SMA_M.1.2.007

Please quote the use of this SOP in your Methods.

Human and murine *SMN* transcript analysis by absolute real time PCR

SOP (ID) Number	SMA_M.1.2.007
Version	1
Issued	February 16th, 2011
Last reviewed	July 8th, 2016
Author	F. Danilo Tiziano Institute of Medical Genetics Catholic University, Roma, Italy
Working group members	Christine Di Donato, Children's Memorial Research Center, Northwestern University Rosa Lomastro, Catholic University, Roma Louise Simard, University of Manitoba, Winnipeg, Manitoba Brunhilde Wirth, Markus Riessland and Julia Schreml, Institute of Human Genetics, University of Cologne, Cologne, Germany
SOP responsible	F. Danilo Tiziano
Official reviewer	Christine Di Donato

SMA_M.1.2.007

TABLE OF CONTENTS

1. OBJECTIVE 3
2. SCOPE AND APPLICABILITY 3
3. CAUTIONS 3
4. MATERIALS 4
5. METHODS 5
   5.1 Preparation of external standards 5
   5.2 Handling and storage of starting material. 10
   5.3 RNA extraction and cleanup 10
   5.4 Estimating RNA concentration; storage of samples 11
   5.5 Preparation of cDNA 12
   5.6 Primers and fluorescent–labelled Taqman-MGB probes 13
   5.7 Running an Absolute Quantification (AQ) plate 15
6. EVALUATION AND INTERPRETATION OF RESULTS 17
7. REFERENCES 17

1. OBJECTIVE

The present SOP is aimed at providing a standardized protocol for *SMN* transcript quantification, both from human and murine *SMN* genes. Recently, a consensus has been reached to apply the absolute real time quantification assay described by Tiziano et al (2010). Although this method is slightly more time-consuming compared to the more commonly used approaches based on relative real time PCR, it offers the advantage of reducing the bias due to different expression levels of housekeeping transcripts that are used as calibrators for normalization of results. In this approach, transcripts are quantified compared to serial dilutions of an external standard with a known number of molecules. This method has already been validated for human blood samples; the validation for murine tissues is currently ongoing. Moreover, the development of a commercial kit based on this method is ongoing.

2. SCOPE AND APPLICABILITY

The purpose of the following procedure is to assess *SMN* transcript levels in different tissues from SMA models. The prerequisite of the applicability of the present assay is that the model of interest expresses *SMN1*, *SMN2* and/or wild type *Smn* genes. The scope of *SMN* transcript level assessment is the identification of potential correlations between gene expression and phenotypic severity of affected animals and/or to evaluate the potential effect of therapeutic candidates on *SMN* transcript levels.

3. CAUTIONS

Three issues are critical in this experimental procedure:

1) RNA degradation: in contrast to semiquantitative relative approaches, this assay is more sensitive and error prone as a result of partial degradation of RNA samples. Thus, it is suggested that the manipulation of tissues and RNA samples be done following standard protocols designed to avoid RNA degradation (general remarks on handling RNA are provided by Qiagen).
2) Genomic DNA (gDNA) contamination: gDNA impairs the quantification of RNA samples when using absorbance-based methods. Although primer and probe sets reported in this assay are located in different exons to avoid gDNA interference in

cDNA amplification, some reduction in PCR efficiency could be observed if gDNA contamination exists. Thus, RNase-free DNase treatment is strongly recommended.

3) RNA quantification: the determination of mRNA levels is strongly dependent on RNA quantification which should be extremely accurate. When using column-based purification strategies, it is common that, after elution, the RNA solution may not be uniformly resuspended. This issue may introduce an error both during RNA sample quantification and during pipetting of the appropriate quantity of RNA for cDNA synthesis.

