Q-PCR Analysis of GATA-1 in Umbilical Cord Blood: Optimizing RNA Extraction and Reverse Transcription Techniques

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Abstract

Rationale: Cord blood GATA-1 mRNA has been shown to serve as a biomarker of eosinophilopoeisis, but yields are often low and the assay challenging to optimize. In this study, we compared three RNA extraction protocols followed by three reverse-transcription assays to optimize our experimental yield.
Methods: Umbilical cord blood non-adherent MNCs (NAMNCs) were stimulated with Interleukin-5 (IL-5) to promote eosinophilopoeisis. NAMNCs were collected at 0, 24, 48 and 72 hours post stimulation and RNA was isolated using the RNeasy® Plus Mini kit (QIAGEN) and Total RNA extraction kit (FroggaBio). Samples were reverse transcribed using three kits: QuantiTect® Reverse Transcription kit (QIAGEN), AffinityScript qPCR cDNA synthesis kit (Agilent), and High Capacity RNA-to-cDNA kit (Applied Biosystems). The samples were also reverse-transcribed using iScript cDNA synthesis kit (BioRad) and three qPCR supermixes were tested: Sso Fast Probe Supermix (BioRad), NoRox Probe Mix (QIAGEN), and iTaq Probe Supermix (BioRad). The mRNA expression of GATA1 was measured using qPCR (BioRad).
Results: We found that the standard curve efficiencies were optimal for Qiagen/Qiagen, Qiagen/Applied Biosystems, Qiagen/iScript and Froggabio/Qiagen, Froggabio/Applied Biosystems, while Qiagen/Agilent and Froggabio/Agilent exhibited suboptimal efficiencies. In addition, there are not differences in efficiency when using various qPCR supermixes.
Conclusions: The combination of Qiagen/Qiagen and Qiagen/Biosystems seem to be more compatible and thus more efficient. In addition, the Sso Fast probe mix appears to be more versatile in the amplification of the GATA1 gene under various conditions.

Methods

Consent was given by expectant mothers and the umbilical cord blood was collected after delivery. The cord blood samples were processed immediately after their collection. All samples were depleted of red blood cells via unit gravity to isolate MNCs and temporarily frozen. All samples were then thawed, depleted of adherent cells and reseeded at 2-3 million cells per condition. Non-adherent MNCs (NAMNCs) were then stimulated with recombinant human Interleukin-5 (1ng/mL) to promote eosinophilopoeisis.
NAMNCs were collected at 0, 24, 48 and 72 hours post stimulation and RNA was isolated and pooled using the RNeasy® Plus Mini kit (QIAGEN) and Total RNA extraction kit (FroggaBio). The samples were then reverse transcribed using three kits: QuantiTect® Reverse Transcription kit (QIAGEN), AffinityScript qPCR cDNA synthesis kit (Agilent), and High Capacity RNA-to-cDNA kit (Applied Biosystems). The samples were also reverse-transcribed using iScript cDNA synthesis kit (BioRad) and three qPCR supermixes were tested: Sso Fast Probe Supermix (BioRad), NoRox Probe Mix (QIAGEN), and iTaq Probe Supermix (BioRad). The mRNA expression of *GATA1* was measured using qPCR (RotorGene, Corbett Life Science) and (CFX Connect, BioRad).

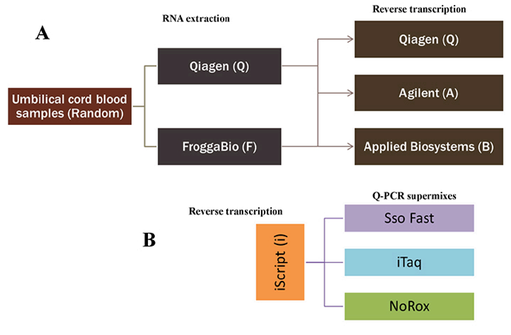
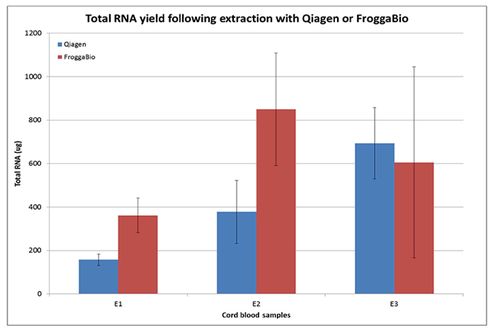


