

# McDonald Lab

## Supplemental Reading

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# Chapter 1

## Rotavirus

### 1.1 Double-stranded RNA viruses

**Double-stranded (ds) RNA viruses** are a diverse group of viruses that vary widely in host range (humans, animals, plants, fungi, and bacteria), genome segment number (one to twelve) and virion organization (T-number, capsid layers or turrets). Members of this group include the rotaviruses, known globally as a common cause of gastroenteritis in young children, and bluetongue virus, an economically important pathogen of cattle and sheep.

Of these families, the *Reoviridae* is the largest and most diverse in terms of host range.

In recent years the increasing knowledge of virus particle assembly, virus-cell interactions, and viral pathogenesis allow approaches for the development of novel antiviral strategies or agents.<sup>[1]</sup>

#### 1.1.1 Taxonomy

Viruses with dsRNA genomes are currently grouped into a number of families, unassigned genera and species.

Three families infect fungi: *Totiviridae*, *Partitiviridae* and *Chrysosiridae*. These families have monopartite, bipartite and quadripartite genomes respectively. They are typically isometric particles 25–50 nanometers in diameter. Based on sequence similarity of the RNA dependent RNA polymerase, the partitiviruses are probably derived from a totivirus ancestor.<sup>[2]</sup> A fourth family — *Alternaviridae* — has recently been described also with quadripartite genome.

Hypoviruses are mycoviruses (fungal viruses) with unen-

capsidated dsRNA genomes. They may have common ancestry with plant positive strand RNA viruses in supergroup 1 with *potyvirus* lineages, respectively<sup>[2]</sup>

A new clade (as yet unnamed) of six viruses infecting filamentous fungi has been reported.<sup>[3]</sup>

#### Taxa

##### Families

- *Alternaviridae*
- *Birnaviridae*
- *Chrysosiridae*
- *Cystoviridae*
- *Endornaviridae*
- *Hypoviridae*
- *Partitiviridae*
- *Picobirnaviridae*
- *Reoviridae*
- *Totiviridae*

##### Unassigned species

- *La France isometric virus*
- *Sclerotinia sclerotiorum debilitation associated virus*

- *Sclerotinia sclerotiorum mitovirus 1*
- *Sclerotinia sclerotiorum mitovirus 2*

### 1.1.2 Notes on selected species

#### Reoviridae

*Reoviridae* are currently classified into nine genera. The genomes of these viruses consist of 10 to 12 segments of dsRNA, each generally encoding one protein. The mature virions are non-enveloped. Their capsids, formed by multiple proteins, have icosahedral symmetry and are arranged generally in concentric layers. A distinguishing feature of the dsRNA viruses, irrespective of their family association, is their ability to carry out transcription of the dsRNA segments, under appropriate conditions, within the capsid. In all these viruses, the enzymes required for endogenous transcription are thus part of the virion structure.<sup>[1]</sup>

**Orthoreoviruses** The orthoreoviruses (reoviruses) are the prototypic members of the virus *Reoviridae* family and representative of the turreted members, which comprise about half the genera. Like other members of the family, the reoviruses are non-enveloped and characterized by concentric capsid shells that encapsidate a segmented dsRNA genome. In particular, reovirus has eight structural proteins and ten segments of dsRNA. A series of uncoating steps and conformational changes accompany cell entry and replication. High-resolution structures are known for almost all of the proteins of mammalian reovirus (MRV), which is the best-studied genotype. Electron cryo-microscopy (cryoEM) and X-ray crystallography have provided a wealth of structural information about two specific MRV strains, type 1 Lang (T1L) and type 3 Dearing (T3D).<sup>[4]</sup>

**Cypovirus** The cytoplasmic polyhedrosis viruses (CPVs) form the genus *Cypovirus* of the family *Reoviridae*. CPVs are classified into 14 species based on the electrophoretic migration profiles of their genome segments. Cypovirus has only a single capsid shell, which is similar to the orthoreovirus inner core. CPV exhibits striking capsid stability and is fully capable of endogenous RNA transcription and processing. The overall folds of CPV proteins are similar to

those of other reoviruses. However, CPV proteins have insertional domains and unique structures that contribute to their extensive intermolecular interactions. The CPV turret protein contains two methylase domains with a highly conserved helix-pair/β-sheet/helix-pair sandwich fold but lacks the β-barrel flap present in orthoreovirus λ2. The stacking of turret protein functional domains and the presence of constrictions and A spikes along the mRNA release pathway indicate a mechanism that uses pores and channels to regulate the highly coordinated steps of RNA transcription, processing, and release.<sup>[5]</sup>

**Rotavirus** Rotavirus is the most common cause of acute gastroenteritis in infants and young children worldwide. This virus contains a dsRNA genome and is a member of the *Reoviridae* family. The genome of rotavirus consists of eleven segments of dsRNA. Each genome segment codes for one protein with the exception of segment 11, which codes for two proteins. Among the twelve proteins, six are structural and six are non-structural proteins.<sup>[6]</sup> It is a double-stranded RNA non-enveloped virus

