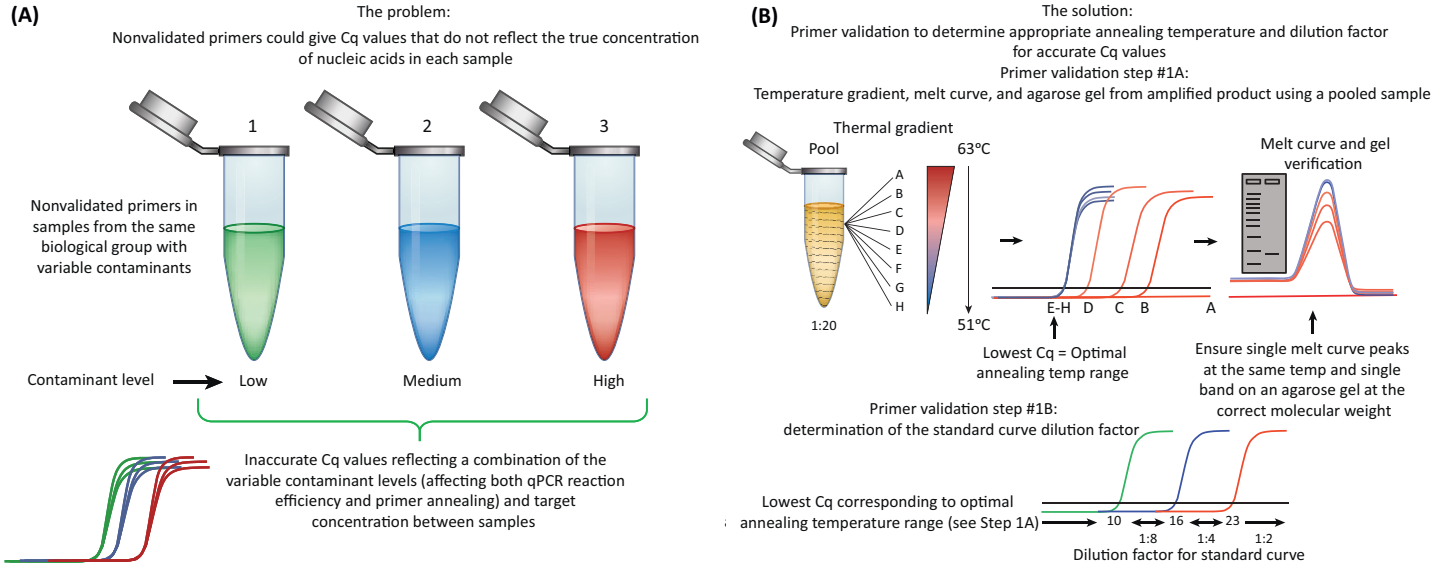
CellPress REVIEWS



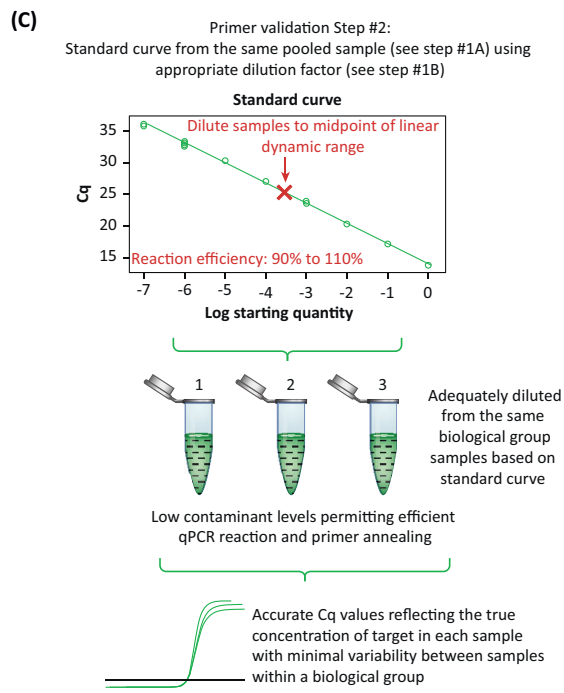


Figure 4. Validating a Quantitative PCR (qPCR) Experiment to Minimize Error and Maximize Data Quality. (A) Nonvalidated primers could give variable and/or artefactual data that do not reflect the true target abundance in each sample. (B) Primer validation using thermal gradient and gel electrophoresis. Step #1A: Thermal gradient and agarose gel validation. To validate primers, an equalized pool of samples from each biological group is diluted 1:20 and initially tested using a thermal gradient to determine the optimal annealing temperature, average level of expression, and unique product for each target from melt curve and gel analysis. Step #1B: The quantitative cycle (Cq) value from the optimal annealing temperature range can be used as a guide to establish thestandard curve dilution factor for each target (i.e., if the Cq value for optimized temperature range is between 10 and 16, use a 1:8 serial dilution series of the pooled cDNA sample in water). (C) Standard curve validation. An eight-point standard curve is tested for each primer pair using the same pooled sample and the appropriate dilution factor as determined from the thermal gradient data (Step #1B) to cover the widest dynamic range possible. Amplification efficiency, as determined from the slope, should range between 90% and 110%. Deleting the highest and/or lowest concentration points from each primer validation standard curve may be necessary to achieve the best efficiency. The dilution factor from the midpoint is then used to dilute the individual experimental samples per target, assuming that the pooled DNA sample represents the average abundance of each target for the experiment (i.e., equalized pool of the same number of DNA samples from each biological group). This ensures minimal presence of contaminants affecting primer efficiency and accurate quantitative data.

