Optimization of Real Time RT-PCR Methods for the Analysis of Gene Expression in Mouse Eggs and Preimplantation Embryos

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ABSTRACT This study was carried out to optimize conditions for using real time RT-PCR as an efficient and precise quantitative method for estimating the transcript levels of genes expressed in samples containing miniscule amounts of RNA, such as single mammalian oocytes and embryos. First, using mouse eggs and blastocysts, we tested three kinds of RNA isolation or collection methods: TRIZOL reagent, oligo-dT conjugated beads, or three freeze/thaw cycles with the reverse transcription buffer. There were no significant differences among three groups in mRNA quantity as assayed by real time RT-PCR analysis. Second, we compared the efficacy of real time analysis between TaqMan fluorescent probes and the SYBR-green dye system. The two systems presented similar real time RT-PCR profiles for the *16s ribosomal protein* gene from oocytes to blastocysts. Third, RNA from mouse embryos at defined stages of preimplantation development were isolated and the levels of transcripts encoded by several housekeeping genes (*GAPDH*, *β-actin*, *ribosomal protein L7*, *16s ribosomal protein*, *histone H2A.Z*) were quantitatively analyzed by real time RT-PCR. The *histone H2A.Z* and *16s ribosomal protein* slightly increased from the egg to blastocyst stages by approximately 10- and 30-fold, respectively. However, other transcripts increased more than 300-fold as a function of developmental stage from eggs to blastocysts. Our results suggest that the simple freezing/thawing method for RNA collection, the economic SYBR-green dye system, and *histone H2A.Z* gene as an internal control should be useful for the real time RT-PCR analysis of single mouse eggs and preimplantation embryos. *Mol. Reprod. Dev.*

Key Words: quantitative real time RT-PCR; eggs; preimplantation embryos; freezing/thawing; SYBR-green dye; *histone H2A.Z*

INTRODUCTION

Quantitative estimations of gene expression at the total mRNA level and of specific genes of interest in particular samples are very informative to evaluate the changes in cell physiology resulting from differentiation or in response to various parameters. Classical techniques of RNA analysis such as Northern blotting (Thomas, 1980), dot- or slot-blotting (White and Bancroft, 1982), and in situ hybridization are not suitable to detect mRNA in single cells, mammalian oocytes, or preimplantation embryos because these techniques lack the sensitivity to detect the small amounts of mRNA in these samples. The reverse transcription-polymerase chain reaction (RT-PCR) allows the amplification of low abundance mRNA and permits the analysis of gene expression in scarce mRNA samples (Rappolee et al., 1988; Brenner et al., 1989). However, due to differential reaction efficiencies and kinetics in RT-PCR, the amount of final product obtained after amplification may not accurately reflect the initial sample mRNA concentration. Competitive PCR procedures have been developed and overcome the problems associated with endpoint analysis (Becker-Andre and Hahlbrock, 1989; Wang et al., 1989; Stieger et al., 1991). However, these procedures are time-consuming because of they require the design of the standardized cDNA templates and the analysis numerous dilution series of competitor.

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Real time RT-PCR with fluorescent monitoring has been introduced to determine the concentration of low abundance mRNAs of interest (Higuchi et al., 1993). The unique feature of this system is the use of fluorescent reporters, such as TaqMan probes and SYBR Green dye (Morrison et al., 1998; Bustin, 2000). By analysis of the fluorescence generated during the course of the reaction, reliable quantitative data can be obtained in the exponential phase of amplification phase. The analysis program determines a threshold cycle (C_t), which is the cycle when the fluorescent signal of the sample exceeded a threshold level significantly rises above the mean of the baseline fluorescence. Compared with conventional RT-PCR, several steps involved in the quantification procedure can be omitted. There is no need for optimization of amplification cycle number, gel electrophoresis of PCR products, and densitometry of band intensity. The successful use of this system has been reported for analysis of gene expression in single oocytes and preimplantation embryos of mammals (Steuerwald et al., 1999, 2000; Hartshorn et al., 2002; Hayashi et al., 2003; Lindeberg et al., 2004). The development of quantitative real time RT-PCR has to be combined with reliable determinations of RNA as internal standards and requires methods for accurate normalization. In general cells and tissues, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), β-*actin*, and *ribosomal RNA* are commonly used as "housekeeping genes" (Suzuki et al., 2000).

