Vol. 11
2022

ASSAYWISE LETTER

LIFE SCIENCE RESOURCES AND APPLICATIONS

Featuring

Overview of DNA Extraction Methods

A Practical Overview of qPCR

Applications of TBNK Antibody Kits & Newest Improvements on Current Standards

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Overview of DNA Extraction Methods

Abstract

DNA extraction is a fundamental method in molecular biology, despite being developed unintentionally. In 1869, the chemist Friedrich Miescher attempted to separate the cytoplasm from the nucleus in human leukocytes. He would filter suspended cells and treat them with diluted hydrochloric acid. This resulted in the discovery of a strange substance he dubbed "nuclein." While Miescher wrongly speculated about the role of "nuclein," his work paved the way for future developments in DNA extraction leading to techniques that are more reproducible, easier and faster to perform, cost-effective, and produce higher yields with minimal impurities.

Introduction

DNA, which is present in almost all organisms, is widely accepted as the blueprint of life. Stored within the lengthy sequences of its polynucleotides are the instructions for making every protein and molecule essential for growth, development, and health. When isolated, DNA can be used in various downstream applications, mainly polymerase chain reaction (PCR) and real-time PCR (qPCR), DNA sequencing, southern blotting, preparation of genomic libraries, and various genetic polymorphism applications. In modern medicine, many of these applications serve as the basis for prescreening and diagnosing genetic disorders and developing novel gene therapies and gene-editing technologies. Although several variations of DNA extraction have been developed since Miescher's initial discovery (figure 1), the principle behind each method consists of the same basic steps: (1) disruption of the cell and nuclear membranes; (2) separation of DNA from the cell lysate (e.g., lipids, proteins, and other nucleic acid species); and (3) concentration and purification of DNA. Determining which DNA extraction method to use is primarily influenced by the intended downstream applications for which the isolated DNA will be used. Besides the quality and quantity of the DNA extracted, other factors to consider are time, cost, yield, laboratory equipment, and the amount of starting material needed for the experiment.

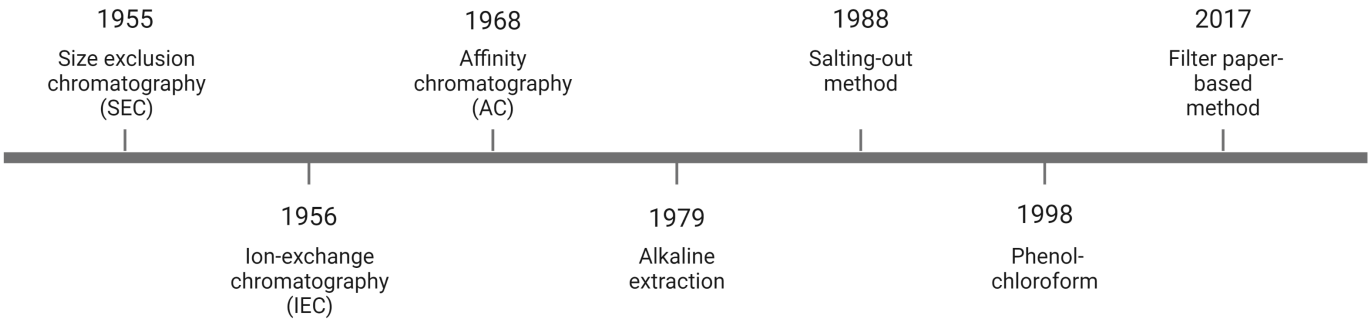

Figure 1. The development of different DNA extraction techniques over the years.

DNA Extraction and Purification Methods

Phenol-Chloroform Extraction

A common way to isolate DNA is via the phenol-chloroform extraction method. This method is suitable for extracting DNA from various samples, including blood, suspension culture, and tissue. It produces relatively high yields and higher purity DNA than conventional extraction methods. Because this technique uses toxic chemicals, such as phenol, to denature proteins and chloroform to solubilize lipids, it should be performed in a fume hood, and the necessary precautions should be taken while handling.

Like all DNA extraction methods, the phenol-chloroform process begins with destroying the cell membrane and non-nucleic acid cellular components. Cells are treated with a lysis buffer typically containing denaturing detergents, such as sodium dodecyl sulfate (SDS), and depending upon the type of DNA (e.g., genomic, mitochondrial, or plasmid), can include other additives. For instance, lysis buffers for extracting plasmid DNA from bacterial cells will contain sodium hydroxide for alkaline lysis and potassium acetate for the renaturation of the plasmid DNA.

A mixture of phenol:chloroform:isoamyl alcohol (PCIA) is then added to the lysate to denature proteins and facilitate the precipitation of DNA. Since phenol is hydrophobic and less dense than water, centrifugation is used to partition the lysate into three distinct layers or phases. The bottom layer, or "organic phase," contains hydrophobic molecules like phenol, lipids, and chloroform. The middle layer, or "interphase," consists of denatured proteins, and the top layer, or "aqueous phase," comprises DNA and other polar molecules. The aqueous phase is then removed and transferred to a clean tube.

Transferring the aqueous layer requires careful pipetting. Disruption of the interphase risks exposing the aqueous phase to the organic phase and may cause contamination. When the aqueous phase is successfully transferred, DNA is precipitated using a solution of ammonium acetate and ethanol. The resulting DNA pellet is separated via centrifugation. Often, multiple ethanol washes are needed to remove contaminants and further concentrate the DNA. Each added wash involves removing the supernatant, resuspending the pellet with ethanol, and centrifuging the tube. Following the final wash, the pellet is air-dried and resuspended in a polar solution, such as an elution buffer.

Silica-Phase Extraction

Besides organic methods, solid-phase extraction using a solid substrate, such as silica resins or beads, is another popular way to isolate DNA. Instead of using solvents to force DNA precipitation, this technique uses a simple lyse-bind-wash-elute process. First, cells are lysed with a buffer solution containing 1% SDS, 0.05 M EDTA, 0.2 M Tris pH 8.0, and Proteinase K. A binding

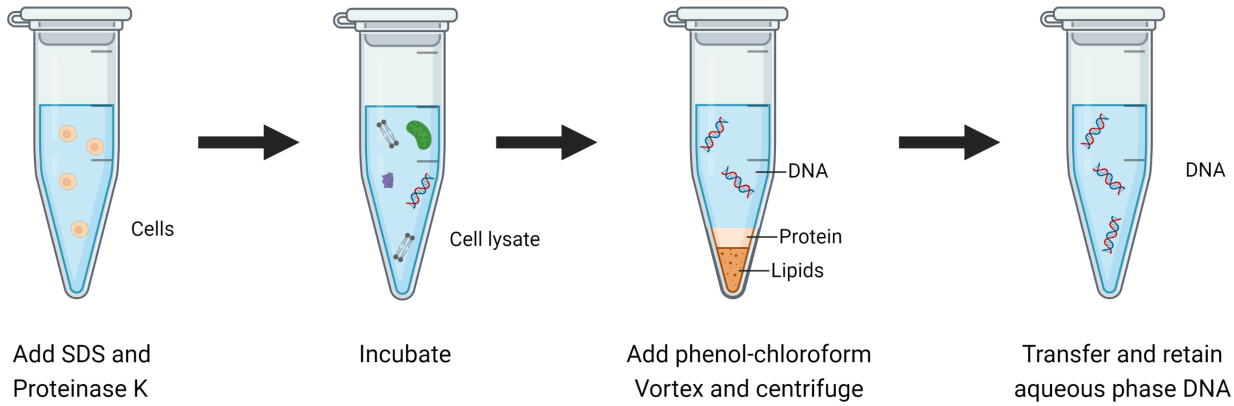

Figure 2. Illustration of organic DNA extraction using the phenol:chloroform:isoamyl alcohol (PCIA) method. PCIA partitions DNA to the aqueous phase while lipids and proteins are partitioned into the organic and interphases, respectively.

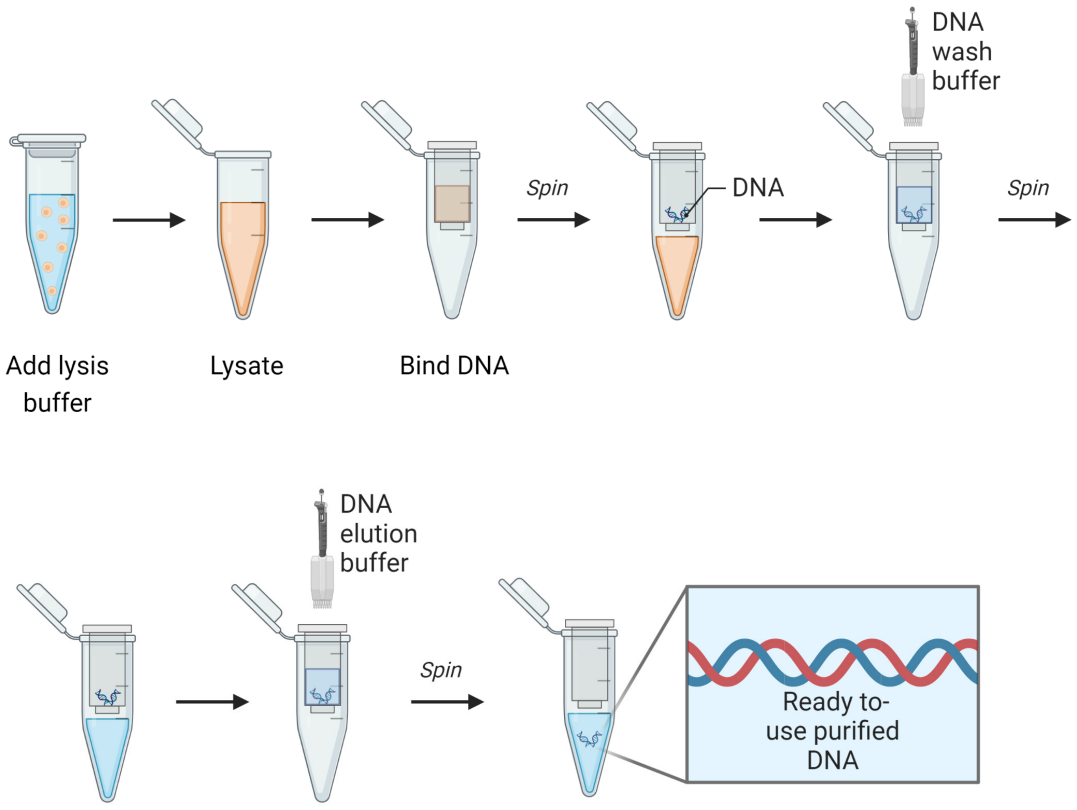

Figure 3. Illustration of solid-phase DNA extraction using silica gel spin columns. In the presence of chaotropes, DNA binds to silica resins while other cellular components are washed away.

buffer made up of various chaotropic salts (e.g., phenol, ethanol, guanidine hydrochloride, urea) is added to the lysate and then transferred to a spin-column and centrifuged. Centripetal forces push the solution through the silica membrane within the spin column, whereby DNA binds to the silica membrane. The rest of the solution (e.g., proteins and organic phase) passes through the column and is discarded.

The spin column undergoes multiple washes with various buffers, each formulated to remove specific contaminants while maintaining DNA binding conditions. Following the last wash step, DNA is selectively eluted under low-salt conditions using TE buffer.

Both techniques will yield high-quality DNA samples, but there are optional treatments to improve sample quality. Enzymes such as proteases and nucleases can be added to the sample to remove cellular contaminants further. Some examples are Proteinase K and various RNases. The use of enzymatic degradation does add to the overall cost, but the increased sample quality often makes it worth it.

Quantitative and Qualitative Analysis of DNA Yield and Purity

Following successful DNA extraction and before the intended downstream application, the yield and purity of the DNA must be assessed. The primary methods to measure DNA yield and purity include absorbance, fluorescence, and gel electrophoresis.