**Bluetongue virus** The members of genus *Orbivirus* within the *Reoviridae* family are arthropod borne viruses and are responsible for high morbidity and mortality in ruminants. Bluetongue virus (BTV) which causes disease in livestock (sheep, goat, cattle) has been in the forefront of molecular studies for the last three decades and now represents the best understood orbivirus at the molecular and structural levels. BTV, like other members of the family, is a complex non-enveloped virus with seven structural proteins and a RNA genome consisting of 10 variously sized dsRNA segments.<sup>[7][8]</sup>

**Phytoreoviruses** Phytoreoviruses are non-turreted reoviruses that are major agricultural pathogens, particularly in Asia. One member of this family, Rice Dwarf Virus (RDV), has been extensively studied by electron cryomicroscopy and x-ray crystallography. From these analyses, atomic models of the capsid proteins and a plausible model for capsid assembly have been derived. While the structural proteins of RDV share no sequence similarity to other proteins, their folds and the overall capsid structure are similar to those of other *Reoviridae*.<sup>[9]</sup>

### The yeast dsRNA virus L-A

The L-A dsRNA virus of the yeast *Saccharomyces cerevisiae* has a single 4.6 kb genomic segment that encodes its major coat protein, Gag (76 kDa) and a Gag-Pol fusion protein (180 kDa) formed by a –1 ribosomal frameshift. L-A can support the replication and encapsidation in separate viral particles of any of several satellite dsRNAs, called M dsRNAs, each of which encodes a secreted protein toxin (the killer toxin) and immunity to that toxin. L-A and M are transmitted from cell to cell by the cytoplasmic mixing that occurs in the process of mating. Neither is naturally released from the cell or enters cells by other mechanisms, but the high frequency of yeast mating in nature results in the wide distribution of these viruses in natural isolates. Moreover, the structural and functional similarities with dsRNA viruses of mammals has made it useful to consider these entities as viruses.<sup>[10]</sup>

### Infectious bursal disease virus

Infectious bursal disease virus (IBDV) is the best-characterized member of the family *Birnaviridae*. These viruses have bipartite dsRNA genomes enclosed in single layered icosahedral capsids with  $T = 13l$  geometry. IBDV shares functional strategies and structural features with many other icosahedral dsRNA viruses, except that it lacks the  $T = 1$  (or pseudo  $T = 2$ ) core common to the *Reoviridae*, *Cystoviridae*, and *Totiviridae*. The IBDV capsid protein exhibits structural domains that show homology to those of the capsid proteins of some positive-sense single-stranded RNA viruses, such as the *nodaviruses* and *tetraviruses*, as well as the  $T = 13$  capsid shell protein of the *Reoviridae*. The  $T = 13$  shell of the IBDV capsid is formed by trimers of VP2, a protein generated by removal of the C-terminal domain from its precursor, pVP2. The trimming of pVP2 is performed on immature particles as part of the maturation process. The other major structural protein, VP3, is a multifunctional component lying under the  $T = 13$  shell that influences the inherent structural polymorphism of pVP2. The virus-encoded RNA-dependent RNA polymerase, VP1, is incorporated into the capsid through its association with VP3. VP3 also interacts extensively with the viral dsRNA genome.<sup>[11]</sup>

### dsRNA bacteriophage Φ6

Bacteriophage Φ6, is a member of the *Cystoviridae* family. It infects *Pseudomonas* bacteria (typically plant-pathogenic *P. syringae*). It has a three-part, segmented, double-stranded RNA genome, totalling ~13.5 kb in length. Φ6 and its relatives have a lipid membrane around their nucleocapsid, a rare trait among bacteriophages. It is a lytic phage, though under certain circumstances has been observed to display a delay in lysis which may be described as a “carrier state”.<sup>[12]</sup>

#### 1.1.3 Anti-virals

Since cells do not produce double-stranded RNA during normal nucleic acid metabolism, natural selection has favored the evolution of enzymes that destroy dsRNA on contact. The best known class of this type of enzymes is Dicer. It is hoped that broad-spectrum anti-virals could be synthesized that take advantage of this vulnerability of double-stranded RNA viruses.<sup>[13]</sup>

#### 1.1.4 See also

- Microbiology
- Virology
- RNA virus
- Virus classification
- List of viruses
- Animal virology

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## 1.2 Rotavirus

**Rotavirus** is the most common cause of severe diarrhea among infants and young children.<sup>[1]</sup> It is a genus of double-stranded RNA virus in the family *Reoviridae*. Nearly every child in the world has been infected with rotavirus at least once by the age of five.<sup>[2]</sup> Immunity develops with each infection, so subsequent infections are less severe; adults are rarely affected.<sup>[3]</sup> There are five species of this virus, referred to as A, B, C, D, and E. *Rotavirus A*, the most common species, causes more than 90% of rotavirus infections in humans.