In mammals, preimplantation embryo development is characterized by various morphological and physiological transitions that occur after fertilization. The embryonic genome becomes functionally active and embryonic transcripts and proteins replace some of the maternal components required for the early developmental process. The semiquantitative or real time RT-PCR is the most frequently used system for the studies of gene expression and regulation during the preimplantation development. It involves normalization of RNA amount by adding a known amount of external control RNA or utilizing endogenous transcript for housekeeping genes as an internal control. A prerequisite for usefulness in normalization is that the expression level of the housekeeping gene does not vary markedly through the preimplantation development or in response to different experimental conditions. The amount of mRNA in preimplantation embryos is variable for most genes, including housekeeping genes, because of dynamics of gene expression in early development (Bilodeau-Goeseels and Schultz, 1997). This variability introduced a considerable bias when the values of the target genes were normalized to the values of an inconsistent housekeeping gene in bovine preimplantation embryos (Robert et al., 2002).

In this study, we compared the efficacy of RNA preparation and fluorescent signal generation methods of real time RT-PCR analysis in mouse eggs and preimplantation embryos. In addition, the present work identified the most suitable of a group of candidate housekeeping genes in mouse eggs and preimplantation embryos by evaluating the expression levels of *GAPDH, β-actin, ribosomal protein L7, 16s ribosomal protein*, and *histone H2A.Z*.

MATERIALS AND METHODS

Collection of Eggs and Preimplantation Embryos

Mouse ovulated eggs and preimplantation embryos (PN-zygote, 2-cell, morula, blastocyst) were collected at the proper time of development. ICR female mice (5–7 weeks old) were induced to superovulate by standard hormonal treatments using an i.p. injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG) followed 48 hr later by an i.p. injection of 7.5 IU of human chorionic gonadotropin (hCG). After mating overnight with males of the same strain, females were inspected for vaginal plugs the next day, and killed by cervical dislocation 48 hr after hCG treatment. Embryos at presumptive late 2-cell stage were recovered by flushing the oviducts with modified HTF medium with 0.4% bovine serum albumin (BSA). The embryos were washed with G1 medium (Vitrolife, Göteborg, Sweden), and then cultured in G1/G2 medium (Vitrolife) for 4-cell or blastocysts at 37°C in an atmosphere of 5% CO_2 and 95% air. At specific times, the eggs and embryos were collected and then washed with Ca^{2+}- and Mg^{2+}-free PBS. They were individually loaded into each PCR tube and stored at −70°C until used.

Isolation and Collection of RNA From Eggs and Preimplantation Embryos

Three methods were employed for isolation or collection of RNA from samples. (1) Total RNA of each individual oocyte or embryo was isolated using the TRIZOL reagent (Gibco BRL, Rockville, MD). The RNA was pelleted by centrifugation, washed using 300 μl of 75% ethanol and dissolved in 10 μl of RNase-free water. (2) Messenger RNA was isolated with oligo-dT conjugated bead using the Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway) according to the manufacturer's instructions. (3) Three freeze/thaw cycles with the only reverse transcription buffer (Promega, Madison, WI) carried out for collection of total RNA.

Reverse Transcription

First-strand complementary DNA (cDNA) synthesis was performed by priming with 20 pmoles oligo-dT in a 20 μl reaction mixture containing 10 mM each of dATP, dCTP, dGTP, and dTTP; 40 U/μl of ribonuclease inhibitor, 10 U/μl avian myeloblastosis virus (AMV) reverse transcriptase (Promega), and 10× AMV-RT buffer. The reaction condition was 42°C for 60 min, 95°C for 5 min using an Eppendorf model 9600 thermocycler (Eppendorf, Norwalk, C_t). The RT reaction was stopped by cooling to 4°C for 10 min. A portion of RT product (2 μl as 0.1 equivalent of single egg or embryo) was used directly for real time PCR.

