl of the reaction volume.
5. Incubate at 65°C for 10 min, then place immediately on ice for 1 min.
6. Add 4 µl of Transcriptor RT Reaction Buffer 5×.
7. Add 0.5 µl of 40 U/µl Protector RNase Inhibitor.
8. Add dNTP mix (final concentration of 1-mM each).
9. Add 0.5 µl of 20 U/µl Transcriptor Reverse Transcriptase (final concentration 10 U).
10. Vortex and quick-spin.
11. If using random hexamers and oligo-dT, incubate at 25°C for 10 min.
12. Incubate at 55°C for 30 min.
13. Incubate at 85°C for 5 min to heat-inactivate the reverse transcriptase.
14. Place on ice and store at 4°C.

2.3.5. VRT-PCR Primer Design

Primers should be designed with the aid of appropriate software, e.g. Roche provides the LightCycler Primer Design software. Attention should be paid to the optimal size of the amplicon produced during the reverse transcription (*see* **Note 12**). Real-time products are usually less than 200 bp.

The following guidelines should be observed for optimal and accurate primer design:

1. The primer's 3 ends should be free from secondary structures, repetitive sequences, palindromes and highly degenerate sequences.
2. Forward and reverse primers should not have significant complementary sequences.
3. Forward and reverse primers should have equal GC contents, ideally between 40 and 70%, with equal melting temperatures (60°C). Moreover, if there is a constant melting temperature for all primers, RT-PCR runs can be performed together in the LightCycler.

2.3.6 . RT-PCR

This protocol uses the Roche LightCycler FastStart DNA Master SYBR Green I kit.

2.3.6.1. Reaction Mix

1. Prepare the LightCycler Reaction mix by pipetting 10 µl from vial 1a, containing FastStart Taq DNA polymerase, into vial 1b, containing reaction buffer, dNTP mix, SYBR Green I and 10-mM $MgCl_2$.
2. For a 20-µl reaction, mix 2 µl of LightCycler Reaction mix, 2 µl of cDNA, forward and reverse primers (at optimised concentration), optional additional $MgCl_2$ (vial 2; *see* **Note 13**) and PCR grade water (vial 3).
3. Negative controls (such as RNA reverse-transcribed in the *absence* of the reverse transcriptase, i.e. no-RT) should always be included in real-time runs and, wherever possible, appropriate positive controls should also be included.
4. Transfer the reaction into 20-µl LightCycler glass capillaries and load it into the LightCycler carousel.
5. Quick-spin in the LightCycler centrifuge and load into the LightCycler instrument.

2.3.6.2. RT-PCR Protocol

The following procedure is optimised for use with the LightCycler 1.5 instrument. The primers used for performing the RT-PCR experiments are given in **Table 2.1**.

The RT-PCR protocol is divided into four steps:

(a) Pre-incubation to activate the FastStart Taq DNA polymerase.

(b) Amplification of the target DNA (denaturation, annealing and extension).

(c) Melting curve for PCR product identification.

(d) Cooling for complete hybridisation.

1. Pre-incubate at 95°C for 10 min to activate the FastStart Taq DNA polymerase.
2. Incubate at 95°C for 10 s to denature the cDNA.
3. Incubate at 55°C for 6 s to start the annealing process with the primers.
4. Incubate at 72°C for 16 s to start extension of the amplicon, and acquire fluorescent reading.

Table 2.1
PCR primers for potassium channel mRNA detection (reproduced from ref. *6*)