Of the three, absorbance methods are the most common and easiest. They do not require additional reagents like fluorescence-based methods, which need DNA-selective dyes. All that is required is a spectrophotometer. Absorbance readings

of the DNA solution are taken at 260 nm, where DNA absorbs light maximally, and at 230, 280, and 320 nm, which correspond to absorbance peaks of common chemical contaminants or chaotropic salts, proteins, and turbidity, respectively. The number recorded at A_{260} can be used to estimate the concentration of the solution and should be within the linear range of the instrument (0.1-1.0). Based on the relationship that A_{260} of 1.0 is equivalent to 50 µg/mL pure dsDNA, the following formula can be used to estimate DNA concentration (C):

C = (A_{260} reading - A_{230} reading) x dilution factor x 50 µg/mL

To determine the total yield, take the calculated DNA concentration and multiply it by the final sample volume:

Yield (µg) = DNA conc. (µg/mL) x total sample volume (mL)

To evaluate DNA purity, calculate absorbance ratios at A_{260}/A_{280} and A_{260}/A_{230}. For pure DNA samples, the A_{260}/A_{280} ratio should be between 1.8-2.0. The ratio A_{260}/A_{230} is used to evaluate the level of organic compounds present in the purified DNA and should range between 2.0-2.2.

A downside to measuring absorbance is that other nucleic acids also peak at 260 nm. The listed concentration from the 260 nm reading cannot differentiate between nucleic acid types in the sample. The concentration could be just DNA or a mix of DNA, RNA, and other nucleic acid species. A workaround is to use DNA-sensitive fluorophores such as Helixyte™ Green. These dyes selectively bind to double-stranded DNA and, when excited, emit strong fluorescence signals proportional to the amount of dsDNA present. Although fluorescence-based methods offer greater sensitivity, specificity, and wider dynamic ranges than absorbance methods, they cannot provide information about contamination or extraction quality.

Protocol for DNA Extraction and Purification

Purpose

The phenol-chloroform extraction is a straightforward method to isolate DNA from the lysate. This technique relies on chemical means to separate DNA from the lysate. Though it is more time-consuming than spin column extraction, phenol-chloroform extraction can yield high-quality DNA with minimal reagent cost.

Materials

- Ammonium acetate, concentrated solution (5M - 7.5M)
- 25:24:1 phenol/chloroform/isoamyl alcohol solution, pH 7.8-8.2
- 24:1 chloroform/isoamyl alcohol solution
- Elution buffer (10 mM Tris-HCl, pH 8.5)
- Glycogen, 20 mg/mL (molecular biology grade)
- 100% Ethanol
- 80% Ethanol

Procedure

1. Add 200 µL of starting material (lysate) into Tube 1. Bring the total volume to 200 µL with the elution buffer if needed.
2. Add 200 µL of the phenol/chloroform/isoamyl alcohol solution into Tube 1.
3. Vortex Tube 1 for 30-60 seconds.
4. Centrifuge Tube 1 at 16,000xg for 5 minutes at room temperature.
5. Remove the top aqueous layer from Tube 1 and transfer it into a new clean tube, Tube 2.
6. Add 200 µL elution buffer into Tube 1.
7. Repeat steps 3 and 4.
8. Remove the top aqueous layer from Tube 1 and transfer it to Tube 2.
9. Add equivalent volumes of the 24:1 chloroform/isoamyl alcohol solution into Tube 2.
10. Vortex Tube 2 for 30-60 seconds.
11. Centrifuge Tube 2 at 16,000xg for 5 minutes at room temperature.
12. Remove the top aqueous layer from Tube 2 and transfer it into a new clean tube, Tube 3.
13. Add ammonium acetate to Tube 3 until the final concentration is 0.75 M.
14. Add 1 µL of the glycogen mixture into Tube 3. Pipette up and down repeatedly to mix.
15. Add 100% ethanol at 2.5 times the volume into Tube 3.

16. Centrifuge Tube 3 at max force for 20 minutes at 4°C.
17. Remove the supernatant without disturbing the pellet.
18. Wash by adding 300 µL of 80% ethanol into Tube 3. Pipette up and down to resuspend the pellet.
19. Centrifuge tube at max force for 15 minutes at 4°C
20. Remove the supernatant without disturbing the pellet.
21. Repeat steps 15 to 17 for a second 80% ethanol wash.
22. Spin Tube 3 on a tabletop centrifuge and remove residual ethanol with a P20 pipette.
23. Let air dry for several minutes
24. Dissolve the pellet in elution buffer or DNase-free molecular-grade water

    1. Keep on ice if measuring concentration in the sample. Otherwise, store at -20°C. Avoid multiple freeze-thaw cycles to preserve sample integrity.

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Applications of TBNK Antibody Kits

& Newest Improvement on Current Standards

Abstract

Lymphocyte cell subsets are major research targets for a huge range of medical and drug discovery research, including the perennial search for better cancer and HIV treatments, as well as more recent Covid-19 therapeutic targets. Recently developed, the ReadiUse™ 6-Color Human TBNK Antibody Kit is designed to provide both the percentages and absolute counts of the lymphocyte subsets T-cells, B-cells, and NK-cells, using gated laser lines commonly present in flow cytometers. This research-use-only (RUO) kit is intended to differentiate the main lymphocyte subsets commonly present in whole blood and PBMCs.

Introduction

Tracking lymphocyte cell populations is a common requirement for monitoring multiple immune disorders in health facilities, as well as long-term medical research projects. Optimized for flow cytometry, the ReadiUse™ 6-Color Human TBNK Antibody Kit has been developed to eliminate multiple 'pain points' of prior TBNK kits, for simpler sample analysis and improved consistency. Additionally, it will do so in a no-wash format, using lyophilized materials for ease of usage and permitting long term RT storage instead of refrigeration.

By having known numbers of dried multicolor beads, and eliminating several steps of the typical protocols for similar kits, inconsistency is reduced as well as time at the bench and the expense of peripheral materials.

Lymphocyte Cell Subsets

The immune system is an incredibly complex network of biochemical pathways and reactive cascades, and immunology is a huge component of medical research. In very general terms, there are 4 types of immunological cells that are the most prominent, generally termed as lymphocytes. These 4 subsets are: T-cells—which are further split into helper or cytotoxic types, B-cells, and Natural Killer (NK) cells.

There are myriad structural and operational differences between these cells, but one of the most common laboratory methods of differentiating them is by detecting the specific surface and transmembrane Cluster of Differentiation (CD) antigens. Numerically assigned in the order they were discovered or first described, these proteins are referred to consistently via their CD nomenclature (i.e. how the CD antigens are named) which is a universally adopted designation system sanctioned by the World Health Organization (WHO). This system was designed for the classification of monoclonal antibodies (mAbs) directed against epitopes on the surface molecules of leukocytes. For difficult to characterize cell surface molecules, or ones only recognized by a single mAb, they are given the designation 'CDw' in which the 'w' stands for 'workshop' (e.g. CDw60 or CDw156). If the surface molecule is well characterized (recognized by 2 or more mAbs), then they are assigned a distinct value, such as CD4, CD8 or CD45.

CD nomenclature is also used to describe lymphocyte and leukocyte subtypes. The presence or absence of a specific

antigen from the surface of the particular cell population is denoted with "+" or "-" respectively. A "+" symbol added to a CD number indicates the presence of that molecule on a cell or cell population, whereas a "-" indicates its absence. For example, a 'CD45+/CD3-' cell is one that expresses CD45, but not CD3. Cell populations can also be defined as 'hi' or 'low' indicating an overall variability in CD expression, particularly when compared to other cells being studied. This can also be written as 'bright' or 'dim', in reference to the strength of fluorescent signal from the fluorophores used to detect them.

Since immune cells express CD antigens at various stages of development or cell activation, they serve as reliable biomarkers for differentiating leukocyte populations and subpopulations. This technique is known as immunophenotyping. By using targeted fluorescent-labeled antibodies, these cell types can be efficiently sorted via flow cytometry. CD antigen expression frequently varies based on the presence or progression of multiple pathologies, including leukemia, and so are often used as diagnostic or prognostic metrics.

The common lymphocyte subsets can be generally described as follows:

T-cells

CD4-expressing leukocytes, also known as helper T-cells, trigger the immune response by recognizing pathogens and secreting cytokines in order to signal to other immune cells, including CD8-expressing leukocytes, commonly referred to as cytotoxic T-cells. Helper T-cells are not directly responsible for the attack of the pathogens, only for identifying targets. Cytotoxic T-cells destroy the infected cells.

B-cells

Primarily produced in the bone marrow, CD19-expressing leukocytes, or B-cells, are an intrinsic part of the immune cascade. These cells produce antibodies against infections specifically targeted to the specific pathogen as identified by the helper T-cells.

NK-cells

Natural killer cells, or NK-cells, are part of the innate immune system, and act as defense not only against foreign pathogens, but also against anomalous native cells throughout the body, such as precancerous and infected cells. These effector cells are of great interest in new types of vaccines.

NK-cells present a variety of surface cell markers, notably CD16 and CD56 among others. There are also Killer Immunoglobulin-like receptors (KIR), as well as natural cytotoxicity receptors (NCR), which are prominent research targets in their own right.

In humans, NK cells are typically defined as CD3-CD56+ cells. This definition will be explained in further detail in the next section. Two different NK-cell subtypes have been identified: CD3-CD56dimCD16+ and CD3-CD56brightCD16-. As a reminder, this common nomenclature of 'dim' and 'bright' refers to the expression levels of these proteins on the cells, with 'dim' signal being detected from low expression, and 'bright' from high.

Other model species have differing NK-cell definitions. For example, in mice, NK-cells are defined as CD3-NK1.1+ cells. Three subsets are characterized based on the differential expression of Integrin alpha M/CD11b and CD27, including CD11dimCD27bright NK-cells, CD11bbrightCD27dim NK cells, and CD11bbrightCD27bright NK-cells.

Research and understanding of these immune cells is ongoing, and contribute to therapies and preventative vaccine development for human health concerns worldwide.

Kit Components & General Usage

The ReadiUse™ 6-Color Human TBNK Antibody Kit is optimized for flow cytometry, and has only 2 components, both of which can be stored at room temperature (RT).

Self-contained tubes (sets of 5 or 25 depending on kit size) containing the TBNK dried reagent. This reagent is a cocktail of fluorescently-labeled monoclonal antibodies directed against human CD3, CD4, CD8, CD16, C19, CD45, and CD56. A known number of dried counting beads are included, permitting absolute lymphocyte subset cell concentration determination without a washing step. This not only eliminates a common source of experimental inconsistency, but also reduces hands-on time at the bench. A viability stain is also included.

Note: The TBNK reagent cocktail contains sodium azide as a preservative, which is a known toxin if swallowed. Additionally, if the reagent comes into contact with acids, the azide group will react and produce harmful gas.

Vials (1 or 5 depending on kit size) of 10x concentrated lyse/fix buffer. The concentrated lysing buffer is simple to dilute prior to use, and permits thorough mixing of the experimental sample.

All of the labeled antibodies are murine (mouse) host monoclonal, with human reactivity, and were purified through affinity chromatography. The specific clones were chosen for their consistency and prominence in research literature, for easy reproducibility and compatibility with known protocols.

Sample Protocol

Depending on instrumental setup and experimental goals, this suggested protocol should be optimized. However, a good starting point, and one that should suit basic usage of the kit is below.

Collect fresh samples if at all possible, ideally within 24 hours of analysis.

Prior to beginning the assay, prepare the 1X Lysis/Fix Buffer by adding 3.0 mL of deionized water to one bottle of the 10X Lyse/Fix Buffer (Labeled as component B in the kit). Mix and relabel the bottle as 1X Lysis/Fix Buffer. This mixed buffer can be stored for up to 1 month at RT away from light. Handle the concentrated or mixed buffer with suitable protection, as it contains formaldehyde.

