Give me a P! P! Give me a C! C! Give me an R! R! What do yo get? Lots and lots of copies of DNA! I LOVE proteins – so I talk most about those – but to get to proteins you need an instruction guide, so DNA you must provide. All aboard the DNA Pol train, with a PCR(Polymerase Chain Reaction) survival guide to fill up your brain! PCR is another fundamental technique that’s become crucial to modern biochemistry (and even modern medicine!) It allows us to make lots of copies of specific regions of DNA allowing us to look closer and answer questions like: “how is a frog related to a dog?,” “is there a mutation in this gene causing this disease?,” and “is this viral gene present, indicating infection?”It also allows us to (after making lots of copies of a protein recipe) stick DNA into cells to get them to make proteins we want! So how does it work? (And how to get it to work if it doesn’t want to…)

The instructions for making proteins (long chains of amino acid letters that fold up into cool shapes and act as molecular workers) are written in a different biochemical language – DNA. Stretches of DNA that hold “protein recipes” are called “genes” and, since the genetic code is universal, you can stick that gene to “any” cell and that cell’s ribosomes (protein-making machinery) will “understand” it and make the corresponding protein. Similarly, if you know what genes an organism (Including a person) has, you can know what proteins they make, whether they have mutated versions of some genes, how they relate to other organisms.

So, DNA’s really important and kinda like how you might love your IKEA table but don’t have a particular fondness for the instruction manual, but you need it, in order to better understand proteins I often have to deal with their DNA instructions, and to do this I need to make lots of copies of specific stretches of DNA, and thankfully PCR provides a way! The discovery of PCR is attributed to Karry Mullis (working for a biotech firm called the Cetus Corporation) who got the first glimmers of it in 1983 and published it as a legit technique in 1985 (and in 1993 won the Nobel Prize for it).

As with basically all “breakthrough” scientific discoveries, he couldn’t have done it with out the work of countless other scientists working before him. (and we couldn’t do the work we do today without the work of him and others!).

For example, the stage was set in part by Arthur Kornberg, who won the Nobel Prize for discovering an [enzyme](https://thebumblingbiochemist.com/glossary/enzyme/) (reaction speeder-upper) that could copy DNA, using a single strand of DNA letters (deoxynucleotides (dNTs)) as a template for linking up (polymerizing) “opposite” letters into a complementary chain (which can then be used as a template for recreating that original template sequence thanks to the 1:1 “oppositeness” of DNA letters (more below). He called this enzyme DNA Polymerase (DNA Pol) and he could get it to make single complementary copies of DNA, but Mullis’ contribution was using that DNA Pol to make lots and lots of copies.

And we still do. But we typically use super-hardy versions of DNA Pol that can withstand the high temps we cycle them through in the process I’m gonna tell you about. And that choice of polymerase is just one decision you have to (I mean get to) make when you go to the DNA copy machine! So hopefully today’s post will help you iron out your routine. I’m going to start with an overview of how it works (so hopefully not too jargony/wonky) but then I’ll get into some details for those who want them. (apologies in advance for formatting – full-time grad student doing full-time lab stuff…) So let’s go!

DNA (DeoxyriboNucleic [Acid](https://thebumblingbiochemist.com/glossary/acids-and-bases/)) is the biochemical language our genetic info’s written in & its alphabet consists of 4 deoxynucleotide (dNT) “letters,” A, T, C, & G which have a “generic” part made up of a deoxyribose sugar with phosphate(s) hooked up on the “left arm” (5’ position) has a hydroxyl (-OH) group as a “left leg” (3’ position) & then the different letters have different “nitrogenous bases” (bases) that stick off as a “right arm” – these bases are the single- or double-ringed parts.

dNTs use their generic parts to link together through phosphodiester bonds to form long single stranded DNA (ssDNA) & 2 complementary single strands “zip together” using their unique base parts (A across from T, C across from G) to form double-stranded DNA (dsDNA). This double-strandedness protects it from damage (the bases are facing in) and allows for easy copying since if you unzip it one strand can be used as a template for making another.