Gene	Accession no.	Primer 5′–3′	Amplicon size (bp)	Amplicon T_m (°C)	Efficiency[a]
β-*actin*	NM_007393	F_1 CACTATTGGCAACGAGC R_1 CGGATGTCAACGTCAC	126	83.1	1.82 ± 0.05
KCNA1 (K_Vα1.1)	NM_010595	F_1 TCTAGCGCAGTGTACTT R_1 GGCTATGCTATTGTTCATA	378	88.3	1.76 ± 0.05
KCNA2 (K_Vα1.2)	NM_008417	F_1 TCGATCCCCTCCGAAA R_1 CTAAGGGCACGTTCACA	114	86.5	1.81 ± 0.03
		F_2 CACCCACAAGACACCT R_2 GGCGGTTGCGATCAAA	192	85.1	1.96 ± 0.02
KCNA3 (K_Vα1.3)	NM_008418	F_1 GCTTCCCGAGTTTCGC R_1 CCCATTACCTTGTCGTTC	298	88.9	1.89 ± 0.06
		F_2 AGGACAGACGCTGAAG R_2 AGTTGGAAACAA TCACAGG	287	87.6	1.86 ± 0.03
KCNA4 (K_Vα1.4)	NM_021275	F_1 CCCTAAGAGCCAGCAT R_1 GGTTAAGACACCCGCA	245	ND	ND
KCNA5 (K_Vα1.5)	NM_008419	F_1 GAGCCGTTGAAGTGGT R_1 AAATGCACTCGTCAGC	215	81.5	2.10 ± 0.05
KCNA6 (K_Vα1.6)	NM_013568	F_1 CGCTGTCTACTTCGCAG R_1 CTCGATGTGGAGTCGG	380	ND	ND
KCNA7 (K_Vα1.7)	NM_010596	F_1 CCTAAGGGTCATCCGA R_1 CCCATAGCCAACCGTG	259	ND	ND
KCNAB1 (K_Vβ1)	NM_010597	F_1 AAATGACGGTGTGAGT R_1 CAGTATGTTATCAATCTCG	127	84.8	2.17 ± 0.01
KCNB1 (K_Vα2.1)	NM_008420	F_1 AGCAATAGCGTTCAACTT	239	87.0	1.78 ± 0.06

ND not determined, *F* forward, *R* reverse

[a]Mean ± SEM efficiency determined for ≥4 independent cDNA templates

5. Repeat **steps 2–4** for 40 cycles.
6. Incubate at 65°C for 15 s.
7. Ramp up the temperature to 95°C at a rate of 0.1°C/s while continuously acquiring fluorescent readings (*see* **Note 14**).
8. Cool the reaction to 40°C and hold for 30 s for complete hybridisation.

2.3.7. RT-PCR Quantification

2.3.7.1. Identification of the Desired Amplicon

Real-time PCR reactions can be analysed by melting curve analysis to differentiate primer dimers from specific PCR products. A typical plot of the dissociation curve is shown in **Fig. 2.2**. However, if the raw data from a melting profile are presented as a first derivative plot of fluorescence units against temperature, this provides a clearer view of the rate of SYBR Green I loss and the temperature range over which it occurs (**Fig. 2.2**). Primer dimers usually melt at lower temperatures than specific PCR products and thus the small peaks are most likely primer dimers of non-specific product formation. It is also useful to analyse the results by agarose gel electrophoresis to correlate product length with melting peaks, to identify PCR artefacts (**Fig. 2.3**) and to sequence the PCR product to validate the result.

1. To pour an agarose gel, agarose powder is mixed with the electrophoresis buffer to the desired concentration (2%). Prevent it from boiling over.
2. Heat in a microwave oven until completely melted.
3. Cool the solution to about 60°C before adding ethidium bromide to the gel (final concentration 0.5 µg/ml).
4. Pour the solution into a casting tray containing a sample comb and allow to solidify at room temperature.
5. After the gel has solidified, remove the comb, taking care not to rip the bottom of the wells. If there are excess lanes

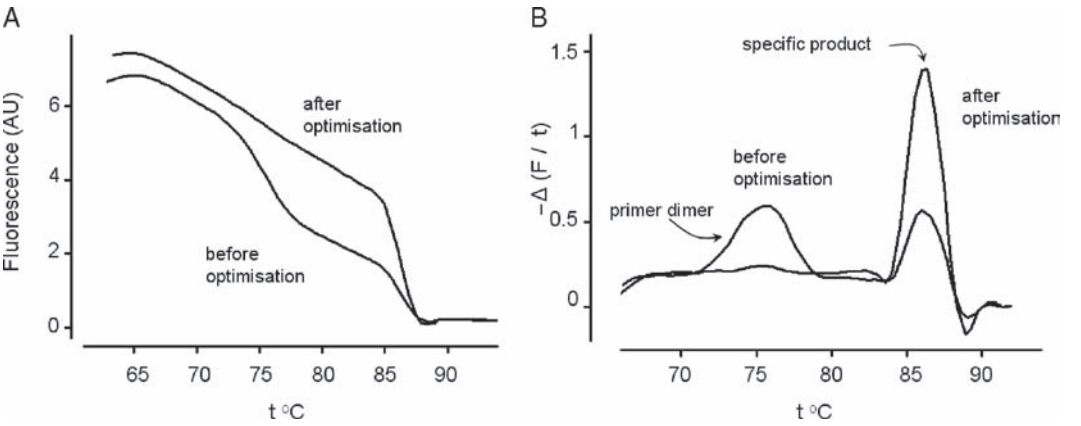

Fig. 2.2. Melting curve for aorta samples investigated by RT-PCR for $K_v\alpha 1.3$. (A) Dissociation curves before and after optimisation of the PCR reaction. (B) First derivative plot of fluorescence against temperature reveals the initial presence of a primer dimer prior to optimisation. This subsequently disappears after improving reaction conditions, leaving a single specific melting curve.