1. Prepare blood cell samples: either fresh blood collected in an EDTA tube or isolated PBMCs at a concentration no greater than 106 cells/mL can be used.

Note: Refrigerated or excessively aged blood samples, or those from individuals taking immunosuppressive medications, are likely to give skewed results. All blood specimens are considered biohazards. Handle them as if they are capable of transmitting infection and dispose of them with proper precautions and in accordance with governmental regulations.

2. Remove the desired number of reagent tubes from the pouch and reseal the pouch.
3. Thoroughly mix the sample and dispense exactly 50 µL of sample into the designated reagent tube.

Note: The accuracy of the sample dispense will affect the accuracy of the absolute cell concentration determined.

4. Gently vortex each tube for 30 seconds to ensure complete solubilization of the dried reagent. This allows for all of the beads in the reagent to be mixed with the sample, ensuring improved accuracy of the absolute counts.
5. Incubate for 20 minutes at room temperature. Protect the tube from direct light.
6. Add 450 µL of 1X Lysis/Fix Buffer to each tube and vortex for 10 seconds. Return tubes to the dark for at least 15 minutes.
7. Vortex sample tube thoroughly at low speeds and load onto cytometer for analysis. The ReadiUse™ 6-Color Human TBNK Antibody Kit is designed to be used in a Lyse/No-wash format.

Note: If the sample is washed before analysis, the ability to determine absolute cell concentrations will be lost.

8. Flow Cytometer Acquisition: start and operate flow cytometer according to manufacturer's instructions. Adjust the threshold to minimize debris and ensure populations of interest are included.

Analyze the data using the appropriate cytometer-specific software.

Relevant Cell Surface Antigens & Conjugated Labels

Because the CD antigens used to identify leukocytes are expressed at varying degrees and cell types, multiparameter flow cytometric analysis utilizing two or more combinations of fluorescently labeled CD antibodies permits clear resolution. This is the reasoning, aside from benchtime efficiency, that the ReadiUse™ 6-Color Human TBNK Antibody Kit, as well as

Visual Representation of Hierarchy

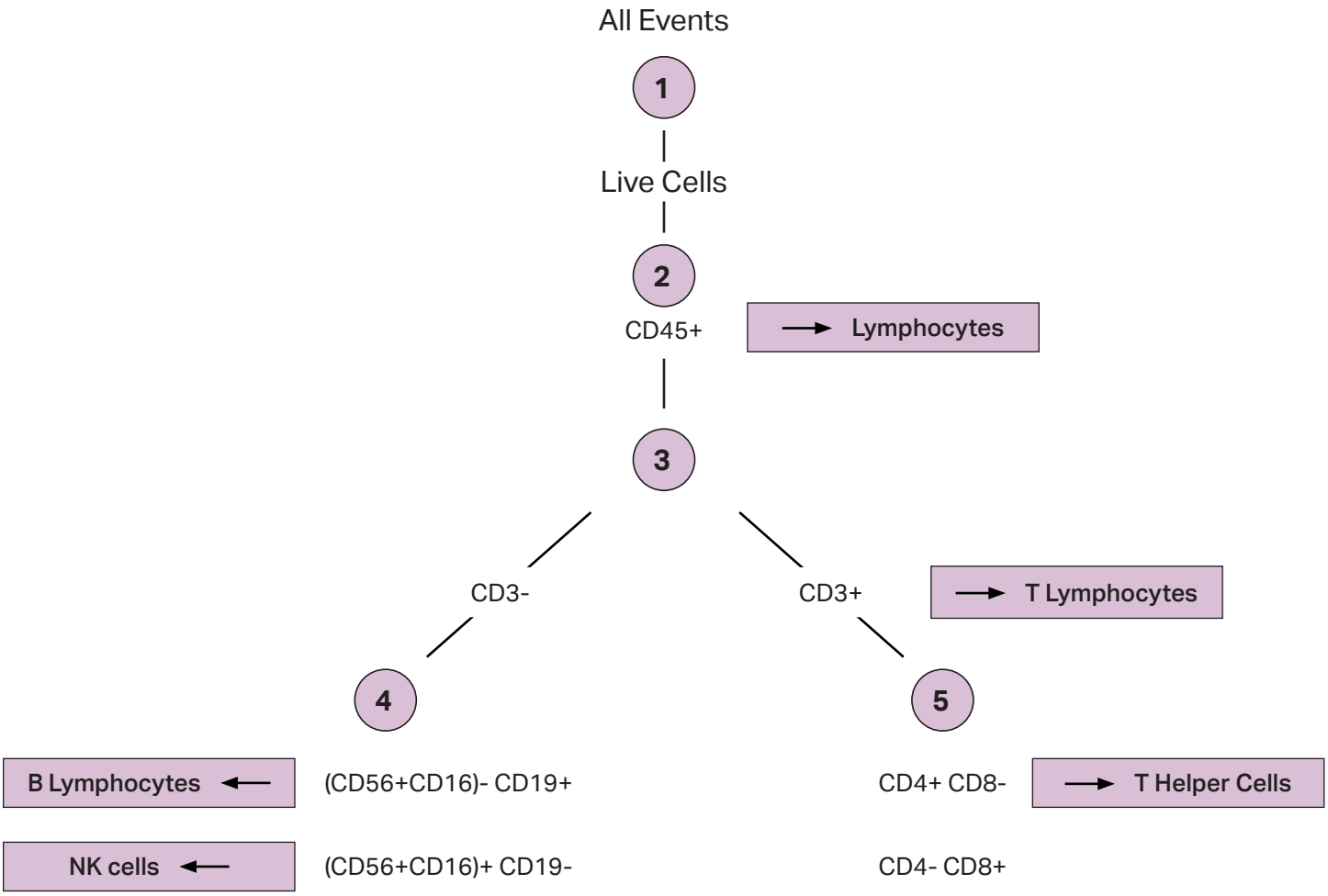

Figure 1. Hierarchical representation of the different lymphocyte subsets.

preceding versions of similar kits, is designed with so many colors.

The kit includes labeled antibodies specific for CD3, CD4, CD8, CD16, C19, CD45, and CD56, which will be used to sort the cell types. These particular proteins are known to be expressed on particular leukocyte types, and their fluorescent labels were selected carefully based on expected signal strength and anticipated target frequency.

A more comprehensive data table of the CD antigens most frequently used for flow cytometry immunophenotyping in both human and mouse models is available here.

The infographic (figure 1) represents a simple logic cascade, and is how the flow cytometer sorts (and counts) the various lymphocyte cells, based on the fluorescent signals from each of the labeled anti-CD antibodies present in the kit's reagent cocktail.

Before beginning the acquisition of any of the dot plots, it will be necessary to adjust the channel thresholds. In simplest terms, this is the separation of the forward from the side-scatter signal, gating appropriately to minimize cell debris. The beads in the kit have low FSC, and so the channel threshold should be adjusted to collect these events. If the debris box is too large, some beads may be lost. Methodology will vary between instruments, but is typically a relatively simple adjustment.

Once the instrument has been gated correctly, this will permit accurate readings of initial 'all events'.

The initial proprietary viability stain NucPO-1 indicates the first event, marking all dead cells present in the sample. This signal will be detected in the violet channel of the flow cytometer, to minimize overlap with other wavelengths. The second step is detection of the signal from CD45, a receptor-type protein which marks all lymphocyte types regardless of category. This requires a bright signal, and so the anti-CD45 antibody (clone 2D1) has been conjugated to PerCP-iFluor™ 680, for extremely bright and sustained fluorescence detected at 700 nm wavelength. By having such extreme difference in emission wavelengths between the viability and lymphocyte markers, crosstalk between them is minimized. CD45 is a glycoprotein also known as leukocyte common antigen (LCA), and is one of the larger proteins used in the kit, ranging from 180-240 kD.

The third step, which is also the first major gating, is marked by either the presence or absence of CD3 glycoproteins. All T-cells will be positive for this receptor, so this signal will also function as a count of the entire T lymphocyte population within the sample. As such a pivotal event, the signal is from the iFluor® 488 fluorescent probe, arguably the best green fluorescent label currently available, conjugated to an anti-human CD3 antibody (clone SK7), a small 20kD chain. An example of flow cytometry analysis of whole blood cells using this conjugate alone is shown in Figure 2.

The fourth plot is marked by the presence or absence of signal from the labeled anti-CD8 antibody (clone SK1) which is conjugated to APC-iFluor® 750. CD3 is present on all T cells, but CD8 is on T cytotoxic cells specifically, although this moderate-sized 65 kD protein is also expressed in lesser amounts on some other cell types.

CD4 marks the fifth of the possible events, and is present on T helper cells among many others. This 55 kDa transmembrane protein is part of the IgG superfamily, and is also the primary receptor for HIV, playing a role in immunosurveillance for many cell and tissue types. It is detected by an anti-CD4 antibody (clone SK3) tandem-labeled with PE-iFluor® 750.

These PE tandems have the longest emission wavelength present in the kit, with a recommended channel of B13 (772-795 nm). The NIR emission of these tandem dyes, with the strength boosted by either the PE or APC phycobiliproteins, give an easily-identifiable signal. This will provide absolute counts for the T cytotoxic or helper cells present in the sample. When calculating overall results post-gating, these dyes will permit precise demarcation of the two subgroups within the T-cell population. The B-cell population is identified in the sixth possible plot, to detect CD19. This 95kD glycoprotein is expressed on both

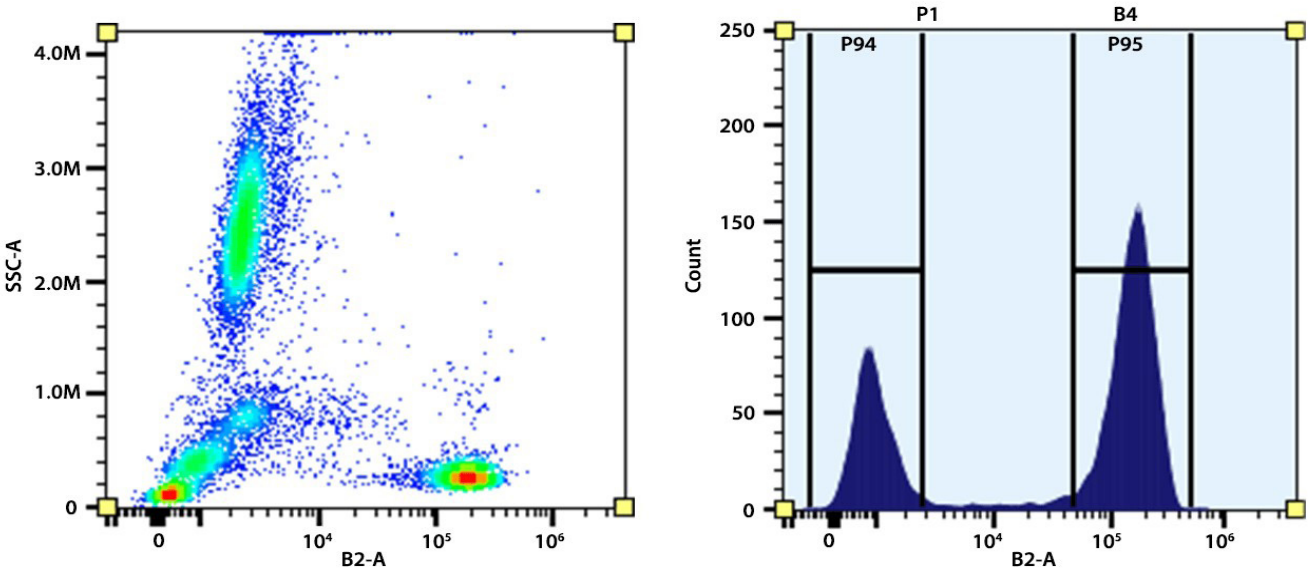

Figure 2. Flow cytometry analysis of whole blood cells stained with iFluor® 488 anti-human CD3 antibody (Clone: SK7). The fluorescence signal was monitored using an Aurora flow cytometer in the iFluor® 488 specific B2-A channel.

developing and mature B cells of all types except plasma cells, and will be identified via an anti-CD19 antibody (clone SJ25C1) labeled with APC. This classic red fluorescent label is a common channel preset on almost all flow cytometers.