This “unzip and copy” is what happens in replication – before a cell divides, it needs to copy all its DNA (its entire genome) so that it can pass on a full set to each daughter cell, and it does this with the help of DNA Pol, which brings together the freely-roaming nucleotides, holding the right ones together & helping them link up, while rejecting the wrong ones (ones that don’t complement each other (e.g. don’t let an A bind a C!)

PCR (Polymerase Chain Reaction) is a way to carry out this process in a (really tiny) test tube & only copy a small section of DNA, which we specify by using PRIMERS. Primers are short pieces of DNA (oligonucleotides, or “oligos”) which we design to “bookend” our region of interest (AMPLICON). So, for each reaction we design and order different primers (thankfully they’re cheap!)

We need these primers because DNA Pol can’t start chain-building from scratch – it needs to start from a short double-stranded stretch. This is just one of its limitations (but sometimes limitations can be good! You don’t want cells copying DNA randomly!) Another limitation of its is that it can only copy DNA in One Direction (5’ to 3’). Before we get too far, let’s make sure we’re NSYNC… (90s girl, sorry!)

The letter-linking is “generic” because it only involves the “backbone” parts that all the letters have (phosphodiester bonds involve the phosphate & hydroxyl merging) so you can link letters in any order (e.g. ATTACA or CAAATT). But the strand-zipping is specific because it occurs through interactions of the unique bases. So the “opposite” of ATTACA is TAATGA, which is different from GTTTAA. But, writing the opposites like this is a bit misleading because opposite direction you should be reading.

If you have ATTACA, and you stick the complementary letters across from it, you get this:

ATTACA

TAATGA

BUT – dsDNA is ANTIPARALLEL – this means that the strands are running in opposite directions (one is 5’->3’ and the other is 3’->5’ with the ‘ pronounced “prime” and referring to whether the left arm (5’) or “left leg” (3’) of that end’s sugar is free). So

5’ ATTACA 3’

3’ TAATGA 5’

And we usually write sequences 5’ to 3’, so the “complimentary sequence” to 5’ ATTACA 3’ is 5’ AGTAAT 3’

This may seem like a mere technicality, but it’s really important in reality! Because DNA Pol can only copy DNA in one direction, 5’ to 3’, and you always have to keep in mind which way the “train tracks run”

Train tracks? This is another weird analogy of mine – I like to think of nucleotides as train tracks and DNA Pol as a train. This train can only travel on double-stranded track, so it has to lay the track down ahead of it as it goes (and it knows what track to lay down by making it “match” the other side of the track (e.g. if the next track across from it (on the other strand) is a T, lay down an A). In PCR, primers provide the starting stations for the train (since DNA Pol needs double-strandedness to start, it’ll only start where you make it double stranded (but shorter than the other strand so there’s still stuff to copy. Basically, you want something like this:

——====—- to make this ——========

PCR is run in cycles of  MELT (heat up dsDNA to unzip strands)  ANNEAL (cool down slightly to allow primers to bind and  EXTEND (starting where primers leave off, add nucleotides complementary to template strand until you reach end of template strand). After the 1st cycle (where Pol goes till it runs out of steam or out of time), this end is determined by other strand’s primer because DNA can only \*copy\* it cannot “compose” so it’ll run off the track corresponding to the position that strand started being copied from in the 1st round. (easier to explain in pics)

You do this over & over  (30 or so times) to get lots of copies (each time you get 2X as many copies because each new strand becomes another template strand).

It might sound like a ton of work – and for the [molecules](https://thebumblingbiochemist.com/glossary/molecule/) it is! – but for us, once we get the reaction set up, the hardest part is just waiting for it to finish! Not for Mullis, however… It’s “easy” these days because we have machines called thermal cyclers that do the rapid heating and cooling and heating and cooling and heating and cooling and…

But these machines didn’t come on the scene until 1987 (and of course not every lab could afford them, etc.) so in the beginning scientists would manually transfer the reaction tubes from one water bath to another over and over again.