Probe and Primer Design for Real Time PCR

The TaqMan probe and primers for mouse *16s ribosomal protein* was designed using the Primer Express program (Applied Biosystems, Foster City,

TABLE 1. Information on the Primers Used for Real Time RT-PCR

Genes	Sequences	Product size (bp)	Annealing temperature (°C)
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	452	62
β-*actin*	5′-GTATGCCTCGGTCGTACCA-3′ 5′-CTTCTGCATCCTGTCAGCAA-3′	499	62
Ribosomal protein L7	5′-TCAATGGAGTAAGCCCAAAG-3′ 5′-CAAGAGACCGAGCAATCAAG-3′	246	62
Histone H2A.Z	5′-CGTCAGAGAGACGCTTACCG-3′ 5′-AAGCCTCCAACTTGCTCAAA-3′	286	63
16s ribosomal protein	5′-AGATGATCGAGCCGCGC-3′ 5′-GCTACCAGGGCCTTTGAGATGGA-3′	163	62

CA). The probe sequences were-5′-VIC-TGCAGTAC-AAGTTACTGGAGCCTGTTTTGCTT-TAMARA-3′. The primers of housekeeping genes (*GAPDH, β-actin, ribosomal protein L7, 16s ribosomal protein* and *Histone H2A.Z*) were designed for real time PCR with SYBR green and listed in Table 1. Prior to the quantitative analysis, optimization procedures were performed by running real time PCRs with or without template to verify the reaction condition, including the annealing temperatures of the primers and specific products.

Real Time PCR

The real time PCR reaction was performed using the ABI PRISM 7700 sequence detection system and TaqMan PCR Core Reagent Kit (Applied Biosystems) or DNA Engine Opticon 2 fluorescence detection system (MJ Research, MA) and Dynamo SYBR green qPCR Kit (Finnzymes Oy, Espoo, Finland). The SYBR reaction mixture consisted of modified Tbr DNA polymerase, SYBR green, optimized PCR buffer, 5 mM MgCl2 and dNTP mix including dUTP. The PCR protocol was used: denaturation program (95°C for 10 min), amplification and quantification program repeated 40–45 times (94°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec with a single fluorescence measurement), melting curve program (65°C ~95°C with a heating rate of 1.0°C per 30 sec and a continuous fluorescence measurement) and finally a cooling step to 4°C. Thereafter, PCR products were identified by generating a melting curve. Since the melting curve of a product is sequence specific, it can be used to distinguish between them. The size of RT-PCR products were confirmed by gel electrophoresis on a standard 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light. For the mathematical analysis, it is necessary to determine the C_t value for each transcript. The C_t value represents the cycle number at which a fluorescent signal rises statistically above background. The relative quantification of gene expression was analyzed by the $2^{-\Delta C_t}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Statistical comparisons were performed by Student's *t*-test and Kruskal–Wallis nonparametric ANOVA test, followed by Dunn's multiple comparison test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Amplification and melting curve profiles of real time PCR from this study are shown in Figure 1. Reaction samples consisted of single mouse eggs and preimplantation embryos. Amplification was evident from all samples while the negative control (no template) responded appropriately. Individual experiments were repeated a minimum of three times to evaluate the