4. MATERIALS

- QIAzol Lysis Reagent (Qiagen, cat. N° 79306) or equivalent
- RNeasy Mini Kit (Qiagen,cat. N° 74106)
- RNase Free DNase Set (Qiagen, cat. N° 79254)
- High Capacity cDNA Reverse Transciptase Kit (Applied Biosystems, cat. N°4368813)
- Qiagen PCR Cloning Kit (Qiagen, cat. N° 2312122)
- QIAprep Spin Miniprep Kit (Qiagen, cat. N° 27104)
- QIAquick gel extraction kit (Qiagen) or equivalent
- Primers and MGB-probes (Applied Biosystems)
- Taqman Universal Master Mix (Applied Biosystems, cat. N°4318157)
- Optical fast 96-well plates for realtime PCR (Applied Biosystems, cat. 4366932)
- TE pH8
- Absolute ethanol (molecular biology grade)
- Chloroform
- Isopropanol
- DNase-RNase-free water
- 1.5 ml centrifuge tubes
- 0.5 ml centrifuge tubes
- 0.2 ml PCR tubes
- 7900 HT real time PCR instrument (Applied Biosytems) or equivalent
- Thermomixer (Eppendorf) or equivalent

- 2700 thermal cycler instrument (Applied Biosystems)
- Genequant Pro II spectrophotometer (Pharmacia) or equivalent

5. METHODS

The protocol includes the following steps:

a) Preparation of external standards
b) Handling and storage of starting material
c) RNA extraction and cleanup
d) Estimating RNA concentration; storage of samples
e) Preparation of cDNA
f) Primers and fluorescent–labelled TAQMAN-MGB probes.
g) Running an Absolute Quantification (AQ) plate

5.1 Preparation of external standards

For the construction of external standards, PCR products amplified from control cDNAs (both human and murine) are cloned using Qiagen PCR Cloning Kit (Qiagen). Amplicons contain the sequence of primers and probes used in real time PCR. The primer pairs we used for cloning are reported in Table 1.

Table 1: Primers pairs

Primer name	Sequence	Location	PCR product size
SMN_exst-F	5'–GCTTTGGGAAGTATGTTAATTTCA–3'	Exon 6	129 bp
SMN_exst-R	5'–CTATGCCAGCATTTCTCCTTAATT–3'	Exon 7-8	
Smn_exst_F	5'-ACTATATGGGTTTCAGACAAAATAA-3'	Exon 6-7	118 bp
Smn_exst_R	5'-GACGTCTGTTCTTGTCGACC-3'	Exon 8	
Gapdh_exst_F	5'-CCTGGCCAAG GTCATCCAT-3'	Exon 3	119 bp
Gapdh_exst_R	5'-CCATCACGCCACAGCTTTC-3'	Exon 4	

To increase the yield of downstream procedures, we suggest purifying PCR products before cloning. In our experience, the most effective method is purification directly from an agarose gel using QIAquick gel extraction kit or equivalent.

For cloning, experimental steps are as follows (extracted from PCR cloning handbook, Qiagen):

5.1.1 Ligation protocol

1) Thaw 2x Ligation Master Mix, pDrive Cloning Vector DNA, and distilled water (provided). Place on ice after thawing.
2) Prepare a ligation-reaction mixture according to the following scheme:

Component	Volume/reaction
pDrive Cloning Vector (50 ng/µl)	1 µl
PCR product (10 ng)	1–4 µl
Distilled water	0-3 µl variable
Ligation Master Mix, 2x	5 µl
Total volume	**10 µl**

3) Briefly mix the ligation-reaction mixture then incubate for 30 min at 4–16°C
4) Proceed with the transformation protocol or store ligation-reaction mixture at –20°C until use.

5.1.2 Transformation protocol

1) Thaw the appropriate number of tubes of QIAGEN EZ Competent Cells on ice. Thaw SOC medium and warm to room temperature. Proceed immediately to the transformation step once the cells have thawed.
2) Add 1–2 µl ligation-reaction mixture per tube of QIAGEN EZ Competent Cells, mix gently, and incubate on ice for 5 min.
3) Heat the tube(s) in a 42°C water bath or thermomixer for 30 s without shaking.
4) Incubate the tube(s) on ice for 2 min.

5) Add 250 µl room temperature SOC medium per tube and directly plate 100 µl each transformation mixture onto LB agar plates containing ampicillin.
6) Incubate the plate at room temperature until the transformation mixture has absorbed into the agar. Invert the plate and incubate at 37°C overnight (e.g., 15–18 h).