The reaction to link nucleotides together (nucleotide polymerization) is the same regardless of whether it’s happening in your cells or in the tube. It’s also the same for RNA (but using a different Pol). but here we’ll speak in DNA terms. So the reaction takes 2 deoxynucleotide triphosphates (dNTPs)(have 3 phosphate (PO₄³⁻) groups linked together) and links them up. And when it does so, it kicks 2 of those phosphates as a molecule pyrophosphate (PPi)

So, same process “in vivo” (in the body) and “in vitro” (in an artificial setting like a test tube), BUT in cells there are lots of helpers, whereas in PCR the process is stripped down to its bare necessities:

TEMPLATE DNA: dsDNA containing the sequence you want to copy (amplify)

PRIMERS: short ssDNA complementing the ends of amplicon (1 to serve as a start site for each strand)

dNTPS: nucleotide building blocks (letters) to be added

BUFFER: liquid combo of salts & [pH](https://thebumblingbiochemist.com/glossary/ph/) stabilizers to keep everything happy

Mg²⁺: magnesium [cation](https://thebumblingbiochemist.com/glossary/cation/) ( charged molecule) to act as “chaperones” to help shield phosphates’  charge

and, drumroll please…  DNA POLYMERASE (DNA Pol)

One of the hardest parts of carrying out a biochemical reaction is often bringing reactants together & keeping them together long enough to interact. Why’s this so hard? Molecules like to be free to move around (they want high ENTROPY) & they don’t like to be tied down. Entropy refers to how many different “states” a molecule can be in (this can refer to being in different places or in slightly different shapes (e.g. bond rotated a bit). It’s sometimes described as “randomness” or “disorder” because the more ways something can move the less likely you are to know exactly where they are at any time.

Getting nucleotides to link up is like getting a bunch of kids running around at recess to link up to form a really long 3-legged race. First, you have to get them to come over, then you have to get them to stay still long enough to convince them to link up, and then once they’re linked up you have to make it “fun” for them even though they can’t run around anymore because their movement is restricted by being linked to the person next to them. And Pol has an even harder time because it has to get the kids to link up in a specified order!

So how can one little protein do all this? By giving the nucleotides something they want in return – ridding them of a couple of their phosphates. The phosphates stick off the 5’ end & they’re basically really concentrated negative charges clamped together like a spring. PHOSPHATE (PO₄³⁻) has a central phosphorous(P) [atom](https://thebumblingbiochemist.com/glossary/atom-2/) connected to 4 oxygen(O) atoms. It has “extra” electrons (e⁻) so it’s  charged. Like charges repel, so phosphates don’t like to be next to each other. So it takes effort (in the form of energy (E)) to bring & hold phosphates together (like compressing a spring) – so we call these bonds “high energy” and when they’re broken apart that E’s freed to be used for other things like paying cost of linking nucleotides together.

So, even though nucleotide polymerization is still energetically costly because you’re tying down molecules,  their freedom ( entropy), this is compensated for by the large  in entropy that occurs when PPi is released & hydrolyzed (split by water) to give you 2 individual orthophosphates (Pi) (and 2 little things moving around freely has even more freedom than the 1 medium-little thing (PPi) that’s initially released – so you get a double-boost

BUT in order to get this benefit, you 1st need to get them to react & this is often where proteins &/or RNA [CATALYSTS](https://thebumblingbiochemist.com/glossary/catalyst/)(reaction speeder-uppers) called ENZYMES come in. They act as a sort of “mediator” bringing the right reactants together, holding them in optimal positions to react, stabilizing reaction intermediates. & providing a friendly environment.