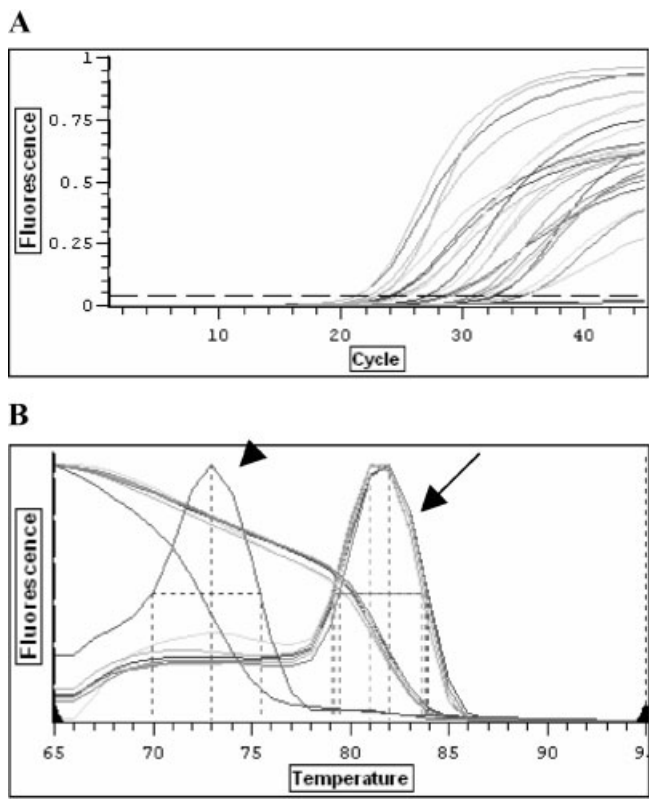

Fig. 1. Amplification and melting curve profile in real time RT-PCR. **A**: Graphic illustration for intensity of fluorescent signals after subtraction of background. **B**: Graphic illustration for melting curve of nonspecific primer dimers (arrow head) and specific PCR products (arrow). Since the melting curves are sequence specific, it can be used to distinguish between nonspecific and specific PCR products.

TABLE 2. C_t Value of Real Time RT-PCR According to RNA Extraction Methods

Genes	Samples	RNA extraction methods		
		Freeze/thaw	TRIZOL	Dynabeads
Histone H2A.Z	Egg	30.9 ± 1.3	28.0 ± 1.6	28.2 ± 0.1
	Blastocyst	27.3 ± 0.3	24.7 ± 1.8	24.3 ± 0.6
16s ribosomal protein	Egg	27.8 ± 1.8	27.0 ± 0.2	26.0 ± 0.2
	Blastocyst	22.4 ± 0.5	21.7 ± 0.8	21.3 ± 0.5
β-*actin*	Egg	31.9 ± 3.5	29.4 ± 0.7	29.9 ± 1.0
	Blastocyst	23.5 ± 1.0	21.8 ± 0.6	22.2 ± 0.5
Ribosomal protein L7	Egg	29.3 ± 1.0	28.8 ± 0.3	31.8 ± 0.4
	Blastocyst	21.1 ± 1.0	21.2 ± 0.8	24.1 ± 0.9

Data of C_t value are collected from triplicate experiments and represented mean ± SD. There was no significant difference by Student's *t*-test and Kruskal–Wallis nonparametric ANOVA test.

degree of variation. The reaction condition including the annealing temperatures of the primers and specific products could be verified by melting curve analysis (Fig. 1). The optimized annealing temperatures of fluorescence acquisition for all the genes in this study are listed in Table 1.

The amounts of mRNAs from mouse eggs and blastocysts with three RNA isolation or collection methods were compared by C_t values in the real time RT-PCR. There were no significant differences of C_t values among three methods (Table 2). Even the simple freeze/thaw method provided comparable C_t values.

Two detection methods are available for real time PCR: specific detection involving fluorescent probes, which will bind the amplified product, and nonspecific detection with SYBR green, which binds to all double-stranded DNAs. For this nonspecific detection, it is important to measure the fluorescence precisely before the predetermined melting temperature of the specific product to avoid the additional fluorescence of the contaminating double-stranded DNA produced by primer dimers. We evaluated the efficacy of real time analysis between TaqMan fluorescent probes and the SYBR green system for *16s ribosomal protein* mRNA. Both methods showed similar results, and were equally effective at detecting product accumulation (Table 3).

TABLE 3. Quantitative Analysis of *16s Ribosomal Protein* mRNA by Real Time RT-PCR Using Two Different Fluorescence Detection Methods

Samples	SYBR-Green		TaqMan probe	
	C_t value	Relative amount[a]	C_t value	Relative amount
Egg	30.3 ± 0.2	1.0	28.1 ± 0.5	1.0
PN-zygote	30.1 ± 0.2	1.2	28.0 ± 0.7	1.1
2-cell	30.7 ± 1.0	0.8	28.6 ± 0.5	0.7
8-cell	29.1 ± 0.4	2.3	26.9 ± 0.5	2.3
Morula	28.2 ± 1.9	4.3	25.2 ± 0.8	7.5
Blastocyst	25.0 ± 0.8	39.4	22.7 ± 0.7	42.2

Data of C_t value are collected from at least triplicate experiments represented mean ± SD.
There was no significant difference between two methods by Student's *t*-test.
[a]Data of relative amount are normalized to the C_t of egg and calculated by the $2^{-\Delta C_t}$ method.