5.1.3. Plasmid DNA extraction and purification

We suggest isolating more than one colony from the LB-agar Petri dish, and to label each clone with different progressive numbers. For plasmid purification, we suggest using the QIAprep Spin Miniprep Kit (Qiagen) or equivalent. The experimental procedure with the Qiagen kit is the following:

1) Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
2) Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times. Mix gently by inverting the tube. Do not vortex as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 min.
3) Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
4) Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge. A compact white pellet will form. Apply the supernatant from step 4 to the QIAprep spin column by decanting or pipetting.
5) Wash the QIAprep spin column by adding 0.75 ml Buffer PE.
6) Transfer the QIAprep spin columns to a microcentrifuge tube. Centrifuge for 1 min.
7) Place the QIAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM TrisHCl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min. EB buffer is preferred to water for elution of DNA as EB buffer provides an environment for long term stability of plasmid DNA.

5.1.4. Quality and quantity evaluation of external standard

As a first step, we strongly recommend that you rule out the presence of possible mutations introduced by Taq polymerase in external standards. This can be done by

performing sequence analysis of the plasmids using a primer located in the vector (T7 or SP6). The orientation of cloned fragments is not relevant for subsequent applications.

Sequence analysis is also essential to differentiate *SMN1* and *SMN2* standards on the basis of the C-T transition in position +6 of SMN exon 7. If starting from a control cDNA, a prevalence of *SMN1* clones is expected. To increase the chance of obtaining *SMN2* clones, we suggest amplifying and cloning a cDNA from a type 2 or type 3 SMA patient.

For quality and quantity evaluation of the external standards, both absorbance and agarose gel electrophoresis with scaling serial dilution of lambda DNA are suggested (ranging from 50 to 400 ng). The relevant aspects which should be evaluated by agarose gel electrophoresis are: 1) absence of gDNA contamination (high molecular weight staining in wells), 2) absence of RNA contamination (smear migrating parallel to the lower molecular weight standards), 3) absence of plasmid degradation which migrates similarly to RNA contamination. Linearization of plasmids by restriction enzyme digestion is suggested, although not crucial.

The plasmid length is 3849 bp; thus, if primers indicated in Table 1 are used for cloning, the expected size of the recombinant plasmids is indicated in Table 2:

Table 2: Size and molecular weight (MW) of External Standards

External standard	Total length	MW (KDa)
SMN	3980 bp	2626.80
Smn	3969 bp	2619.54
Gapdh	3970 bp	2620.20

Assuming that each nucleotide weighs 660 Da, the molecular weight of each plasmid can be estimated as indicated in Table 2. The number of molecules/ng of DNA can be calculated by the following formula:

$$\text{No. of molecules contained in 1 ng} = \frac{6.023*10^{23}\ (\text{Avogadro's number})*10^{-9}\text{grams}}{\text{MW (Da)}}$$

Resulting in an estimation of $2.29*10^{8}$ molecules/ng.

5.1.5 Dilution of external standards

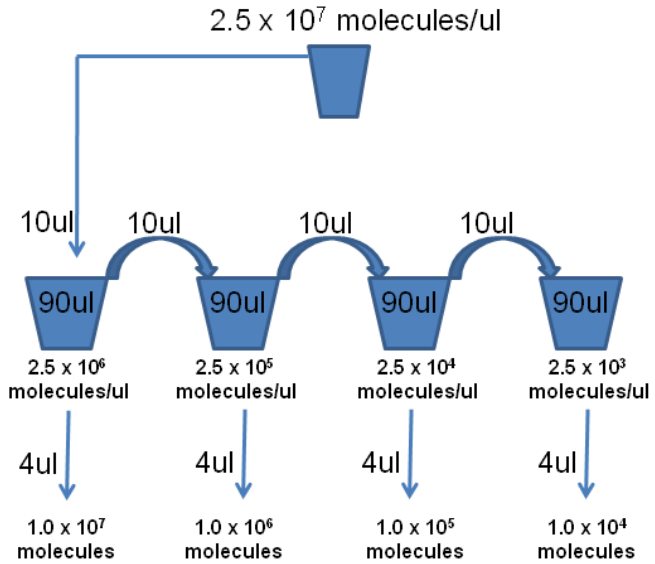
All dilution steps are performed in sterile TE pH8 to preserve DNA for long term storage at 4°C.