Step	Description	Sub steps	Instruction
1	Experimental design	Procedure	List the best targets, samples, and treatments based on previously acquired, vetted, and validated data or literature (Figure 1A).
		Biological groups	Define the appropriate biological groups (i.e., treatments, knockout, time points, etc.) and various combinations thereof (Figure 1).
		Replicates	Biological (samples): number of animals or number of cell culture plates per biological group, which is determined with the aid of a biostatistician and/or a statistical power analysis (highly dependent on the complexity of the organism). Technical: number of wells pipetted per cDNA sample from each biological replicate (typically two or three); (Figure 1).
		Experimental conditions	Carefully note all controllable factors, such as: lot consistency in cell culture media, FBS, BSA, animal feed and drugs or compounds, sex, and phenotype. Take pictures throughout the experiment and carefully note any unusual changes in specific samples or specimens that may become outliers.
2	Tissue and cell harvesting	Sample extraction (complex organisms)	Sacrifice animals, extract and dissect tissue reproducibly and sequentially. Flash freeze in liquid nitrogen immediately upon dissection (Figure 1C).
		Sample extraction (cells)	For adherent cells, quickly remove medium and then add lysis buffer from RNA extraction kit directly to the plate. Generate a stable homogenate by scraping cells and pipetting up and down, then freeze.
		RNA extraction procedure	Research, explore, and test appropriate homogenization methods, which can vary between tissue and cell types to ensure quality, reproducible data. Ensure extraction reagents are nuclease-free and use spin columns to purify RNA from protein and chemical contaminants. DNAse treat the RNA extracts using appropriate procedures [61].
3	Test RNA samples	Minimize protein and chemical contaminants	Nanodrop the RNA samples to ensure an OD 260/280 of 1.8 or higher, and an OD 260/230 of 2.0 or higher.
		Ensure RNA is intact	Run about 400 ng per RNA sample on a bleach gel [62] (28S:18S ribosomal RNA bands should give a ratio of at least one or higher). If samples are precious and limited, use an automated electrophoresis instrument like the Bioanalyzer (RIN number of at least 7.0).
4	Reverse transcription	Dilute RNA samples	Normalize all extracted RNA samples to the same approximate concentration. For tighter data, normalize using RiboGreen fluorescence [63]. Use same amount of RNA from each sample for reverse transcription.
		Use a good kit	Use a good reverse transcription kit containing: (i) a mix oligo(dT) and random hexamers for complete coverage of the mRNA; (ii) RNAse H+ for one-to-one conversion of mRNA to cDNA; (iii) RNAse inhibitor and robust enzyme mix to reverse transcribe mRNA over a broad dynamic range of concentrations. Test different amounts of total RNA for a given sample type to produce optimal quality cDNA – using less total RNA may yield better qPCR data.
5	Primer validation	Thermal gradient	The unique, copurified protein and chemical contaminants in nucleic acid extracts can affect primer annealing. Prepare a 1:20 diluted cDNA sample from a pool of equivalent quantities of each treatment condition, and run a thermal gradient (typically between 51 °C and 63 °C) of annealing temperatures (Figure 4B).
		Visualize the amplicon	Run an agarose gel and sequence the amplicon to ensure primer specificity, purity, and identity (Figure 4B).
		Assess reaction efficiency, linear dynamic range, and include controls	For each primer pair, perform an eight-point standard curve from the pooled cDNA sample used for the thermal gradient. Serially dilute the cDNA based on expression level per primer: High (Cq: 10–16), 1:8; Medium (Cq: 16–23), 1:4; low (Cq: ≥23), 1:2 (Figure 4B). Dilute individual samples to the midpoint of the efficient range (90% to 110%) of the standard curve (Figure 4C). Prepare and plate reactions appropriately with required controls (Figure 3A).
6	Reference gene validation	Survey literature for potential reference genes	Use the search term 'qPCR reference gene [GeNorm or NormFinder or BestKeeper] [Organism and Tissue of interest]' using Google Scholar. Pick seven to ten targets from the articles, and validate the primers (Figure 4B,C). A spike-in RNA or DNA sample into all test samples can help assess reference gene stability and may also be a useful normalization target [47].
		Confirm target stability	Test the validated reference gene primers against three cDNA samples from each biological/treatment group. Test stability using GeNorm, NormFinder and BestKeeper software. Normalize to multiple reference genes (Figure 4D).

[a]Adapted and updated under a Creative Commons license from [8].