The quantitation of transcripts for the selected housekeeping genes revealed the relatively large abundance of these transcripts at the blastocyst stage. As indicated in Table 4, β–*actin*, *ribosomal protein L7*, and *GAPDH* mRNA levels followed this pattern of sharp increase. However, *histone H2A.Z* and *16s ribosomal protein* gene showed slighter increases in abundance from eggs to blastocysts, corresponding to less than 10-fold and 30-fold, respectively. The mRNA level of *histone H2A.Z* was the most stable across entire preimplantation period (Fig. 2).

DISCUSSION

A serious limitation of early quantitative PCR strategies has been their reliance on endpoint analysis. Since variable reaction efficiencies can alter the duration of the log-linear phase of amplification, considerable differences in overall PCR product synthesis may be observed which may not necessarily correlate with input template concentrations. Competitive PCR strategies were developed to overcome this problem; however, these procedures are laborious. Our study shows that real time RT-PCR is a very useful method to detect gene expression, even in very small amounts of sample such as 0.1 equivalent of single egg or preimplantation embryo. This system was used for the analysis of gene expression even in single blastomeres (Hartshorn et al., 2003; Lindeberg et al., 2004). Increased sensitivity to detect transcripts was illustrated by the fluorescence signals from TaqMan probes or SYBR green during single rounds amplification. In particular, fluorescence monitoring allows earlier product detection than possible by conventional gel electrophoresis. In this study, similar results using both TaqMan probes and the SYBR green system were obtained by quantitative real time RT-PCR analysis of *16s ribosomal protein* gene expression from mouse eggs to blastocysts. We suggest that SYBR green can provide for a simple and inexpensive means of monitoring fluorescence during amplification. However, comparing to the TaqMan system, the SYBR green system may be taken times to optimize the primers and PCR condition, which prevent generation

TABLE 4. C_t Value of Internal Standard Genes in Oocyte and Preimplantation Embryos by Real Time RT-PCR

Samples	*Histone H2A.Z*	*16s ribosomal protein*	β-*actin*	*Ribosomal protein L7*	*GAPDH*
Egg	28.1 ± 0.5^{a}	26.5 ± 0.7^{a}	35.5 ± 2.5^{a}	35.8 ± 3.0^{a}	34.5 ± 2.3^{a}
PN-zygote	27.8 ± 0.3^{a}	$25.6 \pm 0.5^{a,b}$	$32.9 \pm 1.2^{a,b}$	34.7 ± 1.4^{a}	35.3 ± 0.2^{a}
2-cell	$25.8 \pm 0.2^{a,b,c}$	$24.4 \pm 0.2^{a,b,c}$	$30.4 \pm 1.7^{a,b,c}$	$29.6 \pm 0.4^{a,b}$	$31.8 \pm 1.1^{a,b}$
Morula	$25.0 \pm 0.2^{b,c}$	$22.2 \pm 0.7^{b,c}$	28.2 ± 1.1^{b}	$28.4 \pm 2.1^{a,b}$	$29.3 \pm 4.2^{a,b}$
Blastocyst	24.8 ± 0.3^{c}	21.1 ± 1.2^{c}	27.2 ± 1.8^{c}	27.1 ± 1.4^{b}	26.4 ± 1.0^{b}

Data of C_t value are collected from five times experiments and represented mean ± SD.
[a–c]Different superscripts in the same column are significantly different by Kruskal–Wallis nonparametric ANOVA test, followed by Dunn's multiple comparison test.

of primer dimmers or nonspecific products. It can make a mistake in the analysis of gene expression.