The final event is to identify NK-cells, and is unique in that it requires the combination of two surface cell antigens, both CD16 and CD56. The NK-cells express both CD56 and CD16 at different levels in their life cycle, thus the kit was designed to detect both. In peripheral blood, the predominant population will express bright CD16, with a dim signal from CD56. For basic whole blood samples, this will be the standard result, whereas NK-cells from secondary lymphoid tissue and from other tissues will primarily show bright CD56. The anti-CD16 antibody is the 3G8 clone, and the anti-CD56 antibody is the MY31 clone. Both antibodies are conjugated with PE, another classic phycobiliprotein. Many instruments have either an automatic PE channel, or can simply be detected with the red laser.

Once the sample has been processed fully, the next phase will be visualizing the results and calculating the absolute cell type concentrations.

Calculations and Example Results

The absolute count for each cell type can be calculated using the following simple equation:

Absolute cell concentration (cells/µL) = [Gated cell count X Total number of beads in tube]/[Number of gated beads X Sample volume aliquoted into tube]

Example: If the bead count per reagent tube is 50,000, the volume of blood tested is 50 µL, the number of gated beads is 3,000, and the number of gated CD4+ T-cells is 1,500 then the absolute CD4+ T-cell count is 500 cells/µL.

Absolute CD4 Cell Count = (1500 x 50000)/(3000 x 50) = 500 cells/µL

In Figure 1, the simple hierarchy of flow cytometry sorting was outlined. A complete version of gated cell populations as sorted by the flow cytometer is in the table below.

Being able to correctly differentiate not only T, B, and NK cells in general, but also T cell subtypes (i.e. T helper and cytotoxic cells), is useful not only for medical research, but immunological studies in general.

For example, CD3+ CD4+ totals as well as total T-cell and B-cell lymphocyte counts are used as metrics to track the progression of multiple autoimmune diseases. NK-cell quantitation (as counted by CD3- CD16+ and CD56+ populations) is a marker for immune response to tumor formation and growth.

Figure 3 shows several dot plots as generated via flow cytometry. Labeled in red, each view is as follows:

- **View 1:** Plot forward scatter versus side scatter to separate the cells and beads from the bulk of the

Table 1. Cell Subsets.

Plots	Population of Interest	Populations of Interest
FSC vs. SSC	All Events	All Events
NUcPO-1 vs. SSC	NUcPO-1 -	Live Cells
Anti-CD45 PerCP-iFluor™ 680 vs SSC	CD45+	Lymphocytes
CD3-iFluor™ 488 vs SSC	CD45+ CD3+	CD3+ CD45+ T Lymphocytes
CD3-iFluor™ 488 vs SSC	CD45+ CD3-	CD3- CD45+ Lymphocytes
CD4-PE-iFluor™ 750 vs CD8-APC-iFluor™ 750	CD45+ CD3+ CD4+ CD8-	T Helper cells
CD4-PE-iFluor™ 750 vs CD8-APC-iFluor™ 750	CD45+ CD3+ CD4- CD8+	T Cytotoxic cells
CD56 CD16-PE vs CD19-APC	CD45+ CD3- (CD56+CD16)+ CD19-	NK cells
CD56 CD16-PE vs CD19-APC	CD45+ CD3- (CD56+CD16)- CD19+	B Lymphocytes

debris.

- **View 2:** Plot Anti-CD45 PerCP-iFluor® 680 (675-715 nm) versus side scatter (SSC–Linear scale) for the cell population to separate cells from remaining debris.
- **View 3:** Plot Anti-CD3 iFluor® 488 fluorescence (515-545 nm) versus side scatter (SSC–Linear scale) for the total cell population to identify the CD3+ T-cell and CD3- lymphocyte populations.
- **View 4:** Plot Anti-CD8 APC-iFluor® 750 (750-810 nm; 635 nm excitation) versus Anti-CD4 PE-iFluor® 750 (750-810 nm; 488 nm excitation) for the events gated as CD3+ to identify the CD4+ T-cell and CD8+ T-cell populations.
- **View 5:** Plot Anti-CD19-APC (655-685 nm) versus Anti-CD16/56-PE (562-587 nm) for the CD3- lymphocyte population to identify the B-cell and NK-cell populations.
- **Center Top (Viability):** Plot NucPO-1 (425-475 nm) versus side scatter for any cell population to determine the viable cells in that population.

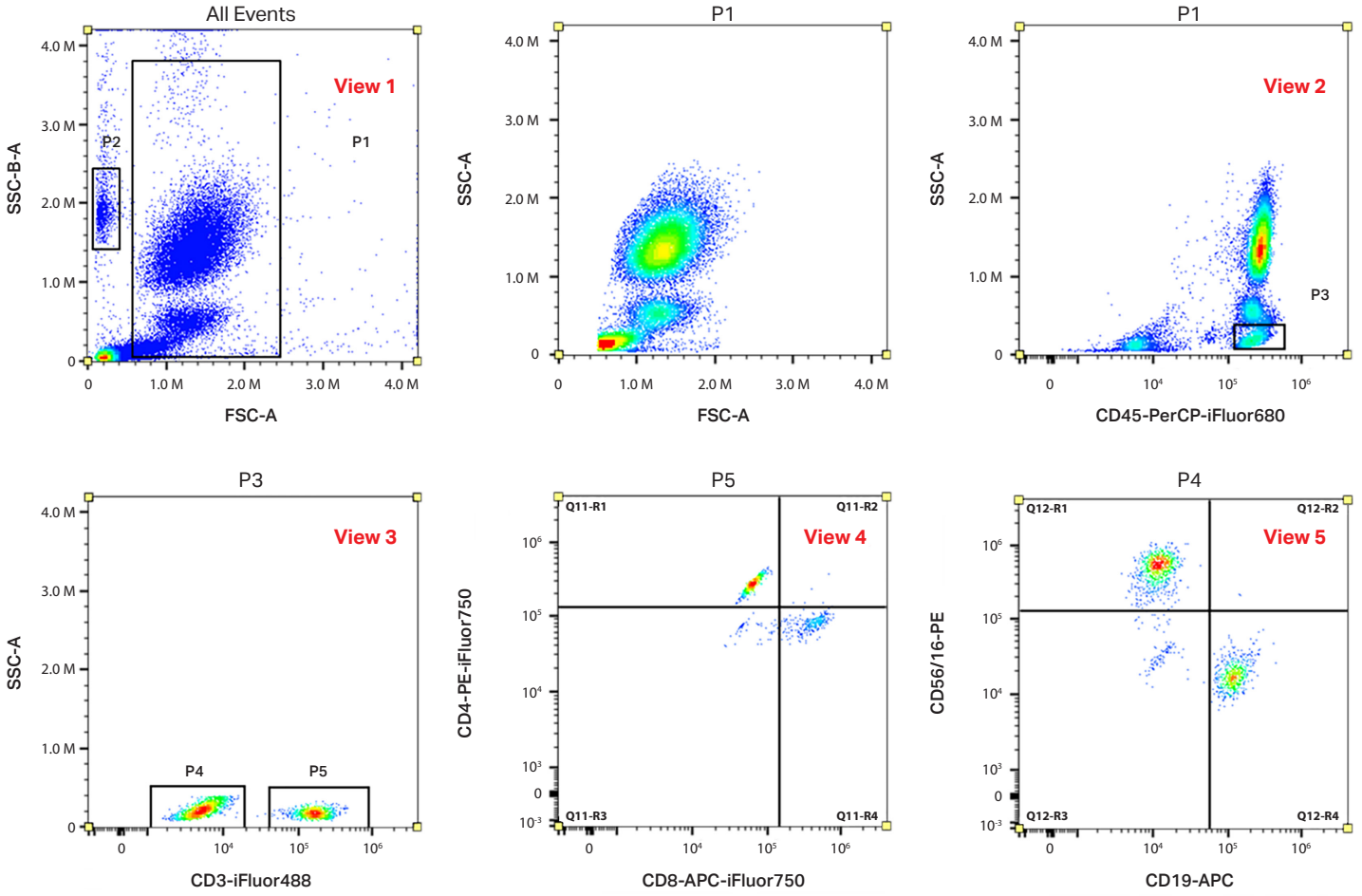

Figure 3. The visualization of a human peripheral blood sample analysis. Human peripheral blood was stained with the ReadiUse™ Human TBNK 6 Color Antibody Kit *Dry Reagent*. Cells were stained at room temperature for 20 minutes, lysed with 1X Lysis/Fix buffer for 15 minutes, and then analyzed by flow cytometry. Live cells were gated on NucPO-1 viability stain. Live cells are gated on CD45+ (2D1)-PerCP-iFluor® 680 for lymphocytes and then gated on CD3+ or CD3- (SK7) iFluor® 488 cell populations. CD3+ cells are shown using CD4 (SK3) PE-iFluor® 750 and CD8 (SK1) APC-iFluor® 750 markers. CD3- cells are shown using CD19 (SJ25C1)-APC and CD56 (5.1H11) / CD16 (B73.1)-PE markers.

For any confusion on the cell groupings as they relate to the sample processing, reference the linear hierarchy (Figure 1) infographic above. Changes to the protocol will of course require commensurate changes to the calculation and examination of assay results.

Conclusion

The ReadiUse™ 6-Color Human TBNK Antibody Kit, with its simplified workflow and lyophilized ingredients for RT storage instead of refrigeration, represents an easier method for lymphocyte immunophenotyping of whole blood or PBMC samples. Accurate quantification of these cell populations is continually necessary in research settings, and by improving the ease of logistical use, this RUO kit provides dependable results with significant time and financial savings.

References

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5. Myeong Sup Lee, Kristina Hanspers, Christopher S. Barker, Abner P. Korn, Joseph M. McCune, Gene expression profiles during human CD4+ T cell differentiation, International Immunology, Volume 16, Issue 8, August 2004, Pages 1109–1124, https://doi.org/10.1093/intimm/dxh112

Product	Unit Size	Cat No.
ReadiUse™ 6-Color Human TBNK Antibody Kit *Dry Reagent Format*	5 Tests	90010
ReadiUse™ 6-Color Human TBNK Antibody Kit *Dry Reagent Format*	25 Tests	90011

A Practical Overview of qPCR

Abstract

The polymerase chain reaction (PCR) is one of the central procedures in multiple areas of research science and has facilitated the expansion and improvement of many applications, including GMO detection or in assessing the presence or absence of a DNA target. For some research applications, all that is necessary is to know whether or not a target of interest is present or absent in a sample, with sensitivity being the primary concern. However, qualitative analysis is necessary to determine how much of the target of interest is in the sample. Real-time qualitative PCR, abbreviated as qPCR, permits more information (such as relative or absolute starting measurements of sample DNA) to be gleaned from small samples. Keeping all procedural steps within a single tube or well improves time efficiency and minimizes the potential for sample contamination. This piece will be an overview of qPCR principles, materials, basic procedures, and common difficulties and challenges.

Introduction

During PCR amplification, each strand of dsDNA is theoretically doubled, so 1-2, 2-4, 4-8, and such. This rarely, if ever, happens, and the efficiency (Q_n) is highly unlikely ever to be 100.

$$Q_0 \times 2^n = Q_n$$

In the equation above, Q_0 is the starting quantity, Q_n is the ending quantity, and n is the number of cycles.

For standard PCR, all nucleic acid detection is done at the very end of the entire set of 40 cycles. Real-time PCR means nucleic acid detection is done at the end of every cycle. This is done by incorporating the fluorescent marker into the nucleic acid at the very beginning of the procedure, and the fluorescence can be measured as the process runs. This permits quantifying the amount of dsDNA in the original sample, leading to the usual acronym qPCR.