Each plasmid is diluted as follows:

100 ng/µl → 30 ng/µl → 3ng/µl

This 3ng/µl solution is further diluted by pipetting 3.7 µl of each plasmid in a final volume of 100 µl to obtain a concentration of $2.5x10^7$ molecules/µl. This tube will be further serially diluted 1/10 four times to obtain final standard concentrations of $2.5x10^6$, $2.5x10^5$, $2.5x10^4$, and $2.5x10^3$ to molecules/µl. Hence, if using this procedure, 4 µl per of each standard concentration noted above will be used during real time PCR to establish the standard curve of 10^7, 10^6, 10^5, 10^4 molecules, respectively (Figure 1). This method reduces pipetting error during the standard curve set-up in real time PCR. We strongly suggest

Figure 1.



preparing a large number of ready-to-use aliquots of plasmids, kept at -20°C, sufficient for several amplifications. This approach will reduce the inter-experimental variability. Finally, when preparing new dilutions of plasmids for standard curves, we suggest verifying that the results obtained with new and old dilutions are reproducible.

Standard curves are diluted separately (exception noted below). It is important that you verify the genotype of the transgenic model you are using when deciding which SMN standard curve(s) is required. The SMN1 standard curve is used to quantify full-length SMN transcripts produced from the SMN1 gene. The SMN2 standard curve is used to quantify full-length SMN transcripts produced from the SMN2 gene. One can mix SMN1 & SMN2 standards together if you are testing samples from a mouse that possesses the SMN1 and

SMN2 transgenes [for example, A2G mice (Jax Stock number 005026) are transgenic for the wild type SMN1 exon 7 sequence with a point mutation in exon 1 and the SMN2 gene]. The SMN2 standard is sufficient for samples from SMA mice harboring only the SMN2 transgene [for example, the 005024 strain of Jax mice has the SMN2 transgene only]. It is preferable that the values obtained for query samples fall within, preferably in the middle, of the standard curves generated; therefore, input cDNA should be adjusted appropriately. This assay, as described in this SOP, does not permit the absolute quantification of SMN-delta 7 transcripts; therefore, SMN-delta7 transcripts will not be detected in SMA mice harboring a SMN2delta7 transgene.

Ready-to-use external standard dilutions can be maintained at 4°C for up to 2 weeks. For longer storage, we suggest storing aliquots at -80°C. **Repeated freeze/thaw cycles results in the degradation of DNA, causing lower concentrations of plasmids, and thus is not recommended.**

5.2 Handling and storage of starting material.

To avoid RNA degradation, it is strongly suggested that tissues be frozen in dry ice or liquid nitrogen immediately upon dissection. Tissues can be stored at -80°C or -150°C.

5.3 RNA extraction and cleanup

To increase the yield of RNA, we suggest that you disrupt or homogenize tissues. To disrupt skeletal muscle and/or spinal cord, samples can be submerged into liquid nitrogen and crushed to a fine powder using a pestle. A tissue homogenizer can be used; however, we suggest keeping samples on ice to avoid overheating and subsequent RNA degradation.

1) Weigh the tissue and use 1 ml of TriReagent (the trademark is not critical) per 100 mg of tissue; pipette until the sample is well resuspended, then keep at room temperature for 5 min.
2) Add 0.2 ml chloroform/ml of TriReagent and shake vigorously (by vortexing) for 15 s. Keep at room temperature for 2-3 min.
3) Centrifuge at 8000 x g for 15 min at 4°C.
4) Transfer the superior, aqueous phase to a new tube and add 0.5 ml isopropanol/ml of TriReagent.
5) Mix by vortexing and keep at room temperature for 10 min.
6) Centrifuge at 8000 x g for 10 min at 4°C.
7) Visualize the pellet and aspirate carefully, discarding the supernatant.
8) Add 1 ml of 70% ethanol/ml of TriReagent and centrifuge at 4500 x g for 5 min at 4°C.

9) Remove the supernatant completely and briefly air-dry the RNA pellet.
10) Resuspend RNA in 100 µl of RNase-free water for the subsequent cleanup procedure.