As I mentioned before, Arthur Kornberg discovered some enzymes that help mediate DNA copying: DNA POLYMERASES (DNA Pols). DNA Pol helps hold an incoming nucleotide (of the triphosphate variety) close to the growing chain it needs to be added to & in the right position. The 3’ hydroxyl (OH) group then goes in for the attack! It latches on to the 1st phosphorus (P) group of the incoming nucleotide -> that P now has too many bonds, so it kicks out the other 2 phosphate groups as the inorganic phosphate molecule PYROPHOSPHATE (PPi) (energy boost 1). PYROPHOSPHATE is then hydrolyzed (broken by the addition of water) into 2 molecules of ORTHOPHOSPHATE (Pi) (energy boost 2).

DNA Pols have that general mechanism in common, but different organisms have slightly different DNA Pols that vary in EFFICIENCY, PROCESSIVITY, FIDELITY, & THERMOSENSITIVITY

* EFFICIENCY: how fast can it go?
* PROCESSIVITY: how many nucleotides can it add before it falls off template?
* FIDELITY: how many typos does it make?
* THERMOSENSITIVITY: how much heat can it take?

You can get higher FIDELITY (fewer typos) if your Pol has a “proofreading” 3′→ 5′ [exonuclease](https://thebumblingbiochemist.com/glossary/nuclease/) (DNA end-chewing) [domain](https://thebumblingbiochemist.com/glossary/domain/" \t "_blank)that can sense errors, “backspace” to remove them, & then put in the correct letter. This profreedng is important because errors will get copied… & copied… & copied… BUT it slows down process so you get lower EFFICIENCY.

Other parts of DNA Pol proteins can also help out. A way to increase EFFICIENCY is by increasing PROCESSIVITY – keep Pol on the template. Constantly falling off & hopping back on surely slows you down! Processivity-enhancing domains (a “domain” is just a protein “section”) or separate processivity-enhancing “subunits” bind dsDNA to help latch Pol on. But importantly they don’t bind “too tightly” & they can bind any sequence – this allows Pol to stay on but slide along

BUT before you can copy strands you have to unzip them & this too is energetically expensive (like peeling apart 2 pieces of stuck together tape). You have to put in energy to give the DNA molecules more energy so they wiggle around more & the strands come apart.

In your cells, enzyme helpers called HELICASES help unzip them using chemical energy from ATP, but our “bare-bones” PCR version doesn’t have these helpers. Instead, we get the needed energy from HEAT. In the MELT step, we physically heat up the dsDNA so the strands come apart. And we have to get it REALLY hot! (~95°C or 200°F). Human DNA Pol would be pretty useless at this temp bc same heat that causes strands of DNA to come apart (yay!) can also cause proteins to unfold (eek!) (just like chains remain chains when you melt DNA (you don’t break up strong [covalent bonds](https://thebumblingbiochemist.com/glossary/covalent-bond/)), heat denaturation of proteins leaves you w/chains of amino acids)

Thankfully there are organisms called THERMOPHILES that have evolved to live in super hot environments (like near thermal vents in the ocean). They have super-strong proteins that can withstand high temps needed PCR. The “classic” PCR Pol is Taq, which was discovered in 1976 and gets its name because it comes from the thermophilic bacterium Thermus aquaticus. Taq really made PCR possible – before that, scientists trying to copy DNA in the lab were using a DNA Pol from e. coli bacteria. That DNA Pol couldn’t take the heat, so after each heat step, they’d have to add more! In fact, the first thermal cycler, named “Mr. Cycle” was designed with an open system so you could keep adding more. <https://bit.ly/2FoUFkD>

So Taq was a major, crucial, discovery. But it’s definitely not “perfect” – Taq tends to make a lot of typos (low FIDELITY). Pfu DNA polymerase (from Pyrococcus furiosus) makes fewer errors (so higher FIDELITY). BUT it has relatively low EFFICIENCY. Could we do better?