The two kinds of quantification are absolute and relative. In absolute, by comparing our unknown sample to the standard/control curve, we can calculate the starting quantity using a calculated log value known as the cycle of quantitation ($C_{q)}$. Relative quantification compares the base versus the treated sample, and the difference between them is calculated. The differences are primarily in sample amounts. In relative quantification, no control curve is required.

In qPCR, adding a fluorimeter and fluorescent labels to the base PCR protocol permits a vast increase in possible research applications. By integrating a fluorescent marker into the denaturation, annealing, and elongation steps, qPCR can give information at every stage of nucleic acid amplification instead of simply being a preliminary step for eventual sample analysis. These real-time readings are not only helpful information in their own right but permit the computation of the amount of original genetic material (the number of copies of the amplified target sequence), as well as the C_q value at the end of each cycle step.

There are two major subtypes of qPCR, which primarily differ by the type of marker used for fluorescent measurement.

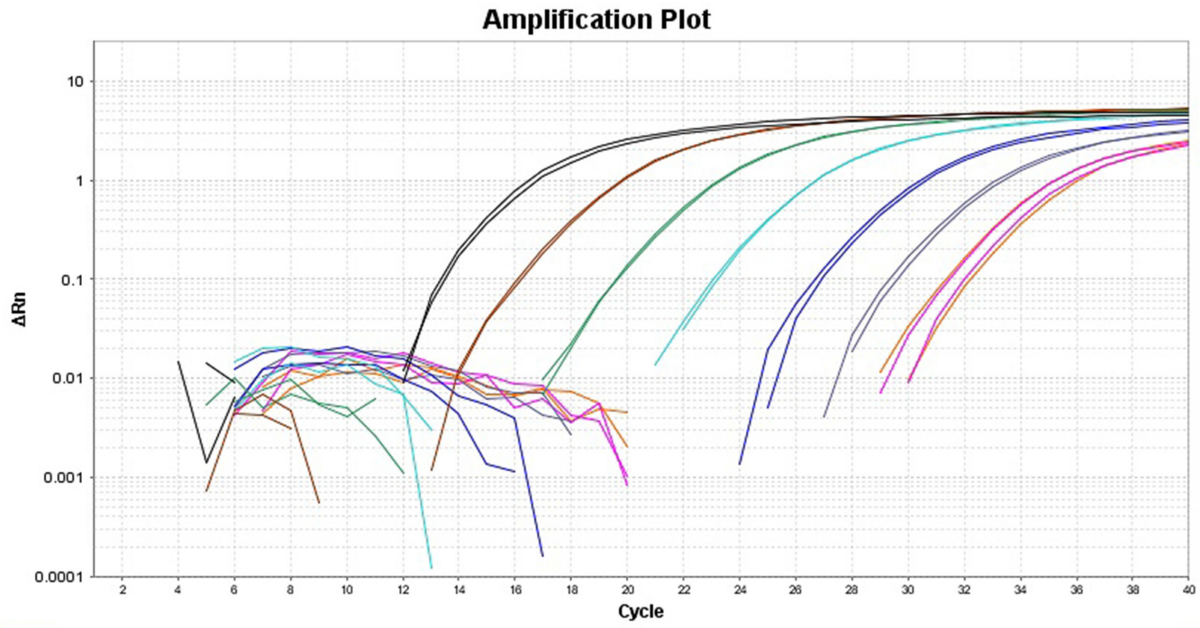

Figure 1. Quantitative PCR results targeting GAPDH with an input of 100 ng–0.00001 ng cDNA were performed using Helixyte™ Green *10,000X Aqueous PCR Solution* and a Fast Advanced Master Mix on an Applied Biosystems® 7500 FAST Real-Time PCR System.

Types of qPCR

What type of qPCR best suits an individual experiment depends on downstream applications and the acceptable costs in materials and time. As mentioned previously, there are two main types: dye-based (SYBR® Green intercalating dye and otherwise) or probe-based (with hydrolysis probes like TaqMan® being the most frequently used).

Dye-based qPCR

Dye-based qPCR is the simpler option of the two and only requires two sequence-specific DNA primers and a DNA-intercalating dye to generate a signal. Compared to microarrays, dye-based qPCR is more sensitive at detecting modest changes in expression levels, making it well-suited for investigating small subsets of genes. It is the most common choice for gene expression validation and DNA quantitation. The fluorescence generated is non-specific, so all the dsDNA present in the sample will generate a signal. This includes non-template amplification (NTC) and secondary structures like primer-dimers.

Dye-based qPCR requires a post-procedure step of a melt-curve analysis to assess the amplification reaction's degree of specificity. It also has the fastest 5′ to 3′ exonuclease activity so that the cycles can be run slightly faster than other qPCR options.

SYBR® Green is the most common DNA-binding dye for measuring PCR amplification, but many alternatives are available, such as Helixyte™ Green, Q4ever™ Green, and EvaRuby™ Dye. All of these dyes bind non-specifically to double-stranded DNA (dsDNA). These intercalating dyes provide a dependable signal, although they require careful handling.

Helixyte™ Green (equivalent to SYBR® Green) exhibits minimal fluorescence when it is free in solution. The fluorescence of the dye increases up to 1,000-fold after binding to DNA. More importantly, the fluorescence output is proportional to the amount of dsDNA present and increases as more PCR product is produced. The fluorescence signal increases exponentially with target amplification until the DNA primers are depleted.

Probe-based qPCR

For probe-based qPCR, the probe oligonucleotide markers are designed to be specific to the target of interest. The most common type of probe is the hydrolysis probe. The probe is broken during the annealing step so that it will fluoresce. Probe-based detection supports multiplexing since different probes (detected in other channels) can be combined to give more information within a single experiment.

A short 30-40 base-pair sequence-specific oligonucleotide is used, which includes a 5′ fluorescent reporter and a 3′ quencher. The reporter and quencher work in tandem. When broken (after the binding to dsDNA during annealing and subsequent breakage during the extension phase), the fluorescent reporter can give a signal without the quencher. The FRET-labeled probe gives sensitive, specific results, but developing specific probes can be time-consuming.

Probe-based qPCR is a favored method for measuring transcript abundance and is most frequently used in diagnostic assays. It is one of the most sensitive detection methods that provide an accurate and reproducible analysis. In probe-based qPCR, fluorescently-labeled target-specific probes are used to measure DNA amplification in real-time, offering the least background fluorescence compared to other (dye-based) chemistries. This method benefits from extreme specificity and allows the end-user to multiplex targets in a single reaction. The number is typically limited by the number of light channels found in the real-time PCR instrument. Several platforms are available that use probe-based chemistry to quantitate transcript abundance. Hydrolysis probes, such as TaqMan® probes and Molecular Beacons, are the most widely used.

Basic Materials, Procedures, and Sample Protocol

A qPCR experiment requires multiple components to run successfully. Mixed thoroughly (to ensure well-to-well or tube-to-tube consistency), the reaction buffer will include the DNA template, primers, and probes (if using), as well as the titular polymerase enzyme, magnesium or other cation, and dNTPs. It is incredibly time-consuming to optimize a qPCR procedure correctly, so using commercially-available master mixes has become common. These master mixes include validated balances of all necessary components except for the template and primers. Some will consist of an intercalating dye for dye-based qPCR.

Given this ever-increasing popularity, the following explanation will include master mixes. Adjusting these mixes is entirely possible, as is assembling an assay mixture from scratch, but it is a more advanced proposition that may be inaccessible for the beginning researcher.

Essential Materials

Successful DNA amplification requires concise control of temperature changes (using a well-maintained instrument) and quality reagents: master mix, primers, template, and lab-grade pure water.

- **Master mix:** A combination of deoxyribose nucleotide triphosphates (dNTPs), buffers, and DNA polymerase, plus any extra components like magnesium or manganese (a master mix is sometimes referred to as a supermix, which usually only has the dNTPs, buffer, and enzymes). Commercially available master mixes vary in performance, but for unusual requirements, a modified DNA polymerase sometimes gives better and more stable results.
- **Primers:** Short, forward, and reverse, usually only a handful of base pairs. These act as an initiation site for amplification to take place. Specificity is the highest priority, with length and other aspects as relevant. There will usually be both ‘upstream’ and ‘downstream’ primers relative to the template, with upstream being towards the 5′ end of the strand and downstream towards the 3′ end.
- **Template:** This is the actual nucleic acid of interest to the procedure. Its composition, length, and purity all can affect the amplification efficiency. An ideal length is usually ~100 base pairs and is typically one of 4 types: genomic, plasmid, viral, or cDNA.
- **Water:** Nuclease-free and lab-grade pure to avoid degradation of the starting template. This is particularly

essential for assays using rare or irreplaceable samples, such as in forensics applications, which may already have some degree of damage.

Material Selection and Issues

The concentrations of materials (aside from premade kits) greatly impact qPCR results, and the ideal balance must be empirically tested. There are some standard ranges that are the most typically chosen and lend themselves well to reproducible results and are a helpful place to begin testing.

Master mix

Probe and dye mixes are similar in that they include everything needed to amplify a single target. Mastermix selection depends on ingredients but should be tested prior to use with any rare or irreplaceable samples since efficiency has a wide variance. Dye-based qPCR typically includes the intercalating dye into this mix at optimal concentration. Probe mixes do not include the fluorescent marker and can be adapted based on marker choice.

Tip: Normalize assay signal with a passive reference dye (such as ROX or higher-performing alternatives like 6-ROXtra™). This helps with the signal variance between wells due to the stationary light source in many instruments. Not all instruments will require a reference dye, but many will so include it as necessary based on your experimental setup. Many universal master mixes will consist of one of these passive reference dyes at differing concentration levels.

When selecting a premade master mix, compare the performances of the available options that contain the materials necessary for the experiment since there is a broad spectrum of performance and dynamic range exacerbated by sample and instrument type.

Examples of Mastermix Comparative Testing of PCR Efficiency

By running serial dilutions of the sample DNA concentration, we can determine the dynamic range, efficiency, and specificity of the qPCR assay. Dilutions are usually done by factors of 10 but may be done using alternative factors if required or desired. Once the amplifications have been run for each starting quantity, the C_t vs. log of the starting quantity can be assessed, with the slope determining the efficiency (E).

$$E = 10^{-1/slope}$$

$$\%E \text{ as } (E-1) \times 100$$

The percent efficiency should be as close to 100% as possible, which equates to a slope value of -3.32. A percent efficiency in the 90-110% range is usually considered optimal. An assay with 100% efficiency is rare and would represent a perfect doubling of the starting target per cycle. Low efficiency is generally caused by poor primer design, expired or low-quality reagents, or incorrect annealing temperature.

The y-intercept is the limit of detection (LOD) of the procedure. Although a qPCR assay could theoretically be sensitive enough to detect a single copy of the target,

Tip: If efficiency is very high (well above 110%), there is likely a primer dimer, and off-target products are present. Assess the lowest concentration point on the dilution assay curve to see if that is changing the slope unduly, at the cost of a smaller dynamic range. Alternatively, check the melt curve for insight into any off-target products that may be present.

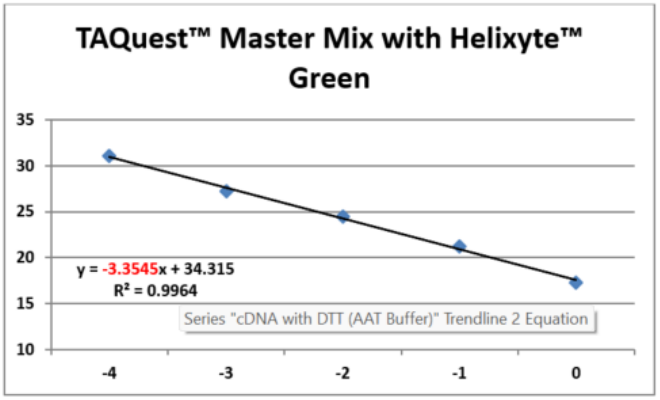

Figure 2. C_t vs. log DNA dilution of TAQuest™ qPCR Master Mix with Helixyte™ Green, run with identical experimental design at different concentrations of sample DNA.