Several methods of RNA isolation from small samples have been used by manual and commercially available kits. Generally, researchers perform RNA isolations by their methods chosen by familiarity rather than an objective assessment of the optimal procedure. We evaluated the efficacy of three methods using the TRIZOL reagent, the Dynabeads, and the mechanical freeze/thaw procedure. There were no significant differences in the results of real time RT-PCR analysis, even with the simple freeze/thaw procedure. We speculated that this result may be related to the relative abundance of mRNA in oocytes and preimplantation embryos compared to tissues or other cultured cells. Oocytes and preimplantation embryos also have low levels of highly glycosylated proteins such as mucins and RNA degrading enzymes that could interfere with RNA isolation and preparation. The simple freeze/thaw procedure had been successfully applied for cloning cDNA amplified from ten mouse oocytes (Revel et al., 1995). We also tested our freeze/thaw procedure using an exogenous standard of rabbit globin mRNA. The C_t value of rabbit globin was constant after freeze/thaw procedure in real time RT-PCR (data not shown). It is important to maintain low temperature during the procedure to prevent the degradation of mRNA.

The exogenous standard was applied to account for the variations caused by manipulation and treatment of samples. Analysis of the global gene expression in microarray experiments would require the exogenous control to verify absolute changes if global shift in mRNA pools occur (van de Peppel et al., 2003). However, the differences in the intrinsic factors and the amount of starting materials could not figure out using only the exogenous standard. The appropriate selection of housekeeping genes as internal controls in real time RT-PCR is critical for the estimation and comparison of mRNA levels in gene expression studies. The ideal internal standard should be expressed at a constant level among different cells/tissues of an organism, at all stages of development, and should be unaffected by various experimental or environmental conditions (Thellin et al., 1999; Bustin, 2000). The present study shows that the most reliable housekeeping gene appears to be *histone H2A.Z* in mouse eggs and preimplantation embryos. Our results correspond with the previous report using bovine preimplantation embryos (Robert et al., 2002). They suggested *histone H2a* was the best internal standard because transcript levels were constant across the preimplantation embryo developmental period. The measurement of alterations in low abundance hypothalamic mRNAs by RT-PCR showed that *histone H3* is an acceptable internal control because its expression is cell-cycle independent and constitutive (Kelley et al., 1993). In human T lymphocytes, *18S rRNA* gave the most reliable results followed by β-*actin* and *GAPDH* mRNA when comparing resting and activated conditions (Bas et al., 2004). The reliability of a housekeeping gene for use as an internal control in the quantitative analysis of gene expression may be dependent on tissue or cell type.

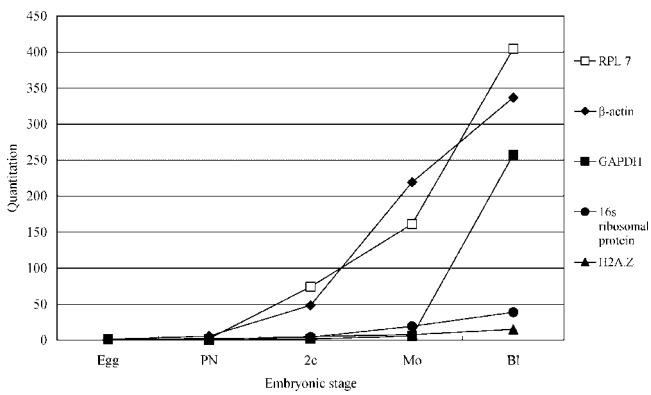

Fig. 2. Quantitative analysis of internal standard genes by real time RT-PCR during the course of mouse preimplantation embryo development. Data of quantitation are normalized to the C_t of egg and calculated by the $2^{-\Delta C_t}$ method. Egg: ovulated oocytes, PN: pronuclear zygote, 2c: 2-cell, Mo: morula, Bl: blastocyst, RPL 7: *ribosomal protein L7*, H2A.Z: *histone H2A.Z*.

Recently, the large number of genes identified as involved in zygotic gene activation and signaling pathways by the microarray analysis of global gene expression in the preimplantation mouse embryos (Hamatani et al., 2004; Wang et al., 2004). Their findings could be robusted by the confirmation of real time RT-PCR in specific genes. Our results suggest that the simple freeze/thaw method for RNA isolation and the economical SYBR green system should be useful for the real time RT-PCR analysis of mRNA from single oocytes and preimplantation embryos. The *histone H2A.Z* gene may be the best internal standard because the transcript levels were similar throughout the preimplantation period. The reliability of these results should be substantiated by the reproducible, consistent quantitative data from experimental samples.

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