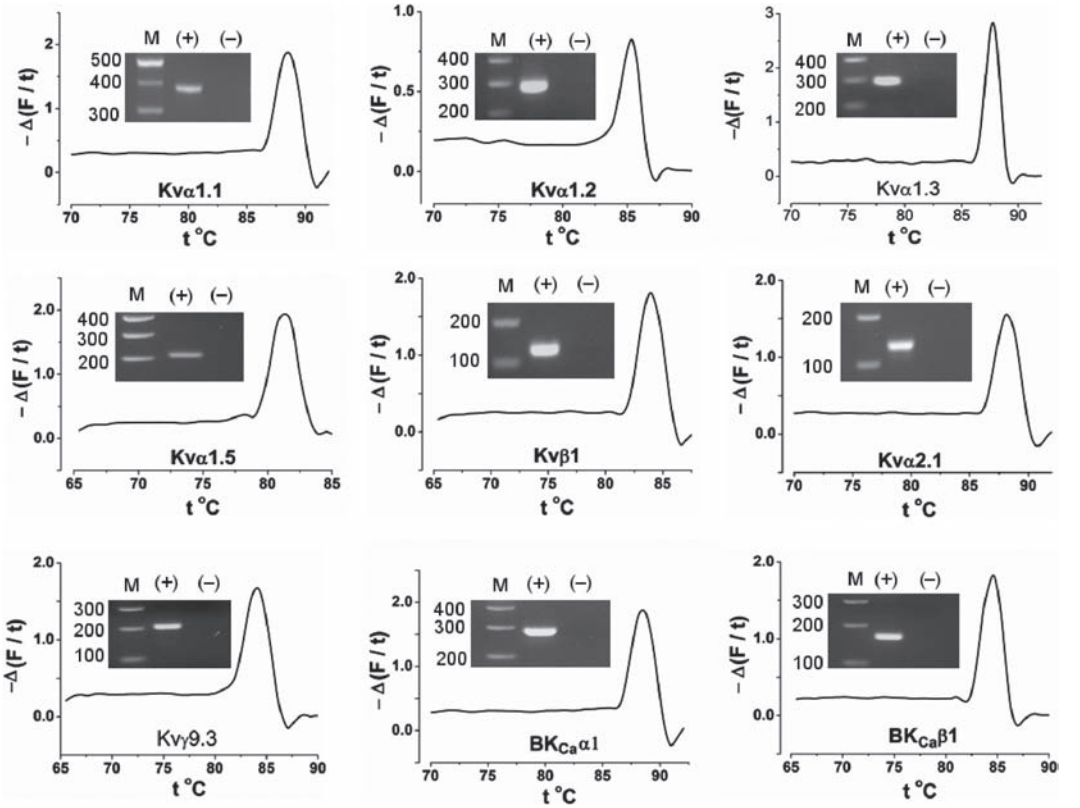

Fig. 2.3. Specificity of detection in aorta samples using F_1/R_1 primer sets (**Table 2.1**). Derivative melting curve analyses are shown with inset agarose gels (reproduced from **ref. 6**).

available, that section of the gel can be cut out with a scalpel blade and stored at 4°C for future use.

6. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and just covered with electrophoresis buffer.
7. Samples mixed with the loading buffer are then pipetted into the sample wells.
8. Close the lid and run the gel at 50–70 V. DNA will migrate towards the positive electrode (usually coloured red).
9. When adequate migration has occurred, DNA fragments are visualised by the ethidium bromide bound between the bases of the DNA.
10. Place the gel on an ultraviolet transilluminator to visualise vthe DNA and take a photograph of the gel.