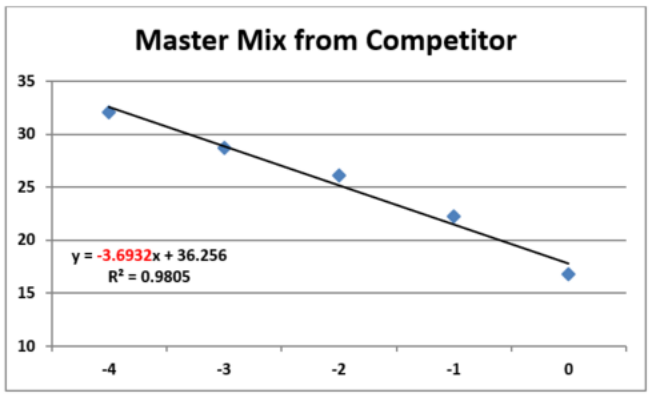

Figure 3. C_t vs. log DNA dilution of a commercial master mix with included intercalating DNA dye, run with identical experimental design at different concentrations of sample DNA.

realistically, 2-10 copies are considered reliable.

R^2 is the best-fit line of the ideal vs. the actual observed plotted line and is referred to either as the correlation coefficient or the coefficient of determination.1 An R^2 value of 0.98 or above is considered acceptable: the higher, the better. It measures the linearity of the relationship between two variables. Although, in theory, the perfect R^2 = 1, in practice, 0.99 and above is more realistic.

Variances usually occur at each end of the dilution range and may be omitted at the cost of a smaller dynamic range. If R^2 is too low, we recommend redesigning the assay with different primers.

For the two example graphs, TAQuest™ Master Mix with Helixyte™ Green and Master Mix from Competitor, both R^2 values are within the acceptable range, with the first TAQuest™ master mix showing a higher (0.9964) and more desirable value than the other (0.9805). Additionally, the second mix shows a slightly steeper slope (associated with over 100% efficiency, suggesting off-target amplification) than the first mix.

When selecting a mix, researchers may need to assess whether accuracy or efficiency is a higher priority and to what extent.

Primers

Primers are short single-stranded stretches of nucleic acids. They are the core concern for any procedure, and their specificity and efficacy are the most common stumbling block during optimization and validation of the assay. Given well-chosen primers, nearly all other aspects of the assay can be adjusted around them. A qPCR procedure will include both 'upstream' and 'downstream' primers, designed to match each end of the targeted DNA sequence (the template). By beginning the DNA synthesis at these endpoints, off-target amplification is minimized.

Assay results are highly dependent on primer sequences, so these sequences must be included along with the associated data in any resultant publications, per MIQE guidelines.[2] Primers that are contaminated, truncated, or have incorrectly chosen bases, can destroy qPCR accuracy.

Tip: Primer pairs should not include complementary sequences/regions. If there is 3' complementarity, it is likely to cause primer-dimer formation.

Generally, primer lengths of between 18-24 base pairs, beginning and ending with at least one G-C pair and containing approximately 40-60% G-C content, give good results. Most available primers will have a T_m of 50-60 °C.

When selecting multiple primers to be used in combination within a single assay (as in multiplex qPCR), ensure that the melting temperature (T_m) is within 5 °C. Mismatched T_m will cause asynchronous dsDNA amplification since they will dissociate at different stages of the thermal cycles.

Template

The template is the piece of DNA that is to be amplified. Ideally, it should be highly pure and of high quality. There are many sample sources where the genetic material may have been gleaned, with qPCR being useful for nearly any type.

Tip: If the genetic material is viral or a plasmid, then a much smaller quantity is needed, often with a final concentration of 1 pg-1 ng being the typical range. For cDNA or genomic DNA, concentrations are typically from 1 ng-1 µg.

The proportion of G-C pairs in a template can be an issue since their stronger bonds can give rise to primer-dimers,

hairpins, and other secondary structures, which can skew assay signals. Although some optimization steps can help with these issues, in general, the development of secondary structures should be avoided if possible.

The template length must be considered when choosing a supermix since longer lengths typically require a polymerase with greater processivity. Polymerase attaches to a small number of bases and then disengages. The processivity measures how many bases that polymerase will process before it unlatches from the DNA strand.

Tip: In terms of efficiency and time at the bench, if the sample template is long, a polymerase with higher processivity should be chosen, so that cycle time doesn't need to be extended.

Additionally, if there is a limited amount of the sample template, researchers may opt to choose a hot-start qPCR procedure (which begins the process at a slightly higher temperature) to minimize other enzyme activities and assist with specific amplification. Most, if not all, instruments will have this functionality.

Water

Lab-grade, enzyme-free pure water is a common requirement for many lab procedures, but it is of particular importance for qPCR since nucleases like RNAse and DNAse can degrade and corrupt samples quickly.

Other consumables associated with qPCR are the sample holders to insert the materials into the experimental instrument and the pipette tips for transferring the samples. Depending on which instrument is used, tubes, tube strips, plates (deep well or microtiter), and their associated seals are the most common options. For selecting these materials, instrument compatibility and quality are the central concerns. The original suppliers/manufacturers of PCR instruments will list what consumable formats are compatible, and there are also online tools available that collate information for researchers.

The bare essentials of any real-time PCR instrument are a thermal cycler and an optical detector. There are a vast number of instruments available, with many lists available comparing them based on the intended use. Although a full exploration of every factor of thermocycler instrument selection is beyond the scope of this introductory piece, significant criteria include:

- Which imaging channels are necessary and how many (to facilitate which dyes your lab uses and how many will be used in combination)
- Adaptability if the custom experimental setup is desired (not required if ready-to-use kits will be the default)
- The intended volume and speed of the assay requirements (small-scale, HTS, or both)

Basic qPCR Procedure and Example Protocol

The actual thermal cycling procedure is relatively simple. The timing and temperatures for an assay are unlikely to need much adjustment.

To begin the amplification process, cDNA is heated up to 95 °C. This is the denaturing step since the strands will break apart into two single-stranded strands (complimentary). Annealing is done by dropping it to 48-72 °C (55-65 °C is most common). Although the annealing temperature varies, it is usually set at approximately 3-6 °C below the primer T_m. The extension is generally done at a similar or slightly higher temperature (68-72 °C) than annealing. This will permit the incorporation of the free-floating dNTPs, starting at the primer sequences, to assemble two new complete double-stranded DNA molecules.

These denaturing, annealing and extension steps are typically repeated approximately 40 times, with each iteration referred to as a cycle. Standard-base amplicons with approximately 100 or fewer base pairs will require a shorter cycle time. Many processes include the 3-step protocol, with the annealing step including an extension temperature of approximately 72 °C. These are ideal for longer amplicons (>150 bp), complex or unknown samples, or low-efficiency amplification. For some very short amplicons or well-validated reagents, the extension step can be shortened or omitted entirely.

Tip: Remember to have the plate be read at the end of the extension step, not the annealing step if you have made manual adjustments to the cycle that your instruments' software does not automatically account for.

Table 1. TAQuest™ qPCR Master Mixes.

Product	Reference Dye	Unit Size	Cat No.
TAQuest™ qPCR Master Mix with Helixyte™ Green	No Rox	1 mL	17270
TAQuest™ qPCR Master Mix with Helixyte™ Green	No Rox	5 mL	17271
TAQuest™ qPCR Master Mix with Helixyte™ Green	Low Rox	1 mL	17272
TAQuest™ qPCR Master Mix with Helixyte™ Green	Low Rox	5 mL	17273
TAQuest™ qPCR Master Mix with Helixyte™ Green	High Rox	1 mL	17274
TAQuest™ qPCR Master Mix with Helixyte™ Green	High Rox	5 mL	17275
TAQuest™ FAST qPCR Master Mix with Helixyte™ Green	No Rox	1 mL	17276
TAQuest™ FAST qPCR Master Mix with Helixyte™ Green	No Rox	5 mL	17277
TAQuest™ FAST qPCR Master Mix with Helixyte™ Green	Low Rox	1 mL	17278
TAQuest™ FAST qPCR Master Mix with Helixyte™ Green	Low Rox	5 mL	17279
TAQuest™ FAST qPCR Master Mix with Helixyte™ Green	High Rox	1 mL	17280
TAQuest™ FAST qPCR Master Mix with Helixyte™ Green	High Rox	5 mL	17281
TAQuest™ qPCR Master Mix for TaqMan Probes	No Rox	1 mL	17282
TAQuest™ qPCR Master Mix for TaqMan Probes	No Rox	5 mL	17283
TAQuest™ qPCR Master Mix for TaqMan Probes	Low Rox	1 mL	17284
TAQuest™ qPCR Master Mix for TaqMan Probes	Low Rox	5 mL	17285
TAQuest™ qPCR Master Mix for TaqMan Probes	High Rox	1 mL	17286
TAQuest™ qPCR Master Mix for TaqMan Probes	High Rox	5 mL	17287
TAQuest™ FAST qPCR Master Mix for TaqMan Probes	No Rox	1 mL	17288
TAQuest™ FAST qPCR Master Mix for TaqMan Probes	No Rox	5 mL	17289
TAQuest™ FAST qPCR Master Mix for TaqMan Probes	Low Rox	1 mL	17290
TAQuest™ FAST qPCR Master Mix for TaqMan Probes	Low Rox	5 mL	17291
TAQuest™ FAST qPCR Master Mix for TaqMan Probes	High Rox	1 mL	17292
TAQuest™ FAST qPCR Master Mix for TaqMan Probes	High Rox	5 mL	17293

Table 2. Overview of the basic steps in the qPCR cycling reaction**.**

Step	Temperature	Time	Process
Denaturation	95°C	~20 to 30 seconds	Double-stranded DNA (dsDNA) template is heated to high temperature. This disrupts the hydrogen bonds between the complementary base pairs causing dsDNA to separate into single-stranded DNA (ssDNA). • **Note:** The required denaturation time may increase if template GC content is relatively high.
Primer Annealing	48 to 72°C	~20 to 40 seconds	After denaturation, the reaction temperature is lowered to ~48 to 72°C. This promotes the binding of forward and reverse primers to each of the ssDNA templates and the subsequent binding of DNA polymerases to the primer-template hybrid. • **Note:** It is critical to determine a proper temperature for the annealing step to ensure optimal efficiency and specificity. A typical annealing temperature is ~5°C below the melting temperature (T_m) of the primer.
Extension	68 to 72°C	~1 to 2 minutes	After annealing, the reaction temperature is raised to ~68 to 72°C. This enables DNA polymerase to extend the primers, synthesizing new DNA strands complementary to the ssDNA template in the 5′ to 3′ direction.

For a more thorough step-by-step explanation of PCR in general, consult 'A Practical Guide for the Detection and Analysis of PCR Products'

Example Protocol

Note: This is a simple sample protocol, and while helpful as a guideline or a starting point, it should be adjusted or changed entirely based on your experimental requirements and optimization results.

The materials used are linked if more information is desired, and their requirements will be similar to others available on the market.

1. Thaw the TAQuest™ qPCR Master Mix (select the correct level of included passive reference dye if any is necessary) at room temperature, and vortex thoroughly before use.
2. Prepare one of the following reaction mixes as indicated below, selecting which one is applicable to the type of procedure being run.
3. Carefully mix the regents with a gentle vortex followed by a brief centrifuge.

Note: Even if volumes are small, always balance the centrifuge according to best practices for safety.

4. Set up the plate, tubes, or strips in your qPCR instrument and run as indicated with the thermal cycling parameters in the bottom table.