Fig 1. Kits chosen for RNA isolation, reverse transcription and q-PCR supermixes of random umbilical cord blood samples. (A) Random umbilical cord samples were selected and the RNA was extracted using the Qiagen or FroggaBio kits. The RNA samples were then subdivided into 3 groups. Reverse transcription was performed using the following kits: Qiagen, Applied Biosystems or Agilent. (B) Cord blood samples were reverse transcribed using the iScript cDNA synthesis kit and three qPCR supermixes were compared: NoRox, Sso Fast and iTaq.

Preliminary Results



RNA Isolation Kit	RT kit	Efficiency [%]	Range of Cq values	No-RT control contamination
Q	Q	98	28-35	No
	A	89	28-35	No
	B	103	27-33	No
F	Q	101	25-31	Inconclusive
	A	85	25-32	Yes
	B	100	24-30	Yes

Tab 1. The combination of QQ, QB, FQ and FB resulted in optimal qPCR efficiencies. In addition, although all the samples isolated with the Qiagen kit show a higher range of Cq values, the No-RT controls as expected did not amplify *GATA1*. All FroggaBio samples displayed lower Cq values however, all No-RT controls amplified the gene.

Fig 2. Total RNA isolation following extraction with Qiagen and Froggabio. Shown are the total RNA yields after isolation with Qiagen or FroggaBio extraction Kits. RNA was isolated for 4 individual samples per experiment (SEM).

RNA Isolation Kit	RT kit	q-PCR Probe Mix	Efficiency [%]	Range of Cq values	No-RT control contamination
Q	i	Sso Fast	103	29-37	No
		iTaq	79	29-37	No
		NoRox	99	29-36	No

Tab 2. The Sso Fast and NoRox supermixes have ideal qPCR efficiencies under an extended 3-step protocol. Samples isolated with the Qiagen kit were then reverse transcribed using the iScript kit. Three supermixes were tested and all Cq values were of similar range. Sso Fast and NoRox mixes showed optimal efficiencies.

RNA isolation	RT kit	q-PCR Probe mix	Efficiency [%]	Range of Cq values	No-RT control contamination
Q	i	Sso Fast	98	29-37	No
		iTaq	97	29-37	No

Tab 3. Both Sso Fast and iTaq supermixes show optimal efficiencies and Cq values under a shortened qPCR protocol. The samples were reverse transcribed using the iScript kit and two supermixes were compared. Both Sso Fast and iTaq supermixes show optimal efficiencies.