The virus is transmitted by the faecal-oral route. It infects and damages the cells that line the small intestine and causes gastroenteritis (which is often called "stomach flu" despite having no relation to influenza). Although rotavirus was discovered in 1973<sup>[4]</sup> and accounts for up to 50% of hospitalisations for severe diarrhoea in infants and children,<sup>[5]</sup> its importance is still underestimated within the public health community, particularly in developing countries.<sup>[6]</sup> In addition to its impact on human health, rotavirus also infects animals, and is a pathogen of livestock.<sup>[7]</sup>

Rotavirus is usually an easily managed disease of childhood, but worldwide more than 450,000 children under five years of age still die from rotavirus infection each year,<sup>[8]</sup> most of whom live in developing countries,<sup>[9]</sup> and almost two million more become severely ill.<sup>[6]</sup> In the United States, before initiation of the rotavirus vaccination programme, rotavirus caused about 2.7 million cases of severe gastroenteritis in children, almost 60,000 hospitalizations, and around 37 deaths each year.<sup>[10]</sup> Public health campaigns to combat rotavirus focus on providing oral rehydration therapy for infected children and vaccination to prevent the disease.<sup>[11]</sup> The incidence and severity of rotavirus infections has declined significantly in countries that have added rotavirus vaccine to their routine childhood immunisation policies.<sup>[12][13]</sup>

### 1.2.1 Signs and symptoms

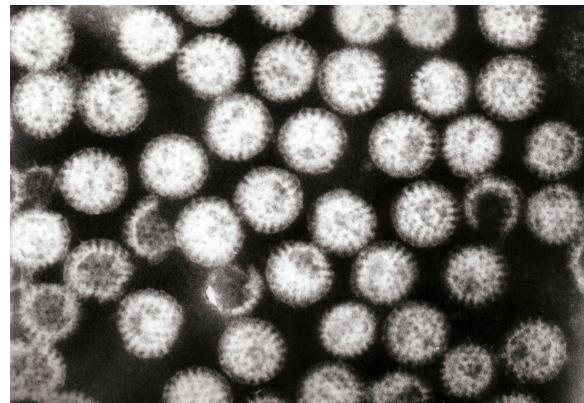
Rotavirus gastroenteritis is a mild to severe disease characterised by vomiting, watery diarrhea, and low-grade fever. Once a child is infected by the virus, there is an incubation period of about two days before symptoms appear.<sup>[14]</sup> Symptoms often start with vomiting followed by four to eight days of profuse diarrhoea. Dehydration is more common in rotavirus infection than in most of those caused by bacterial pathogens, and is the most common cause of death related to rotavirus infection.<sup>[15]</sup>

Rotavirus A infections can occur throughout life: the first usually produces symptoms, but subsequent infections are typically mild or asymptomatic,<sup>[16][17]</sup> as the immune system provides some protection.<ref name="isbn0-471-49663-4">Offit PA (2001). *Gastroenteritis viruses*. New York: Wiley. pp. 106–124. ISBN 0-471-49663-4.</ref><sup>[18]</sup> Consequently, symptomatic infection rates are highest in children under two years of age and decrease progressively towards 45 years of age.<ref name="isbn0-89603-736-3">Ramsay M and Brown D (2000). Desselberger, U.; Gray, James, ed. *Rotaviruses: methods and protocols*. Totowa, NJ: Humana Press. p. 217. ISBN 0-89603-736-3. Free ebook </ref> Infection in newborn children, although common, is often associated with mild or asymptomatic disease;<sup>[3]</sup> the most severe symptoms tend to occur in children six months to two years of age, the elderly, and those with compromised or absent immune system functions. Due to immunity acquired in childhood, most adults are not susceptible to rotavirus; gastroenteritis in adults usually has a cause other than rotavirus, but asymptomatic infections in adults may maintain the transmission of infection in the community.<sup>[19]</sup>

### 1.2.2 Transmission

Rotavirus is transmitted by the faecal-oral route, via contact with contaminated hands, surfaces and objects,<sup>[20]</sup> and possibly by the respiratory route.<sup>[1]</sup> The faeces of an infected person can contain more than 10 trillion infectious particles per gram;<sup>[17]</sup> fewer than 100 of these are required to transmit infection to another person.<sup>[3]</sup>

Rotaviruses are stable in the environment and have been found in estuary samples at levels up to 1–5 infectious par-