It’s hard to get all 4, but that hasn’t stopped scientists from trying! Scientists can stitch together parts they like from different Pols to get Pol “chimeras” w/enhanced functions. Our lab uses a chimera called Phusion Polymerase (not a paid endorsement, just what we use!) It’s based off of a Pfu-like DNA Pol (w/proofreading capability for increased fidelity) fused to a small dsDNA-binding protein called Sso7 (from Sulfolobus sulfactaricus) which serves as a processivity-enhancing domain. It can add 1000 nucleotides (1 kb) in only 15 seconds w/few errors! So we time out the extension step accordingly (e.g. if we want to copy a 4kb segment, we’ll set the extension step for 4×15=60s

So, DNA Pol was one choice we need to make, but there are others too….

Where what do you want your start & stop sites to be? Design primers to match – but not just any sequence… It’s really important to design the stations (primers) carefully. Like many things in biochemistry, it’s largely a matter of [AFFINITY](https://thebumblingbiochemist.com/glossary/affinity/) & SPECIFICITY

SPECIFICITY. We want the primers to bind ONLY where we want them to bind. Say you ask some friends to buy you a train ticket for a trip from Kansas City to NYC Is that Kansas City, Kansas? Or Kansas City, Missouri? Some friends might think Kansas, others might think Missouri & you end up with 2 types of train tickets..

Similarly, if your primer can bind at multiple sites on the DNA, you end up copying different stretches of train track giving you a mix of “nonspecific products” which show up as multiple bands on an [agarose gel](https://thebumblingbiochemist.com/glossary/agarose-gel-electrophoresis/) you use to separate the DNA pieces by size & visualize them: [http://bit.ly/agarosegelrunning](http://bit.ly/2SDKE8I)

When you design primers, you want to make sure they match your site of interest & ONLY that site. Like a computer password, the longer the sequence, the more likely it is to be “unique.” If there are multiple occurrences of sequence you initially choose you might have to lengthen it to include more of the surrounding sequence (like saying “find a blue house w/a red house on the left & a green house on the right” instead of just saying “find a blue house”) You can use free software programs like NCBI BLAST or Primer3 to help you check for specificity & design good primers

BUT too long a primer and you can face other problems that lead to a “PCR TLDR” (too long didn’t read)…

 PRIMER DIMERS: this is where the primer binds to itself instead of to your template. These can be self-dimers (where 2 “start stations” or “stop stations” bind to themselves or cross dimers (a start & a stop)

[SECONDARY STRUCTURE](https://thebumblingbiochemist.com/glossary/secondary-structure/): a single primer can fold up into “hairpins” & bind itself

This leads to less primer available to bind template, so lower yield (less copies made) & DNA Pol can end up using primers as a (really short) template, amplifying primer “artifacts” instead of desired amplicon. And the high primer concentrations needed to prevent template-template zipping, make such primer pairing more likely because there are more primer fish & fewer template fish in the sea

Secondary structure in the \*template\* can also be a problem – some regions of DNA are tightly wound up, making it hard to get to the site to bind (it’s hard to build a train station in the middle of a mountain pass). You also want to avoid repetitive stretches (things like “AAAAAA”) because it makes it easier to “slip” & misprime

Typical primers are usually ~20 nucleotides (nt) long, but it depends on experiment type, etc. There’s free software available (like NCBI Primer-BLAST, Primer3, & AmplifiX) to help you design primers to fit your needs.