Table 3. Components for Dye-Based qPCR Procedure.

Components	Final Concentration
TAQuest™ qPCR Master Mix with Helixyte™ Green	1X
Upstream primer, 10 µM	0.1-1.0 µM
Downstream primer, 10 µM	0.1-1.0 µM
DNA template	Optimized conc.
Nuclease-Free Water	Depends on well/tube volume

Table 4. Components for Probe-Based qPCR Procedure.

Components	Final Concentration
TAQuest™ qPCR Master Mix for TaqMan Probes	1X
Upstream primer, 10 µM	0.1-1.0 µM
Downstream primer, 10 µM	0.1-1.0 µM
TaqMan Probes, 10 µM	100-250 nM
DNA template	Optimized conc.
Nuclease-Free Water	Depends on well/tube volume

Table 5. Basic thermal cycling parameters.

Parameter	Polymerase Activation	PCR (40 cycles)		
	Hold	Denature	Anneal	Extend
Temperature	95 °C	95 °C	55-65 °C	68-72 °C
Time (m:ss)	0:20	0:30	1:00	1:00

Instrument Readouts Associated With Real-Time Amplification

Real-time data is displayed with an amplification curve, taken by measurements at the end of each of the 40 amplification cycles. It always begins low and will grow quickly.

The three segments of the amplification curve will be the baseline phase, the exponential phase (made of the take-off and linear stages), and the plateau phase. After all 40 cycles are done, a melt curve is run if necessary (usually between 65-95 °C).

- **Baseline:** amplification has barely begun at this point, shown by a flat line, since the fluorescence signal is too low to be detected, even though there will be an increase in PCR product.
- **Take-Off (usually around cycle 10 or so):** There is an abundance of PCR reagents at this stage, marked by the exponential increase in amplification and commensurate signal. The threshold will be set in this region of the amplification curve, and it represents the best amplification efficiency and the best precision.
- **Linear:** Amplification decreases (usually because freely available dNTPs are diminished).
- **Plateau:** Amplification flattens, PCR reagents are depleted, and along with the self-annealing of PCR products, will show minimal or no additional amplification. This area also represents the lowest degree of specificity of the amplification.

There will be one curve for each labeled target. A single threshold line will be used for each of them, marking the point at which the detected fluorescent signal positively changes from the background signal. This will be the Cq point for each curve.

There are very few circumstances in which researchers might elect to run fewer than 40 cycles for a qPCR procedure. It is also unusual for more than 40 cycles to be necessary. If the amplification curve displays all expected phases a bit early, researchers may decide to remove the extras in the interest of time. That said, this reduction won't necessarily yield better data, and publication/reproducibility concerns may require running the full 40 cycles to completion.

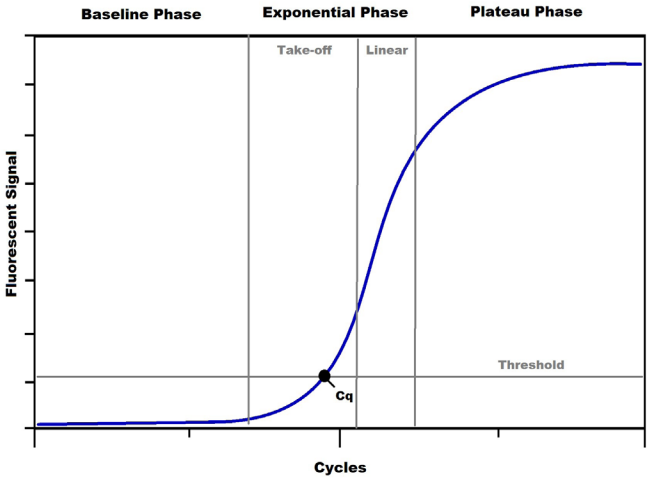

Figure 4. Amplification Curve Phases, Threshold, and C_q Point.

Tip: If adding extra cycles (more than 40) is desired, be sure that you run excellent negative controls since nonspecific amplification is a concern.

Unless the assay has severe issues, amplification graphs will be a sigmoidal curve. Shallow curves show poor amplification, with steep ones being optimal. Different dyes will give different results depending on factors such as template base pairs and stability. Corrupted sample material can be an issue as well.

Tip: If amplification efficiency is a major issue, this can be caused by degraded samples, those high in inhibitors, or excessively long amplicons. In those cases, droplet digital PCR (ddPCR) can give significantly greater precision and better results than qPCR.[3]

Optimization, Validation, and Challenges

Depending on the experimental goals and sample variability, an assay protocol will likely need to be adjusted during initial development and in response to non-ideal results.

Controls

Including controls in a qPCR procedure is essential to experimental best practices, expected by MIQE guidelines and any reputable peer-reviewed journal dedicated to scientific reproducibility.

The four basic qPCR controls are:

- **Extraction control** - a sample that has been extracted before and has had positive results. Keep some sample matrices from prior successful extractions. This is a useful measure of whether or not the current target sample has been successfully extracted and to what extent.
- **Positive control** - includes a sample that is known to express well. This should always give a dependable signal, which assists in normalization and identifying any major issues with the overall procedure.
- **Negative control** - includes base reagents and water, but no sample. This should always have no signal, but it can often indicate sample contamination or poor experimental protocols if it does.
- **No-template control (NTC)** - includes everything (mix, etc.) except the template. This can also be useful for normalization but is most frequently used for identifying any inhibitors present or issues with the template itself.

Ideally, every type of control will be used in every experiment, but not every type will apply to each procedure. If a previously-validated assay is going to be run with a new primer or probe, revalidation is unlikely to be required, but run a positive control to confirm that it is working.

Differences in background fluorescence can lead to differences in fluorescence levels of individual wells. By subtracting this, we can increase the precision of our technical replicates. This is one of the main reasons passive reference dyes are so widely used since they are a simple method of removing this background.

Figure 5 of a comparison of two intercalating dyes, as a researcher might run during the materials selection process of developing a new qPCR assay. In this case, the Q4ever™ Green dye shows a significantly brighter signal than SYBR® Green and will be the likely choice for an experiment. Once the dye has been chosen, the amplification curve can be processed further to eliminate the baseline (which is no longer necessary) and background fluorescence so that a more useful and precise curve can be seen.

Figure 6 shows a processed amplification plot of ΔRn the vs. number of cycles using the integrated instrument software.

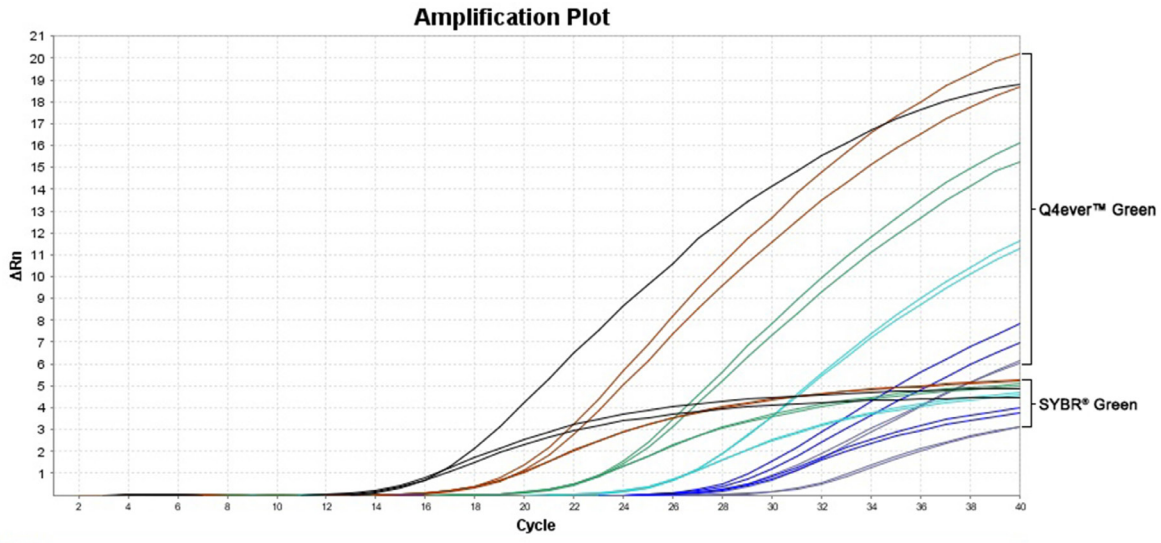

Figure 5. Example Amplification Plots: Q4Ever™ Green and SYBR® Green Fluorescent Signal Comparison

Verification by manually calculating one or two randomly-selected data points is recommended for unusual experiments or simply for emphasis on reproducibility.

Determining Ideal Annealing Temperature

Run a few sample assays using slightly different annealing temperatures. The usual range is between 55-65 °C, read at half-degree increments. This temperature gradient can then be analyzed using a standard curve to determine the optimal annealing temperature (T_a).

Tip: If your instrument does not have automatic software processing, or if you wish to confirm the optimal T_a manually, assess the C_q values at each temperature run using the positive control sample and choose the highest temperature before the C_q value begins to rise exponentially.

Many instruments have settings to make this process easier, usually only requiring information about the primer sequence and buffer ion concentrations.

Post-Amplification

For dye-based qPCR, it is essential to perform a melt curve analysis after the procedure to evaluate the amplification specificity. If there are extra lane bands when running a post-assay gel, than running a melt curve analysis (always expected, per MIQE standards[2]) is absolutely essential. When assessing the melt profile, all unknowns should show a single melt peak, with the NTC showing no peak at all.

By analyzing the melt curve and assessing at what temperature the melt peak occurs, you can determine to what extent off-target products are being produced. For example, primer-dimers are short, and so the Tm will be low, often within the 60s to low 70s, so an unexpected peak in that temperature range strongly suggests the presence of those secondary structures.

Note: If there is a single melt curve but with 'double top's, then that represents two distinct but similar amplification products. This usually represents either a pseudo-gene or splice products. This may or may not be an issue, for example when different variants of a single gene are expected.

If multiple peaks are present, then redesign the assay with a new primer set, or raise the Ta slightly to see if the off-target product can be eliminated. Higher annealing temperatures

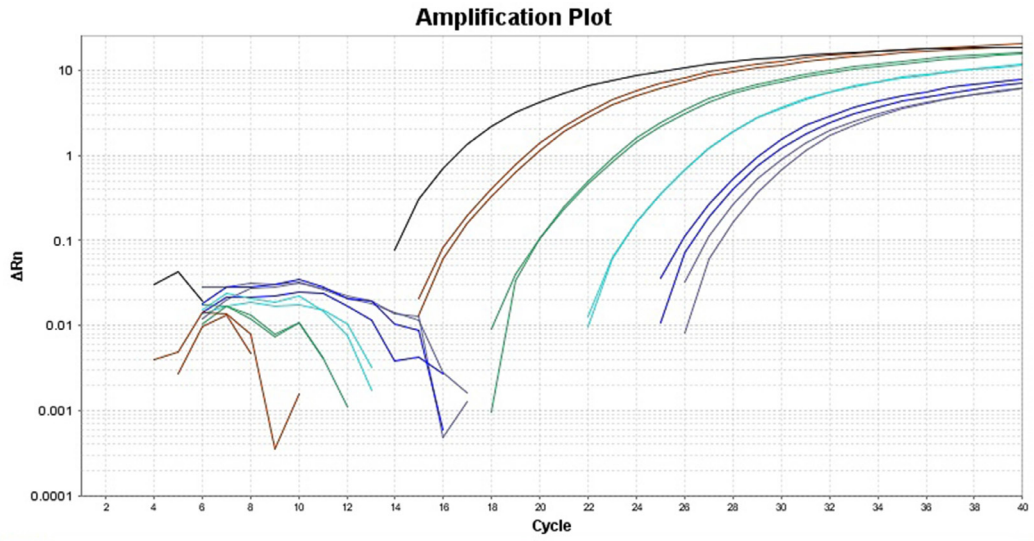

Figure 6. Quantitative PCR results targeting GAPDH with an input of 100 ng–0.00001 ng cDNA were performed using Q4ever™ Green and a Fast Advanced Master Mix on an Applied Biosystems® 7500 FAST Real-Time PCR System.

improve specificity in many cases, although the assay will be slightly less efficient.