*Rotaviruses in the faeces of an infected child*

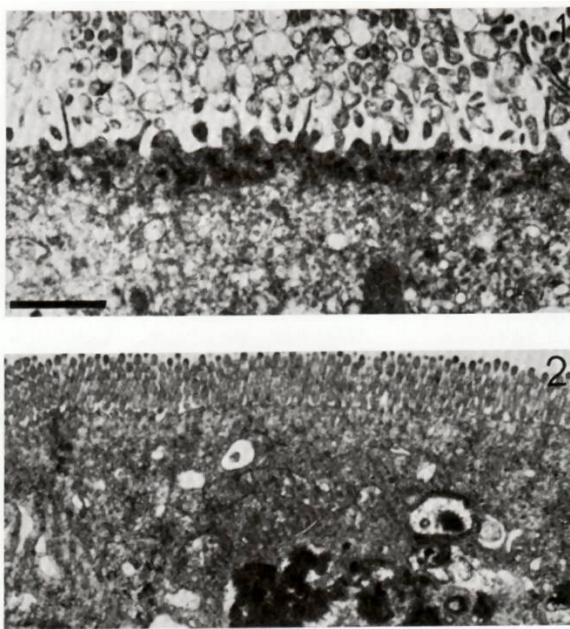
ticles per US gallon, the viruses survive between 9 and 19 days.<sup>[21]</sup> Sanitary measures adequate for eliminating bacteria and parasites seem to be ineffective in control of rotavirus, as the incidence of rotavirus infection in countries with high and low health standards is similar.<sup>[1]</sup>

### 1.2.3 Disease mechanisms

The diarrhoea is caused by multiple activities of the virus. Malabsorption occurs because of the destruction of gut cells called enterocytes. The toxic rotavirus protein NSP4 induces age- and calcium ion-dependent chloride secretion, disrupts SGLT1 transporter-mediated reabsorption of water, apparently reduces activity of brush-border membrane disaccharidases, and possibly activates the calcium ion-dependent secretory reflexes of the enteric nervous system.<sup>[22]</sup> Healthy enterocytes secrete lactase into the small intestine; milk intolerance due to lactase deficiency is a symptom of rotavirus infection,<sup>[23]</sup> which can persist for weeks.<sup>[24]</sup> A recurrence of mild diarrhoea often follows the reintroduction of milk into the child's diet, due to bacterial fermentation of the disaccharide lactose in the gut.<sup>[25]</sup>

### 1.2.4 Diagnosis and detection

Diagnosis of infection with rotavirus normally follows diagnosis of gastroenteritis as the cause of severe diarrhoea. Most children admitted to hospital with gastroenteritis are



Electron micrograph of a rotavirus infected enterocyte (top) compared to an uninfected cell (bottom). The bar = approx. 500 nm

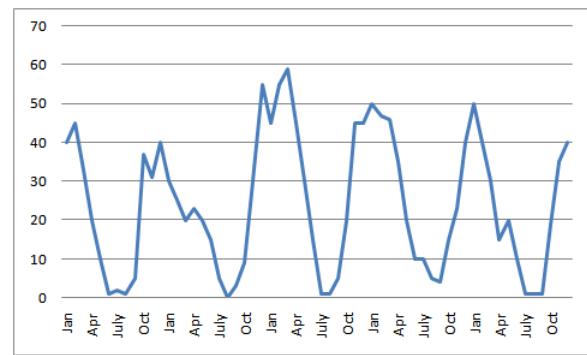
tested for rotavirus A.<sup>[26][27]</sup> Specific diagnosis of infection with rotavirus A is made by finding the virus in the child's stool by enzyme immunoassay. There are several licensed test kits on the market which are sensitive, specific and detect all serotypes of rotavirus A.<sup>[28]</sup> Other methods, such as electron microscopy and PCR, are used in research laboratories.<sup>[29]</sup> Reverse transcription-polymerase chain reaction (RT-PCR) can detect and identify all species and serotypes of human rotavirus.<sup>[30]</sup>

## 1.2.5 Treatment and prognosis

Treatment of acute rotavirus infection is nonspecific and involves management of symptoms and, most importantly, maintenance of hydration.<sup>[11]</sup> If untreated, children can die from the resulting severe dehydration.<sup>[31]</sup> Depending on the severity of diarrhea, treatment consists of oral rehydration, during which the child is given extra water to drink that contains small amounts of salt and sugar.<sup>[32]</sup> In 2004, the WHO and UNICEF recommended the use of low-osmolarity oral

rehydration solution and zinc supplementation as a two-pronged treatment of acute diarrhoea.<sup>[33]</sup> Some infections are serious enough to warrant hospitalization where fluids are given by intravenous drip or nasogastric tube, and the child's electrolytes and blood sugar are monitored.<sup>[26]</sup> Rotavirus infections rarely cause other complications and for a well managed child the prognosis is excellent.<sup>[34]</sup>

## 1.2.6 Epidemiology



The seasonal variation of rotavirus A infections in a region of England: rates of infection peak during the winter months.