RNA cleanup is strongly recommended to remove contaminating traces of phenol and, most importantly, gDNA. We obtained the best results for subsequent downstream applications by using the RNeasy Mini Kit (Qiagen) which allows the cleanup of up to 100 µg total RNA.

The procedure outline is as follows:

1) Add 350 µl of RLT Buffer and mix well.
2) Add 250 µl of absolute ethanol and mix well by pipetting.
3) Transfer the sample to an RNeasy Mini spin column and centrifuge for 15 s at 6000 x g at room temperature. Discard the flow-through.
4) Add 350 µl RW1 Buffer to the column and centrifuge for 15 s at 6000 x g. Discard the flow-through
5) Add 80 µl of freshly prepared DNase-RNase free solution (Qiagen, 10 µl of DNase and 70 µl of DNase buffer), cover carefully the entire membrane surface of the purification column. Keep at room temperature for 15 min.
6) Add 350 µl RW1 Buffer to the column and centrifuge for 15 s at 6000 x g. Discard the flow-through.
7) Pipet 500 µl RPE Buffer to the RNeasy spin column and centrifuge for 15 s at 6000 x g.
8) Pipet an additional 500 µl RPE Buffer to the RNeasy spin column and centrifuge for 2 min at 6000 x g.
9) Place the RNeasy Mini spin column in a new collection tube and centrifuge at maximum speed for 1 min.
10) Place the RNeasy Mini spin column in a new 1.5 ml collection tube, add 30 µl RNase-free water directly onto the spin column membrane and centrifuge for 1 min at 6000 x g.
11) Re-apply the eluted RNA onto the RNeasy Mini spin column and centrifuge for 1 min at 600 x g to increase RNA yield without diluting the sample.
12) RNA samples can be stored for long periods at -80°C.

5.4 Estimating RNA concentration; storage of samples

Before RNA quantification, ensure that the RNA is very well resuspended by incubating

samples at 65°C for 15 min or at 37°C for 30 min. **RNA quantity and quality is the critical step for an accurate transcript level determination by absolute real time PCR.**

RNA quality and quantity is determined by absorbance at 260 and 280 nm in a spectrophotometer, a NanoDrop instrument, or Agilent Bioanalyzer or methods based on RNA specific dyes, like Quant-iT RNA Assay Kit (Invitrogen). If using a spectrophotometer or NanoDrop for quantification, agarose gel electrophoresis is strongly encouraged to evaluate the quality of RNA samples (i.e. excluding degradation and/or gDNA contamination). A ratio 260/280 OD absorbance $\geq$1.8 should be considered acceptable. All RNAs are stored at -80^{o}C until quantitative analyses.

5.5 Preparation of cDNA

Quantification of *SMN*, *Smn* and *Gapdh* transcripts is carried out following a two-step procedure involving cDNA synthesis (step 1) and real time qRT-PCR (step 2). The advantage of a 2-step procedure is that it is amenable to amplification of different transcripts from a single cDNA sample.

cDNA is synthesized using the High Capacity cDNA Reverse transcription Kit (ABI Catalogue No. 4368813). cDNA reactions are prepared in 200µl PCR tubes and contain 1µg total RNA, 1X RT buffer, 4mM dNTPs, 1X random primers, and 2.5U MultiScribeTM reverse transcriptase in a total volume of 25µl. Refer to the Manufacturer's manual for details. The final volume is adjusted with RNase-free H_2O. All reagents and mixes must be kept on ice during preparation.

Prepare the RT Master Mix (volumes shown are for a single reaction):

	Volume (ul)
Buffer 10x	2.5
Random primers	2.5
dNTPs	1.0
Enzyme	1.25
Total	7.25

Set up RT reactions in the order given.

RNase-free H_2O to V_f = 17.75µl.

7.25 µl Master Mix.

RNA (1µg), pipet up and down to mix.

When starting with 1µg RNA, a yield of about 40ng/µl cDNA is expected.

cDNAs are kept on ice while setting up qRT-PCR reactions. Subsequently, cDNAs can be stored at -20^{o} C. Avoid repeated freezing and thawing cycles since this can cause degradation of cDNA samples. Aliquots of cDNAs sufficient to carry out single experiments can be kept frozen at -20°C.