Statistics and Calculations

Once the qPCR procedure has been completed, the researchers' work will take them away from the bench for the next phase: analyzing the gathered data to assess the success of the assay. In general, all calculations should be included in publications to the greatest extent permitted by length limitations.

For common types of analysis, many open-access calculators exist, and many other equations can simply be named without having to show every line of the work. What calculations are necessary, and the extent to which they need to be shown, will vary widely based on the assay and publication requirements.

Relative Gene Expression

To calculate gene expression, calculate the difference between C_q from the control vs. the treated sample. The ΔC_q will then permit a comparison to show the relative difference.

$$\Delta C_q = C_q(\text{treated}) - C_q(\text{control})$$

Relative Quantity (RQ) refers to a full change in expression. It is calculated very simply:

$$RQ = 2^{-\Delta Cq}$$

However, to calculate well, we need to use a reference gene to show variances in sample volumes and quality. Reference genes are usually 'housekeeping' genes and will dependably express. This will permit the calculation of the Δ-ΔC_q.

Δ-ΔC_q is the difference of C_q (target minus treated) and C_q(reference genes). It only works when the assay amplifies the target at 100% efficiency. This is known as the Livak equation.[4]

Other equations include other variants, as well as normalization for multiple reference genes. For data that may not be normally distributed, when it is skewed to the either left or right, a Shapiro-Wilk normality test may be required. This determines the likelihood that the expression values do not represent a normal curve.

Common Types of Comparative Analyses

Students' T Test: Compares two groups, to determine of the averages of the two samples differ, and it assumes normal distribution of each. This is frequently seen in graphs that compare a control group vs. a treated group.

Analysis of Variance (ANOVA): compares more than two groups, and assumes normal distribution of means as well as equal variance. A common use is in showing individual comparisons between populations. It is a very popular method of analysis. The two types of ANOVA are the one-way and two-way. If the experiment is only testing one variable, no matter how many groups (such as in a time course), that is a one-way ANOVA. A two-way ANOVA will include more than one variable.

Data Analysis and Visualization

For qPCR data analysis, bar graphs, box-and-whisker, and dot plots are the most common methods of visualizing data sets.

- **Standard deviation (SD)** measures the dispersion of a dataset relative to its mean, and represents the variability of the data set. It does assume a normal distribution of data.
- **The standard error of the mean (SEM)** measures how much discrepancy there is likely to be in a samples' average compared to the population average. It is essentially the standard deviation of the two averages. It is intended to demonstrate the precision of the data, and will always be smaller than the SD. To calculate this fully, the experiment must be run multiple times and the standard deviation calculated each time. An easier method simply calculates the SEM by dividing the standard deviation by the square root of the number of samples.
- **Bar graph:** easily shows the mean/average, as well as the standard error. It does not provide any information on the relative distribution of the data, however.

- **Box-and-whisker:** shows median value, upper and lower quartiles, as well as min and max value, and outliers. However, it doesn't show anything about the average or the error.
- **Dot Plot:** shows individual data points are all shown, permitting full display of the results to the reader. It can be difficult to assess easily, however.

Multiplex and Multi-Plate Analysis

Multiplex assays nearly always require extra time and work to validate at the beginning. Reagents are often difficult to choose, and often must be added in higher concentrations. When running any multiplex procedure, make all possible efforts to minimize any negative interactions. Run the assays in various combinations and assess if the C_q values are similar, which represents low to no interference. If those values vary widely, then those cannot be run in the multiplex assay. Additionally, be sure to assess the spectral overlap of the selected fluorescent dyes carefully.

Variability between plates may be caused by user error, sample degradation, differences in measured concentration, instrument fluctuation, or even degradation of PCR components due to improper or extended storage.

The number of samples and targets must be considered, and should include replicates as necessary as well as controls. If only using a single plate (if all will fit into a single plate) then no inter-run calibration will be necessary.

Tip: Technical replicates increase precision by identifying outliers. The usual standard deviation value for replicates is between 0.2-0.3.

If using two or more plates, if there are direct comparisons, no inter-run calibrations are required, since all plate-to-plate differences will be identical.

However, if the plates encompass no direct comparisons, then there must be an inter-run calibration. This is often necessary with larger experiments, where it is impossible to have references on every plate, or where samples must be divided between plates. This is not an issue, as long as an inter-run calibrator is run on every plate to allow for variability.

There are several types of inter-run calibrators. Pooled nucleic acid samples are common, or reference materials such as stock cDNA or linearized plasmids.

Frequent Challenges

One of the most frequent preventable errors made, leading to multiple retracted papers, is inaccurate C_q calculation. C_q and C_t values are technically different, but are frequently used interchangeably. C_q stands for 'quantification cycle'. A Ct value is a 'threshold cycle'. There is Top (take-off point) as well, but Cq is the most common and best-accepted.

It is almost always possible to calculate the C_q values automatically with the instrument, but in the interest of reproducibility and for publication standards of extremely strict journals, doing the calculations manually as well may be required for verification.

Tip: A good rule of thumb is to set the C_q point to no more than 10 times the SD of the threshold noise.

If your instrument has the Cq calculations showing an unexpected value (far lower or higher than estimated), there is likely a deeper issue. Confirm that the positive and negative controls are performing as expected.

Another common issue is not taking the template sample type into account. A well-performing protocol for one type of sample may or may not behave equally well for another kind. Adjustments or substitutions are likely to be necessary, even if the original protocol can serve as an excellent starting guideline.

Example amplification curves for the same test run using different kinds of DNA

As is clear from the above two amplification plots, although the observed data is similar to an extent, the type of DNA has a major effect on the procedure. Always be sure to confirm if the selected materials are compatible with the sample type you will be using, such as genomic, plasmid, viral or cDNA templates.

If assay signal is low, one possible cause is suboptimal primer concentration.

Tip: *Try going outside the usual concentrations of 100-500nM.*

It may be necessary to run some test amplifications to find a balance between low concentrations (and commensurate low signal) and the production of off-target products (such as primer-dimers) at high concentrations.

Summary and Closing Remarks

The qPCR assay is a core procedure with ever-expanding applications for researchers. It has contributed to significant advances in disease detection, gene expression, genotyping, and cloning, among many other applications.

If researchers want to multiplex multiple targets per well or

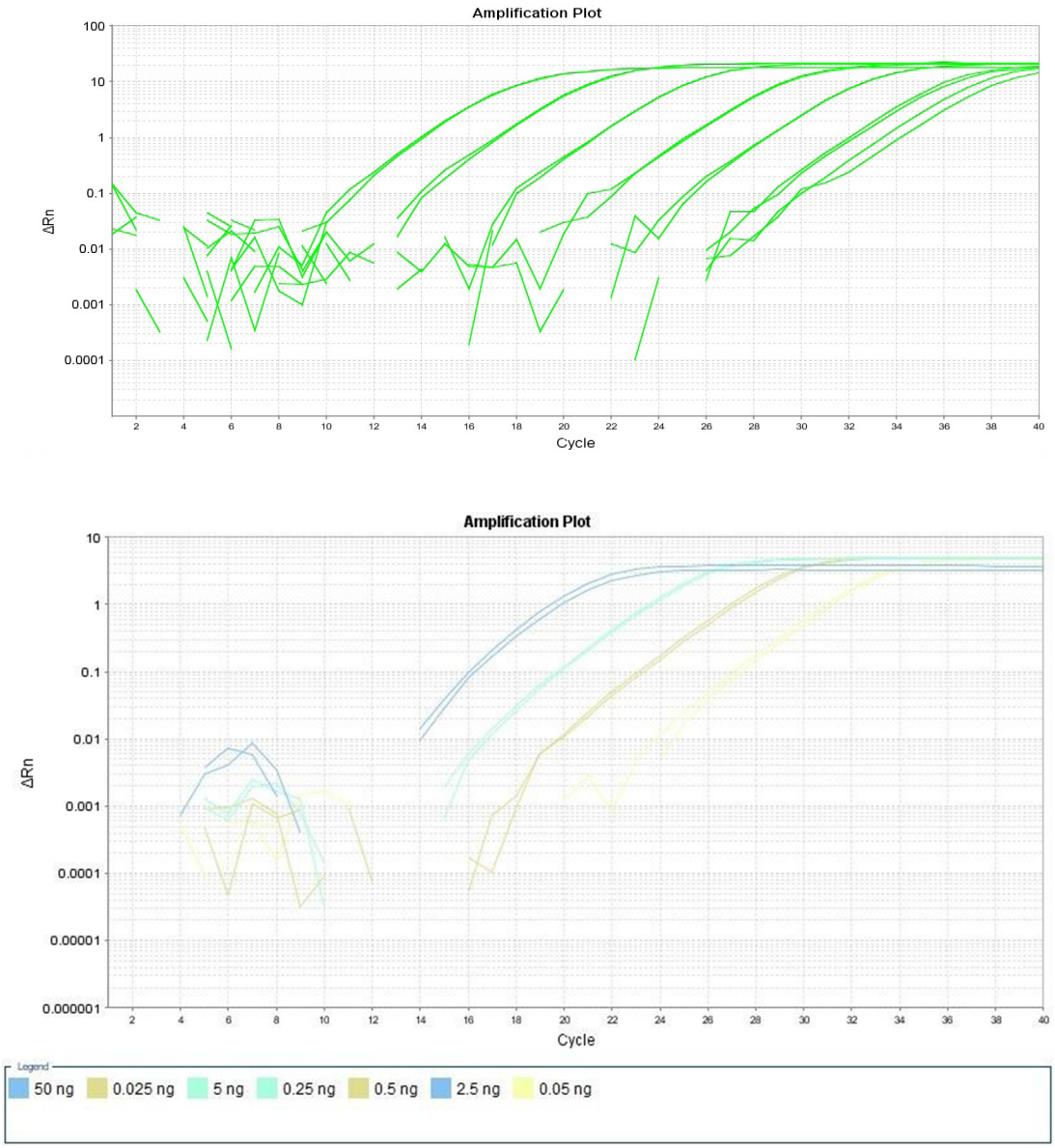

Figure 7. Top: Amplification plot for a dilution series of HeLa cells cDNA amplified in replicate reactions to detect GAPDH using TAQuest™ FAST qPCR Master Mix with Helixyte™ Green *Low ROX*. Bottom: Amplification plot for a dilution series of genomic DNA amplified in replicate reactions to detect GAPDH using TAQuest™ FAST qPCR Master Mix with Helixyte™ Green *Low ROX*.

have multiple similar targets, probes might be the best choice. However, if cost is an issue, then intercalating dyes might be a more economical choice.

The procedure itself is highly adaptable but requires care and consistency to ensure dependable and reproducible results. By ensuring that all necessary optimization steps and controls are represented, a researcher can be confident in the validity of their amplification.

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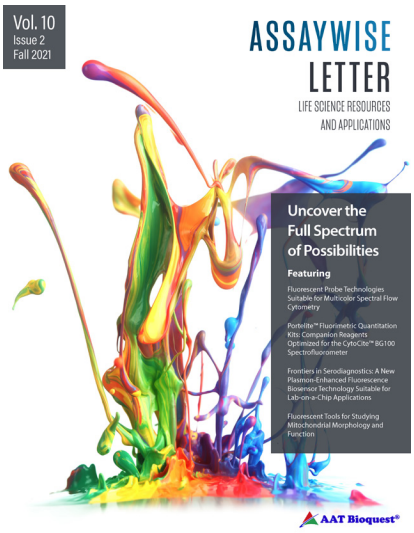

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