Rotavirus A, which accounts for more than 90% of rotavirus gastroenteritis in humans,<sup>[35]</sup> is endemic worldwide. Each year rotavirus causes millions of cases of diarrhoea in developing countries, almost 2 million resulting in hospitalization<sup>[6]</sup> and an estimated 453,000 resulting in the death of a child younger than five,<sup>[8]</sup> 85 percent of whom live in developing countries.<sup>[9]</sup> In the United States alone—before initiation of the rotavirus vaccination programme<sup>[36]</sup>—over 2.7 million cases of rotavirus gastroenteritis occurred annually, 60,000 children were hospitalised and around 37 died from the results of the infection.<sup>[10]</sup> The major role of rotavirus in causing diarrhoea is not widely recognised within the public health community,<sup>[37]</sup> particularly in developing countries.<sup>[6]</sup> Almost every child has been infected with rotavirus by age five.<sup>[38]</sup> It is the leading single cause of severe diarrhoea among infants and children, being responsible for about 20% of cases, and accounts for 50% of the cases requiring hospitalization.<sup>[6]</sup> Rotavirus causes 37% of deaths attributable to diarrhoea and 5% of all deaths in children

younger than five.<sup>[8]</sup> Boys are twice as likely as girls to be admitted to hospital.<sup>[5][39]</sup> Rotavirus infections occur primarily during cool, dry seasons.<sup>[40][41]</sup> The number attributable to food contamination is unknown.<sup>[42]</sup>

"Rotavirus is estimated to cause about 40 per cent of all hospital admissions due to diarrhoea among children under five years of age worldwide—leading to some 100 million episodes of acute diarrhoea each year that result in 350,000 to 600,000 child deaths."

**UNICEF and World Health Organization** <sup>[43]</sup>

Outbreaks of rotavirus A diarrhoea are common among hospitalised infants, young children attending day care centres, and elderly people in nursing homes.<sup>[44]</sup> An outbreak caused by contaminated municipal water occurred in Colorado in 1981.<sup>[45]</sup> During 2005, the largest recorded epidemic of diarrhoea occurred in Nicaragua. This unusually large and severe outbreak was associated with mutations in the rotavirus A genome, possibly helping the virus escape the prevalent immunity in the population.<sup>[46]</sup> A similar large outbreak occurred in Brazil in 1977.<sup>[47]</sup>

Rotavirus B, also called adult diarrhoea rotavirus or ADRV, has caused major epidemics of severe diarrhoea affecting thousands of people of all ages in China. These epidemics occurred as a result of sewage contamination of drinking water.<sup>[48][49]</sup> Rotavirus B infections also occurred in India in 1998; the causative strain was named CAL. Unlike ADRV, the CAL strain is endemic.<sup>[50][51]</sup> To date, epidemics caused by rotavirus B have been confined to **mainland China**, and surveys indicate a lack of immunity to this species in the United States.<sup>[52]</sup>

Rotavirus C has been associated with rare and sporadic cases of diarrhoea in children, and small outbreaks have occurred in families.<sup>[53]</sup>

## 1.2.7 Prevention

Main article: **Rotavirus vaccine**

Because improved sanitation does not decrease the prevalence of rotaviral disease, and the rate of hospitalizations remains high despite the use of oral rehydrating medicines,

the primary public health intervention is vaccination.<sup>[2]</sup> Two vaccines against Rotavirus A infection are safe and effective in children:<sup>[13]</sup> Rotarix by GlaxoSmithKline<sup>[54]</sup> and RotaTeq by Merck.<sup>[55]</sup> Both are taken orally and contain attenuated live virus.<sup>[13]</sup> In 2009, the **World Health Organization** (WHO) recommended that rotavirus vaccine be included in all national immunisation programmes.<sup>[56]</sup> The incidence and severity of rotavirus infections has declined significantly in countries that have acted on this recommendation.<sup>[12][13]</sup> In Mexico, which in 2006 was among the first countries in the world to introduce rotavirus vaccine, diarrhoeal disease death rates dropped during the 2009 rotavirus season by more than 65 percent among children age two and under.<sup>[57]</sup> In Nicaragua, which in 2006 became the first developing country to introduce a rotavirus vaccine, severe rotavirus infections were reduced by 40 percent and emergency room visits by a half.<sup>[58]</sup> In the United States, rotavirus vaccination since 2006 has led to drops in rotavirus-related hospitalizations by as much as 86 percent. The vaccines may also have prevented illness in non-vaccinated children by limiting the number of circulating infections.<sup>[59]</sup>

Rotavirus vaccines are licensed in more than 100 countries, but only 28<sup>[60]</sup> countries have introduced routine rotavirus vaccination.<sup>[61]</sup> Following the introduction of routine rotavirus vaccination in the US in 2006, the health burden of rotavirus gastroenteritis "rapidly and dramatically reduced" despite lower coverage levels compared to other routine infant immunizations.<sup>[62]</sup> Clinical trials of the Rotarix rotavirus vaccine in South Africa and Malawi, found that the vaccine significantly reduced severe diarrhoea episodes caused by rotavirus, and that the infection was preventable by vaccination.<sup>[63]</sup> Safety and efficacy trials of Rotarix and RotaTeq in Africa and Asia found that the vaccines dramatically reduced severe disease among infants in developing countries, where the majority of rotavirus deaths occur.<sup>[64]</sup> A 2012 Cochrane review of 41 clinical trials that included 186,263 participants concluded Rotarix and RotaTeq are effective vaccines.<sup>[65]</sup> Additional rotavirus vaccines are under development.<sup>[66]</sup> In September 2013, the vaccine will be offered to all children in the UK, aged between two and three months, and it is expected to halve the cases of severe infection and reduce the number of children admitted to hospital because of the infection by 70 percent.<sup>[67]</sup>