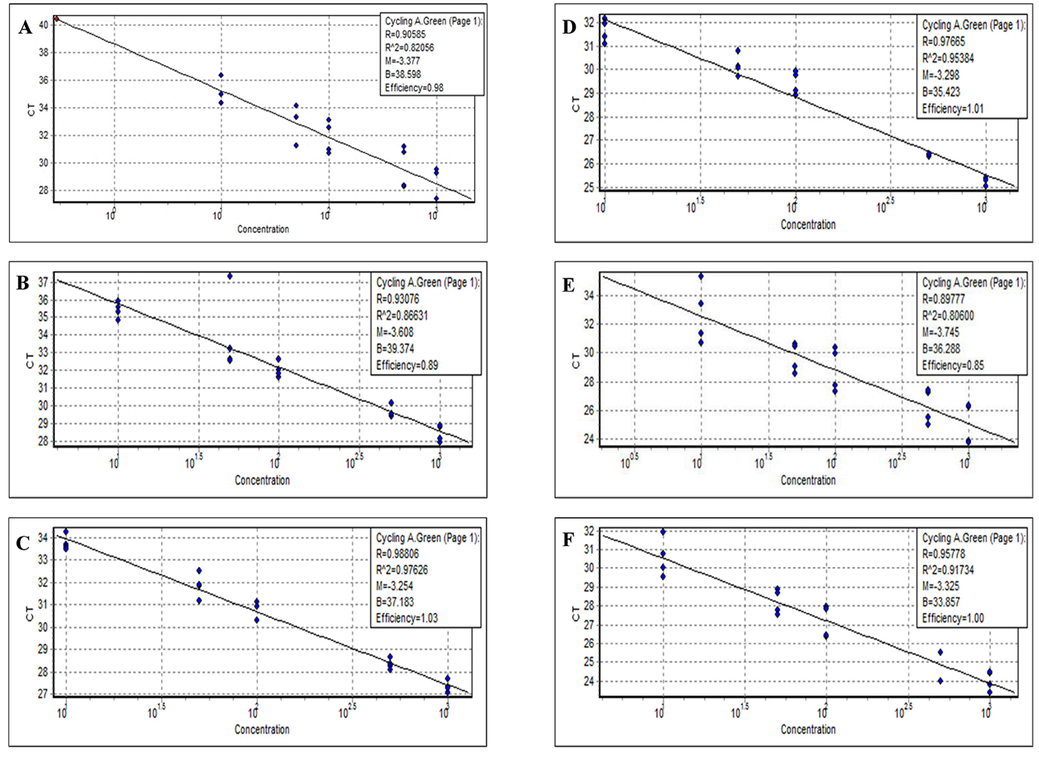


Fig 3. The combination of QQ, QB, FQ and FB resulted in optimal qPCR efficiencies. QA and FA exhibited suboptimal qPCR efficiency. Shown are the standard curves for the following combination of RNA isolation and RT kits: (A) QQ, (B) QA, (C) QB, (D) FQ, (E) FA, (F) FB. Each standard curve was derived using cDNA from two different UCB samples in duplicates.

Discussion

In this study, we found there was a higher RNA yield when using the FroggaBio extraction kit on NAMNCs compared to the Qiagen extraction kit. However, this difference in yields was quite variable between experiments. It is worth noting that the Froggabio extraction kit does not include a DNase elimination step could result in gDNA contaminated samples, which would not be discriminated when determining the nucleic acid yields at 260 nm (Fig 2). We also observed *GATA1* amplification in all Froggabio No-RT controls except for samples reverse-transcribed using the Qiagen kit (gDNA wipe-out buffer included).
Standard curves were generated for each of the combinations of RNA extraction and RT kits. It was found that the qPCR efficiencies were optimal for QQ, QB and FQ, FB (Fig 3. A, C, D, F), while QA and FA samples exhibited suboptimal efficiencies (Fig 3. B, E). We infer that the Agilent RT kit may not be a compatible with both Qiagen and FroggaBio extraction kits. Interestingly, all ranges of Cq values from FQ, FA and FB combination were ideal in the *GATA1* amplification. However, these Cq values could be an artifact of the gDNA present in all the samples.
We also tested three different qPCR supermixes and found that in using an extended 3-step qPCR method both Sso Fast and NoRox show ideal efficiencies of 98 and 103%, respectively. Unfortunately, under extended qPCR protocol the *GATA1* primer pair could potentially bind any gDNA contaminant and amplify the gene. Therefore, using a shortened qPCR protocol would be more suitable for the analysis of *GATA1* expression. Under these parameters both Sso Fast and iTaq supermixes showed optimal efficiencies and Cq values (Tab 2, Tab 3)
In conclusion, the combination of QQ and QB seem to be more compatible and thus more efficient in the RNA extraction and RT analysis of *GATA1* mRNA expression. In addition, the Sso Fast probe mix appears to be more versatile in the amplification of the *GATA1* gene under various conditions.

Acknowledgements

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