2.3.7.2. Quantification Analysis

Real-time PCR results can be analysed in a variety of ways depending on the application. Analyses of quantitative standards can be used to generate PCR curves and from these the

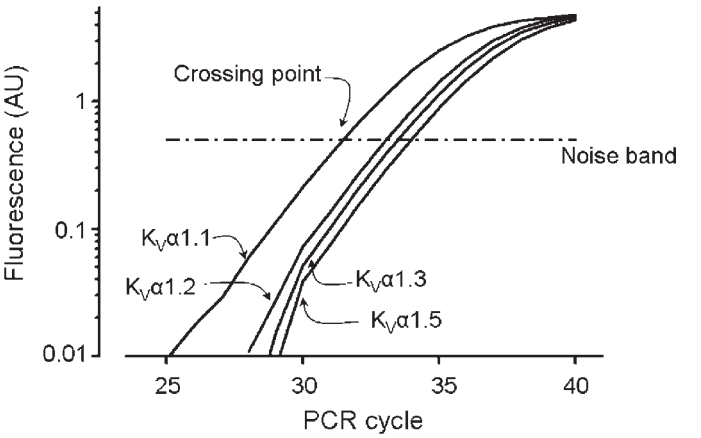

Fig. 2.4. SYBR Green fluorescence-cycle plots for *KCNx* genes in aorta. Crossing point (C_P) is the intersection with the horizontal dotted line (set at 0.5).

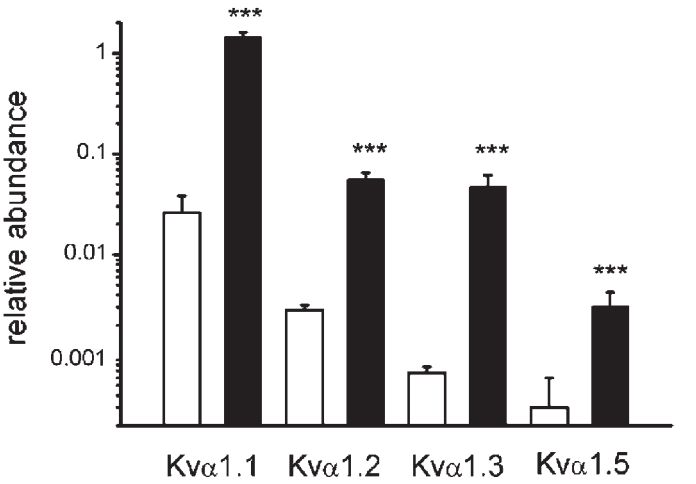

Fig. 2.5. Conduit vs. resistance artery for RNA encoding $K_V\alpha1$ (reproduced from **ref. 6**).

cycle number at which the fluorescent signal increases above a predetermined background value (C_P, crossing point) can be determined (**Fig. 2.4**). This crossing point can be used for the comparison of results from sample to sample and can be used to generate quantitative results.

2.3.7.3. Relative Quantification

1. Record the crossing point of the gene of interest (e.g. *KCNA1*) and an appropriate housekeeper gene (e.g. *β-actin*).
2. Use the equation $2^{C_P(\beta\text{-actin})} / 2^{CP(KCNA1)}$ to calculate the relative abundance of *KCNA1* to *β-actin* (*see* **Note 15**).

Fig. 2.5 shows the SYBR Green-cycle plots for *KCNx* genes in the samples from the two different vascular beds: the aorta and the mesenteric. Analysis of the relative abundance of the RNA shows that there is significantly more $K_V\alpha1.1$, 1.2, 1.3 and 1.5 in the mesenteric artery than in the aorta.