International non-governmental organization PATH, the

WHO, the U.S. Centers for Disease Control and Prevention, and the GAVI Alliance are working to bring rotavirus vaccines to developing countries, where children face the greatest burden. Through the Rotavirus Vaccine Program and the Accelerating Vaccine Introduction initiative, these groups are partnering with research institutions and governments to reduce child morbidity and mortality from diarrhoeal disease by making a vaccine against rotavirus available for use in developing countries.<sup>[68]</sup>

### 1.2.8 Infections of other animals

Rotaviruses infect the young of many species of animals and they are a major cause of diarrhoea in wild and reared animals worldwide.<sup>[7]</sup> As a pathogen of livestock, notably in young calves and piglets, rotaviruses cause economic loss to farmers because of costs of treatment associated with high morbidity and mortality rates.<sup>[69]</sup> These rotaviruses are a potential reservoir for genetic exchange with human rotaviruses.<sup>[69]</sup> There is evidence that animal rotaviruses can infect humans, either by direct transmission of the virus or by contributing one or several RNA segments to reassortants with human strains.<sup>[70][71]</sup>

### 1.2.9 Virology

#### Types of rotavirus

There are five species of rotavirus, referred to as A, B, C, D and E. Humans are primarily infected by species A, B and C, most commonly by species A. All five species cause disease in other animals.<sup>[72]</sup> Within rotavirus A there are different strains, called serotypes.<sup>[73]</sup> As with influenza virus, a dual classification system is used based on two proteins on the surface of the virus. The glycoprotein VP7 defines the G serotypes and the protease-sensitive protein VP4 defines P serotypes.<sup>[74]</sup> Because the two genes that determine G-types and P-types can be passed on separately to progeny viruses, different combinations are found.<sup>[75]</sup>

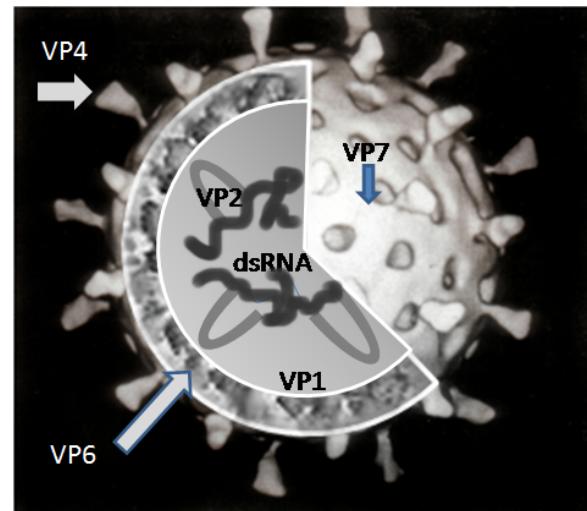
#### Structure

The genome of rotavirus consists of 11 unique double helix molecules of RNA which are 18,555 nucleotides in total.

Each helix, or segment, is a gene, numbered 1 to 11 by decreasing size. Each gene codes for one protein, except genes 9, which codes for two.<sup>[76]</sup> The RNA is surrounded by a three-layered icosahedral protein capsid. Viral particles are up to 76.5 nm in diameter<sup>[77][78]</sup> and are not enveloped.

#### Proteins

There are six viral proteins (VPs) that form the virus particle (virion). These *structural* proteins are called VP1, VP2, VP3, VP4, VP6 and VP7. In addition to the VPs, there are six *nonstructural* proteins (NSPs), that are only produced in cells infected by rotavirus. These are called NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6.<sup>[72]</sup>

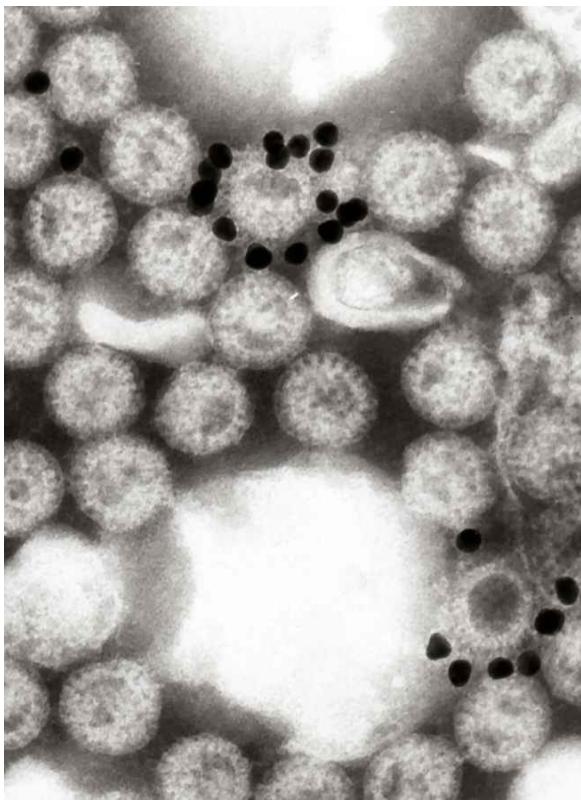


A simplified diagram of the location of rotavirus structural proteins

At least six of the twelve proteins encoded by the rotavirus genome bind RNA.<sup>[79]</sup> The role of these proteins play in rotavirus replication is not entirely understood; their functions are thought to be related to RNA synthesis and packaging in the virion, mRNA transport to the site of genome replication, and mRNA translation and regulation of gene expression.<sup>[80]</sup>