And remember – get the reverse compliment! <http://bit.ly/sequencetermstools>

AFFINITY: We want the primers to have high affinity (attractiveness & stickiness) for the site we want them to bind so that they’ll bind there stably & not fall off randomly during the annealing or extension steps. BUT we don’t want the affinity to be too high or it won’t come off during the melt steps

Affinity is largely dependent on the primer length (longer primers have more interstrand bonds working together to keep the strands glued shut) & “base composition,” When G’s & C’s are across from each other they can form 3 [H-bonds](https://thebumblingbiochemist.com/glossary/hydrogen-bond/). But when A’s & T’s are across from each other they can only form 2 H-bonds, so G-C pairs are stronger than A-T pairs. So a higher “G-C” content (typically given as a % of bases) means stronger binding. Ideal is usually ~40-60%

note: this is why origins of replication (ORIs) (where DNA strands come apart to be copied before cells divide) tend to be “A-T rich” because it makes it easier to melt them apart

Just like it’s easier to pull off a piece of tape from the end than the middle, it’s easier to pull of primers from the end, so you might want to put a“G-C” clamp at the 3’ end (1 C or G) to help latch it on tight

A common measure used to calculate/report this affinity is the Tm (melting temperature). It’s the temperature at which 1/2 the primer is bound – the higher the temp, the more energy the molecules have & the harder it is to get the DNA to “stay still” & bind. A high Tm means the affinity’s high enough to hold down the wriggling DNA. Sp higher Tm, higher affinity. You typically want ~60°C & you want the Tm of the 2 primers to be similar to one another (within ~5°C)

You want to know these Tms because they’ll help you decide what temp to use for your annealing temperature – they temperature you program the thermal cycler to be at during the primer-binding step of each cycle.

How do decide? The higher the temp, the more energy the DNA molecules have so it’s harder for them to be “tied down.” At higher temps, they have to really like their binding partner in order to sacrifice the freedom to move freely. But at lower temps, they have less energy, so they’re less “picky” & more likely to “settle” for less-optimal pairing. As a result, if the annealing temp is too low, your primers can “misprime” & bind at the wrong sites giving you a mixture of “nonspecific” products.

So you want to choose a Goldilocks ANNEALING TEMPERATURE where the DNA molecules have enough energy to seek out their soulmate, BUT not so much that they can’t “tie the knot” once they find it. To help you choose, you can use free software to calculate the melting temperature (Tm) of the primers based on their length & sequence (since G’s & C’s bind each other more strongly than As & Ts (thanks to their 3rd H-bond), higher GC content increases the Tm. Common annealing temps are ~55-80°C, and you want the Tms for both primers to be similar so that they both get to be Goldilocks-happy at the same time.

How long should the extension step be? Basically, each cycle, you need to give DNA Pol enough time to copy the region between the primers. So the optimal time depends on length of the sequence you want copied (AMPLICON SIZE) & the speed (efficiency) of the DNA Pol. The “classic” Taq polymerase (a DNA Pol that can tolerate the high temps required) can add ~360nt/min, but “newer models” (either from other organisms &/or mutated) like Pfu Turbo can go faster (~1000nt (1kb)/min)) So, for example, if you’re using Pfu Turbo to copy something 2kb long, you’d want to make sure your extension is a little over 2 minutes. This is *per cycle* so with longer times, be prepared to do some waiting (a great time to pour that agarose gel you’ll use to check that it worked!)

Just how long you’ll have to wait depends on how many cycles you choose. And that depends in part on how many copies you want made (each cycle you increase # of copies exponentially (e.g. 2, 4, 8, 16, 32, 64…), so by 35th cycle you’d have 68 billion copies! You might think, the more the better, right? BUT the more copies you make, the more chance for errors to occur (& be copied) Common cycle #s are ~25-30 (but even if you have to wait you don’t have to manually move the tube over and over!)⠀

more on DNA sequencing: <http://bit.ly/DNAsequencingmethods>

more on topics mentioned (& others) [#365DaysOfScience](https://www.instagram.com/explore/tags/365daysofscience/) All (with topics listed)  http://bit.ly/2OllAB0

* A picture containing text, indoor

  Description automatically generated
* A picture containing timeline

  Description automatically generated
* Timeline

  Description automatically generated with medium confidence
* Diagram

  Description automatically generated
* Diagram

  Description automatically generated
* Text

  Description automatically generated with low confidence
* Calendar

  Description automatically generated
* A picture containing timeline

  Description automatically generated
* Timeline

  Description automatically generated
* Diagram

  Description automatically generated with medium confidence
* A picture containing timeline