2.4. Notes

1. Tri-Reagent is toxic and prolonged exposure to phenol fumes or contact with skin can be hazardous. If possible, please use a fume hood.
2. RiboGreen is an ultrasensitive fluorescent nucleic acid stain for measuring RNA concentration in solution.
3. SYBR Green I is a minor groove-binding dye which typically exhibits 20–100-fold fluorescence enhancement on binding to double-stranded DNA.
4. Tris–borate–EDTA (TBE) is used for small (<1 kb) DNA and shows increased resolution of small DNA whereas Tris–acetate–EDTA (TAE) is used for DNA recovery and electrophoresis of large (>12 kb) DNA.
5. Ethidium bromide is an intercalating agent commonly used as a nucleic acid stain in molecular biology laboratories for techniques such as agarose gel electrophoresis. When exposed to ultraviolet light, it will fluoresce with a red-orange colour, intensifying almost 20-fold after binding to DNA. Ethidium bromide may be a very strong mutagen, and may possibly be a carcinogen or teratogen, although this has never been definitively proved. High concentration of ethidium bromide (e.g. when the colour of the gel is dark pink or red) should be placed in the biohazard box for incineration.
6. Sylgard is a silicone elastomer. Mix the base part to the curing part at a ratio of 10:1, pour sufficient volume into a 10-cm culture dish with a sufficient depth of 5 mm and leave for 4 h at 65°C before use. The use of the Sylgard-coated surface will prolong the life of the dissection forceps and provide a base for pinning the entomological pins.
7. The act of removing the endothelial layer can damage the tissue. A wire myography experiment can be done to validate the vessel health and complete the endothelial removal. Phenylephrine (10 μM) and acetylcholine (1 μM) will induce contraction in the intact aorta but not in the endothelial-denuded vessel.
8. Co-staining of aorta sections with an antibody against smooth muscle α-actin and a nuclear stain such as DAPI will validate the removal of the endothelial and adventitial layers.
9. DNA and protein can be recovered from the remaining solution by further precipitation.
10. The Microsoft EXCEL macrofunction is a very handy tool to work out the solutions to the RNA concentration equation.

11. Priming of the cDNA reaction from the RNA template can be done using random primers, oligo-dT, or gene-specific primers. The melting temperature T_m of random primers and oligo-dT is low, and hence neither of these can be used with thermostable RT enzymes without a low temperature pre-incubation step. Random primers are by definition non-specific, but yield the most cDNA and are the most useful for transcripts with significant secondary structure. cDNA synthesis with oligo-dT is more specific than random priming, although it will not prime RNAs that lack a poly-A tail such as ribosomal 16S. Gene-specific primers synthesise the most specific cDNA and provide the most sensitive method for quantification, but the RT-PCR reactions are restricted to the genes primed for. In the case of *KCNx* genes in smooth muscle cells, we synthesised cDNA using gene-specific primers to improve detection.
12. Optimal primer concentration should be determined, with a final concentration ranging from 0.1 to 0.5 μM for both primers. For some assays, the optimal concentration for the two primers will not be the same, and it will be necessary to use asymmetric priming, where a lower concentration of one primer is used, to achieve good melting curves. If the problem persists, it is advisable to design new primers.
13. A key variable is the magnesium chloride ($MgCl_2$) concentration since Mg^{2+} affects the specificity and the yield of PCR *(13)*. Concentrations that are too high may lead to incomplete denaturation and low yields, and can also lead to increased production of non-specific products and primer artefacts including primer dimers. Levels that are too low reduce the ability of polymerase to extend the primers. Optimum $MgCl_2$ concentration in the LightCycler instrument ranges from 1 to 5 mM. It is recommended that a $MgCl_2$ titration be performed for each primer set.
14. This will dissociate the SYBR Green from any double-stranded DNA, including any primer dimers, contaminating DNA, and PCR products from mis-annealed primers.
15. Quantitative RT-PCR can be expressed relative to an internal standard. Relative quantification determines the changes in steady-state mRNA levels of a gene relative to the levels of an internal control, usually a housekeeping gene such as *β-actin*, GAPDH or ribosomal 16S. Therefore, relative quantification does not require standards with known concentrations and all samples are expressed as an *n*-fold difference relative to the housekeeper gene, and the number of target gene copies are normalised to the housekeeper gene. If the aim is to compare the relative abundance of *1* gene in *different* tissues, knowing the PCR efficiency is not important,

as it will cancel out. Hence, remembering that DNA amplification is exponential and assuming a 100% PCR efficiency (i.e. each cycle brings a doubling of the product and hence an efficiency value of 2), the equation for the relative abundance of *KCNx* to a housekeeper gene is as described under **Subheading 2.3.7.3**.

However, if the aim is to compare the relative abundance of *2* genes in the *same* tissue, it is necessary to determine the PCR efficiency when using each primer set. The PCR efficiency for a given primer set can be easily obtained by performing RT-PCR using the primer set on serial DNA dilutions. The PCR efficiency is then calculated from the slope value for the derived standard curve:
Efficiency E_{geneX} using X primer set = $10^{-1/\text{slope}}$.
Thus,

$$\text{Relative abundance of } X = \frac{E_{housekeeper}^{C_p(housekeeper)}}{E_{genex}^{C_p(KCNx)}}.$$

Acknowledgements

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