**Structural proteins** VP1 is located in the core of the virus particle and is an RNA polymerase enzyme.<sup>[81]</sup> In an infected cell this enzyme produces mRNA transcripts for

the synthesis of viral proteins and produces copies of the rotavirus genome RNA segments for newly produced virus particles.



*Electron micrograph of gold nanoparticles attached to rotavirus. The small dark circular objects are gold nanoparticles coated with a monoclonal antibody specific for rotavirus protein VP6.*

VP2 forms the core layer of the virion and binds the RNA genome.<sup>[82]</sup>

VP3 is part of the inner core of the virion and is an enzyme called guanylyl transferase. This is a capping enzyme that catalyses the formation of the 5' cap in the post-transcriptional modification of mRNA.<ref name="isbn0-12-375147-0"2>Angel J, Franco MA, Greenberg HB (2009). Mahy BWJ, Van Regenmortel MHV, ed. *Desk Encyclopedia of Human and Medical Virology*. Boston: Academic Press. p. 277. ISBN 0-12-375147-0.</ref> The cap stabilises viral mRNA by protecting it from nucleic acid degrading enzymes called nucleases.<sup>[83]</sup>

VP4 is on the surface of the virion that protrudes as a spike.<sup>[84]</sup> It binds to molecules on the surface of cells called receptors and drives the entry of the virus into the cell.<sup>[85]</sup> VP4 has to be modified by the protease enzyme trypsin, which is found in the gut, into VP5\* and VP8\* before the virus is infectious.<sup>[86]</sup> VP4 determines how virulent the virus is and it determines the P-type of the virus.<sup>[87]</sup>

VP6 forms the bulk of the capsid. It is highly antigenic and can be used to identify rotavirus species.<sup>[17]</sup> This protein is used in laboratory tests for rotavirus A infections.<sup>[88]</sup>

VP7 is a glycoprotein that forms the outer surface of the virion. Apart from its structural functions, it determines the G-type of the strain and, along with VP4, is involved in immunity to infection.<sup>[77]</sup>

**Nonstructural viral proteins** NSP1, the product of gene 5, is a nonstructural RNA-binding protein.<sup>[89]</sup>

NSP2 is an RNA-binding protein that accumulates in cytoplasmic inclusions (viroplasms) and is required for genome replication.<sup>[90][91]</sup>

NSP3 is bound to viral mRNAs in infected cells and it is responsible for the shutdown of cellular protein synthesis.<sup>[92]</sup>

NSP4 is a viral enterotoxin to induce diarrhoea and was the first viral enterotoxin discovered.<sup>[22]</sup>

NSP5 is encoded by genome segment 11 of rotavirus A and in virus-infected cells NSP5 accumulates in the viroplasm.<sup>[93]</sup>

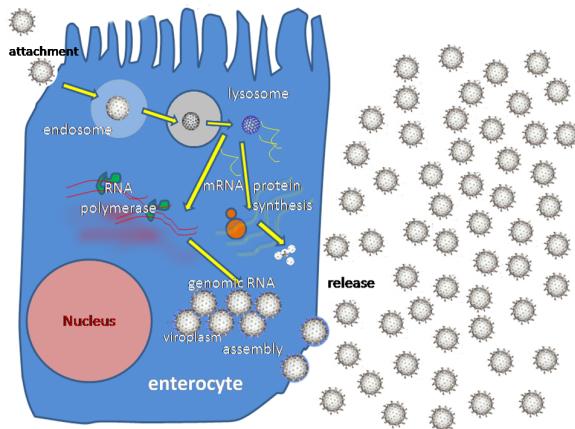
NSP6 is a nucleic acid binding protein,<sup>[94]</sup> and is encoded by gene 11 from an out of phase open reading frame.<sup>[95]</sup>

This table is based on the simian rotavirus strain SA11.<sup>[96][97][98]</sup> RNA-protein coding assignments differ in some strains.

## Replication

Rotaviruses replicate mainly in the gut,<sup>[99]</sup> and infect enterocytes of the villi of the small intestine, leading to structural and functional changes of the epithelium.<sup>[100]</sup> The triple protein coats make them resistant to the acidic pH of the stomach and the digestive enzymes in the gut.