  Description automatically generated
* Timeline

  Description automatically generated
* Diagram

  Description automatically generated

Top of Form

Bottom of Form

# **RT-qPCR overview – with video**

* [Home](https://thebumblingbiochemist.com)
* [365 Days of Science](https://thebumblingbiochemist.com/category/365-days-of-science/)
* RT-qPCR overview – with video

[June 12, 2021](https://thebumblingbiochemist.com/365-days-of-science/rt-qpcr-overview-with-video/)

[Quantitative PCR (qPCR) is a way to “count copies” of specific pieces of DNA – and when those specific pieces are DNA copies of RNA (like the RNA copies of protein recipes) we call it Reverse Transcription (RT) qPCR – RT can also stand for Real Time, which refers to the measuring – the products are detected as they’re made because of the fluorescence they cause (either because of generic dyes or specific probes) – unlike traditional PCR (Polymerase Chain Reaction) which you use just to make copies of defined stretches of DNA, with the goal of making as many copies as possible, RT-qPCR counts the copies as they’re made to figure out how many copies you started with.](https://thebumblingbiochemist.com/365-days-of-science/rt-qpcr-overview-with-video/)

[In addition to the video, I’m going to give a brief overview below, and you can find much more detail in this past post:](https://thebumblingbiochemist.com/365-days-of-science/rt-qpcr-overview-with-video/)<http://bit.ly/rtrtqpcrprimer>

https://youtu.be/Q5njEkhmxo4

In the beginning there aren’t enough copies to measure directly, so you stabilize the recipe copies and make more copies of them to amplify the signal to a detectable level – the more copies you start with, the fewer amplification cycles you’ll need to do this – so you can compare how many cycles are required for different recipes, a number referred to as the quantification cycle (Cq) value – a lower Cq value means more recipes, and likely more protein.

In PCR, we can use DNA Polymerase (DNA Pol) to copy DNA in little tubes. More here: http://bit.ly/pcrtrain

We don’t want it to copy everything, but thankfully for us, we can utilize its limitations to our advantage to only copy defined stretches of DNA. You see, polymerases are a bit like trains that can only travel on double-stranded track, so if a Pol wants to travel along single-stranded DNA or RNA it has to lay down the complementary track ahead of it as it goes. This limitation also applies to the starting – it can only start from double-stranded track – so if you have single-stranded stuff (like mRNA or DNA after you unzip the strands by heating them up (melting) you want to copy you need to provide start “stations” which you can do with short pieces of DNA called primers that you design to match where you want Pol to start. Another limitation of its is that it can only travel in 1 direction (5’ to 3’) more here: http://bit.ly/sequencetermstools

I’ve been talking about DNA Pol because that’s what we use in ReverseTranscription qPCR – even though we’re interested in RNA not DNA.

So the first step in RT-qPCR (after you isolate the RNA) is making DNA copies of the RNA copies of the DNA recipes through REVERSE TRANSCRIPTION. Normal transcription goes DNA->RNA. REVERSE transcription goes RNA->DNA. It uses a different polymerase (instead of the usual DNA-RNA or DNA-DNA Pols you need an RNA-DNA Pol – we call such Pols reverse transcriptase) – and we call the DNA copies of the mature mRNAs complementary DNA (cDNA)

The reverse transcriptase can make DNA copies of RNA, but it still has the limitation of needing a double-stranded starting platform – so you need to provide primers for it, such as oligo(dTs) or random hexamers (more details in link). After you make the cDNA copies of “everything” or at least all the mRNAs, you want to make copies ONLY of the recipe you’re interested in.  So instead of aiming for genericness, you want your primers to be super specific. And you’ll need primerS now  Because now we want to amplify – with reverse transcription we only made 1 strand of cDNA, but now we want to make lots of copies. So we need to make a second strand from that first strand and then we can use those strands as templates for the other strands so you can make more and more and more…

Since you’re now just copying DNA to DNA, you can use a “normal” DNA Pol. And just like in normal PCR, qPCR is performed in cycles of temperature changes – MELT (heat up to separate strands) -> ANNEAL (cool down to let primers bind & Pol latch on) -> EXTEND (let Pol lay down complementary track) -> REPEAT.