5.6 Primers and fluorescent–labelled Taqman-MGB probes.

The assay that we describe for quantifying *SMN1*/*SMN2* transcripts has been validated and published (Tiziano et al., 2010). The two different probes are specific and distinguish the C-T transition in exon 7. When using *SMN1* NED-labelled probes (which are optimized for ABI instruments), no cross-hybridization with *SMN2* transcripts has been observed; however, if *SMN1* probe is FAM labelled, a weak cross-hybridization can be found in patient samples (below 5% of *SMN2* transcript levels). This finding does not hamper *SMN2* transcript analysis. Since most transgenic SMA models express *SMN2* genes, the same assay validated for human samples can also be applied to tissues isolated from SMA animal models. In the case of heterozygous animals which have at least one *Smn* allele, wild type mouse transcripts do not interfere with human transgene mRNA quantification due to the specificity of the *SMN2* probe. *Smn* transcripts are amplified by the human SMN primer pair and recognized only by the *SMN1* specific probe. However, since 2 nucleotide variants are present in the primer pair and 1 nucleotide variant in the 3' end of the *SMN1* probe compared to murine sequence, we recommend using the specific set of probe, primers, and external standards designed for murine transcripts to quantify *Smn* mRNA. *GAPDH* probe and primers set do not amplify *Gapdh* transcripts. The sequence of probes and primers are reported in Table 3. *SMN1*-fl, *SMN2*-fl and *GAPDH* primers are published in Tiziano et al (2010). Murine transcript probes and primers are still unpublished.

Table 3. Oligonucleotide primers and probes for SMN QRT-PCR

Transcript	Position	PCR primers
SMN1-fl	forward	5' – TACATGAGTGGCTATCATACTGGCTA - 3'
SMN2-fl	reverse	5' – AATGTGAGCACCTTCCTTCTTTTT - 3'
		72 bp PCR product
Smn	forward	5' – AAGAAGGAAAGTGCTCACATACAAATT - 3'
	reverse	5' – GGACACCGACACCCCATCT – 3'
		70 bp PCR product
Gapdh	forward	5' – ATCCATGACAACTTTGGCATTG - 3'
Gapdh	reverse	5' - TCTTCTGGGTGGCAGTGATG - 3'
		70 bp PCR product
		Fluorescent-labeled TAQMAN probes
SMN1-fl	exon6-exon7	5'-FAM-TATGGGTTTCAGACAAA-NFQ-3'
SMN2-fl	exon6-exon7	5'-VIC-ATATGGGTTTTAGACAAAA–NFQ-3'
Smn	exon7-exon8	5'-FAM- AGTTCAGCTCTGTCTCAG–NFQ-3'
Gapdh	exon3-exon4	5'-FAM- AAGGGCTCATGACCACAG-NFQ-3'

5.6.1 Preparation of TAQMAN-MGB probes.

The concentration of each probe should be 100µM.

Aliquot 5 µl per tube and store at -20^{o}C.

Before use, add 95 µl of RNase-free H_2O to get a final concentration of 5µM. Mix well.

Avoid direct exposure to light and minimize light-exposure time while preparing stock solutions

5.6.2 Preparation of Forward and Reverse primers

SMN-fl forward- and reverse primers should be 100µM. Aliquot 20 µl per tube and store at -20^{o}C. Before use, add 30 µl of RNase-free H_2O to get a final concentration of 40 µM. Mix

well.

Smn forward and reverse primers should be 100µM. Aliquot 20 µl per tube and store at -20°C. Before use, add 30 µl of RNase-free H_2O to get a final concentration of 40 µM. Mix well.

For *Gapdh*, forward and reverse primers should be 100 µM. Aliquot 10 µl per tube and store at -20°C. Before use, add 90µl of RNase-free H_2O to get a final concentration of 10µM. Mix well.

5.7 Running an Absolute Quantification (AQ) plate

It is generally suggested by ABI technical support to avoid keeping plates on ice since the sudden rise in temperature during the PCR may result in the formation of micro-bubbles which can impair fluorescence reading. Prepare an AQ document as per manufacturer's specifications for the real time apparatus that you are using before setting up the master mix and real time reactions.