So you need 2 primers – one for each strand – one will define the start & the other the stop for the region you want to copy. The first primer will bind the cDNA (at where you want to start) and Pol will start copying it 5’ to 3’ until it falls off the end of the cDNA or it runs out of steam, etc. And then in the next cycle that second strand needs a primer that bind it – and where it binds will define the start of where that strand starts. And it’ll go to where the 1st primer started because that’s as far as the strand its copying goes. So from then on, your strands will be the same length, bookended by those primer sites.

Each round of PCR, another copy can be made from each copy, so you increase exponentially. In the very beginning you can’t tell this though because the levels are so low you’re below the background & just see “noise.” But soon you’ll enter the exponential phase where you get measurable doubling each cycle – and since you start with way more supplies (primers, dNTPS, etc.) than you need, you don’t have to worry about running out. But later on you do start running out, so copy # stops growing exponentially, and your curve plateaus.

How do the copies get measured? Fluorescence – this is where a [molecule](https://thebumblingbiochemist.com/glossary/molecule/) absorbs a certain wavelength of light and release a different wavelength. More here: http://bit.ly/fluorescentstains

If you can directly couple the amount of light given off to the number of copies you make, such as with Taqman probes, and you use a special PCR machine with a fluorescence detector, you can read out – in “Real Time” – the number of copies you’re making.

You can plot cycle # vs fluorescence and – in either type of measuring – what you’re looking for is a value called the Cq value (quantification cycle) which is the # of cycles it takes to pass a “threshold line” corresponding to the background fluorescence level – the more copies you start with, the fewer cycles it will take (lower Cq) and the more “left-shifted” your curve will be.

* [[Diagram

  Description automatically generated](https://thebumblingbiochemist.com/wp-content/uploads/2020/02/qPCRoverview-100.jpg)](https://thebumblingbiochemist.com/wp-content/uploads/2020/02/qPCRoverview-100.jpg)
* [[A picture containing timeline

  Description automatically generated](https://thebumblingbiochemist.com/wp-content/uploads/2021/05/qpcrfromcdna-100-1.jpg)](https://thebumblingbiochemist.com/wp-content/uploads/2021/05/qpcrfromcdna-100-1.jpg)
* [[Timeline

  Description automatically generated](https://thebumblingbiochemist.com/wp-content/uploads/2021/06/rtprimers-100.jpg)](https://thebumblingbiochemist.com/wp-content/uploads/2021/06/rtprimers-100.jpg)
* [[A picture containing timeline

  Description automatically generated](https://thebumblingbiochemist.com/wp-content/uploads/2020/04/UNADJUSTEDNONRAW_thumb_9ca7.jpg)english](https://thebumblingbiochemist.com/wp-content/uploads/2020/04/UNADJUSTEDNONRAW_thumb_9ca7.jpg)
* [[Diagram

  Description automatically generated](https://thebumblingbiochemist.com/wp-content/uploads/2019/10/qpcr3-100.jpg)](https://thebumblingbiochemist.com/wp-content/uploads/2019/10/qpcr3-100.jpg)
* [[Diagram

  Description automatically generated](https://thebumblingbiochemist.com/wp-content/uploads/2021/03/measuringexpression-100.jpg)](https://thebumblingbiochemist.com/wp-content/uploads/2021/03/measuringexpression-100.jpg)

**[Related Terms:](https://thebumblingbiochemist.com/wp-content/uploads/2021/03/measuringexpression-100.jpg)**