Cycling conditions are as follows:

1 cycle	50°C, 2 min
1 cycle	95°C, 10 min
40 cycles	95°C for 15 sec
	60°C for 1 min

5.7.1 Preparation of real time Master Mixes

Briefly, individual real time reactions are prepared for

1) *SMN1*-fl and/or *SMN2*-fl,
2) *Smn*
3) *Gapdh*

A total of 16µl of reaction mix is required for each well and contains:

- 10 µl Taqman® Universal Master Mix (Applied Biosystems),
- appropriate concentration of forward and reverse primers,
- 100nM of the appropriate TAQMAN probe(s), and
- RNase-free H_2O to a V_f of 16µl per reaction

The working concentrations of PCR primers and fluorescent-labeled TAQMAN probes are provided in Table 4.

Table 4. Working concentrations of primers and probes.

Oligonucleotides	Working Concentration
SMN1-fl/*SMN2*-fl forward and reverse primers	900 nM each
SMN1-fl 5'-FAM (or NED)-3'NFQ probe	100 nM
SMN2-fl 5'-VIC-3'NFQ probe	100 nM
Smn forward and reverse primers	700 nM each
Smn probe 5'-FAM-3'NFQ probe	100 nM
Gapdh forward and reverse primers	100 nM each
Gapdh 5'-VIC-3'NFQ probe	100 nM

5.7.2 Preparation of 96-well AQ plates.

- Add 4µl of sample (corresponds to 10^7, 10^6, 10^5, or 10^4 molecules of plasmid or 40ng cDNA). Adding template before the reaction mix will minimize light exposure.
- Add 16µl of appropriate reaction mix.
- Each 96-well plate contains a maximum of 12 wells for the targeted *SMN* standard curve (each point in triplicate) and 36 wells for the query samples (each in triplicate). In addition, 12 wells are included for the *Gapdh* standard curve (each point in triplicate) and 36 wells for the query samples (each point in triplicate) for quality control regarding RNA preparation, cDNA synthesis, and qRT-PCR. We recommend amplifying each sample (both external standards and cDNA samples) in triplicate (or even in quadruplicate, until acquiring enough familiarity with the assay). Finally, we suggest testing each sample at least twice. Some variability can be observed from one run to the other. This can be related to the quality of RNA/cDNA samples or to the efficiency of amplification, rather than to standard curve preparation.
- Start the run.

6. EVALUATION AND INTERPRETATION OF RESULTS

a) Quality control

No template control (NTC) for each of the qRT-PCR assays should be done on the plate run to ensure that assay reagents are not contaminated with either RNA or DNA. Sporadic no RT controls (NRTC) can also be run separately to ensure that the products that are detected by the fluorescent probes arise from amplification of RNA and not from DNA contamination. In addition, random qRT-PCR reactions can be run on acrylamide gels to verify the length of PCR products. These should not exceed the expected 72-bp and 75-bp size of the *SMN* fragments.

b) Data analysis

Data are analyzed using software supplied for your real time apparatus. The report includes critical information with regards to slope of standards indicating PCR efficiency (~-3.3 if 100% efficiency) and the correlation coefficient (R^2=0.99) which measures the reproducibility of the amplification.

Absolute quantification of *SMN1*-fl, *SMN2*-fl, *Smn*, and *Gapdh* transcripts in test cDNA samples is determined with standard curves of plasmids prepared by plotting linear regressions of the mean quantities from triplicate reads of each serial dilution. Linear regression curves of the standards must have an R^2~0.98 and their slopes must be ~-3.3 which corresponds to a PCR efficiency of 1 (i.e. 100%). The quantity of each transcript in test cDNA samples is presented as the mean ± SD of the triplicate runs.

7. REFERENCES

Tiziano FD, Pinto AM, Fiori S, Lomastro R, Messina S, Bruno C, Pini A, Pane M, D'Amico A, Ghezzo A, Bertini E, Mercuri E, Neri G and Brahe C. SMN transcript levels in leukocytes of SMA patients determined by absolute real time PCR Eur J Hum Genet 2010; 18:52-58.

QIAquick gel extraction kit manual

High Capacity cDNA Reverse Transciptase Kit manual

Qiagen PCR Cloning Kit manual

QIAprep Spin Miniprep Kit manual