The virus enters cells by receptor mediated endocytosis and



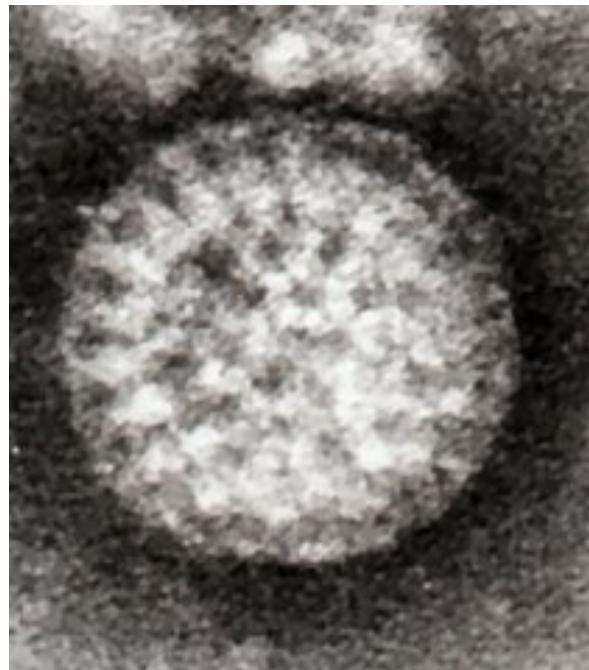
A simplified drawing of the rotavirus replication cycle

form a vesicle known as an **endosome**. Proteins in the third layer (VP7 and the VP4 spike) disrupt the membrane of the endosome, creating a difference in the calcium concentration. This causes the breakdown of VP7 trimers into single protein subunits, leaving the VP2 and VP6 protein coats around the viral dsRNA, forming a double-layered particle (DLP).<sup>[101]</sup>

The eleven dsRNA strands remain within the protection of the two protein shells and the viral **RNA-dependent RNA polymerase** creates mRNA transcripts of the double-stranded viral genome. By remaining in the core, the viral RNA evades innate host immune responses called **RNA interference** that are triggered by the presence of double-stranded RNA.

During the infection, rotavirus produces mRNA for both **protein biosynthesis** and gene replication. Most of the rotavirus proteins accumulate in **viroplasm**, where the RNA is replicated and the DLPs are assembled. Viroplasm is formed around the cell nucleus as early as two hours after virus infection, and consists of viral factories thought to be made by two viral nonstructural proteins: NSP5 and NSP2. Inhibition of NSP5 by RNA interference results in a sharp decrease in rotavirus replication. The DLPs migrate to the **endoplasmic reticulum** where they obtain their third, outer layer (formed by VP7 and VP4). The **progeny** viruses are released from the cell by lysis.<sup>[86][102][103]</sup>

## 1.2.10 History



One of Flewett's original electron micrographs

In 1943, Jacob Light and Horace Hodes proved that a filterable agent in the faeces of children with infectious diarrhoea also caused scours (livestock diarrhoea) in cattle.<sup>[104]</sup> Three decades later, preserved samples of the agent were shown to be rotavirus.<sup>[105]</sup> In the intervening years, a virus in mice<sup>[106]</sup> was shown to be related to the virus causing scours.<sup>[107]</sup> In 1973, Ruth Bishop and colleagues described related viruses found in children with gastroenteritis.<sup>[4]</sup>

In 1974, Thomas Henry Flewett suggested the name *rotavirus* after observing that, when viewed through an **electron microscope**, a rotavirus particle looks like a wheel (*rota* in Latin);<sup>[108][109]</sup> the name was officially recognised by the **International Committee on Taxonomy of Viruses** four years later.<sup>[110]</sup> In 1976, related viruses were described in several other species of animals.<sup>[107]</sup> These viruses, all causing acute gastroenteritis, were recognised as a collective pathogen affecting humans and animals worldwide.<sup>[108]</sup> Rotavirus serotypes were first described in 1980,<sup>[111]</sup> and in the following year, rotavirus from humans was first grown

in cell cultures derived from monkey kidneys, by adding trypsin (an enzyme found in the duodenum of mammals and now known to be essential for rotavirus to replicate) to the culture medium.<sup>[112]</sup> The ability to grow rotavirus in culture accelerated the pace of research, and by the mid-1980s the first candidate vaccines were being evaluated.<sup>[113]</sup>

In 1998, a rotavirus vaccine was licensed for use in the United States. Clinical trials in the United States, Finland, and Venezuela had found it to be 80 to 100% effective at preventing severe diarrhoea caused by rotavirus A, and researchers had detected no statistically significant serious adverse effects.<sup>[114][115]</sup> The manufacturer, however, withdrew it from the market in 1999, after it was discovered that the vaccine may have contributed to an increased risk for intussusception, a type of bowel obstruction, in one of every 12,000 vaccinated infants.<sup>[116]</sup> The experience provoked intense debate about the relative risks and benefits of a rotavirus vaccine.<sup>[117]</sup> In 2006, two new vaccines against rotavirus A infection were shown to be safe and effective in children,<sup>[118]</sup> and in June 2009 the World Health Organization recommended that rotavirus vaccination be included in all national immunisation programmes to provide protection against this virus.<sup>[119]</sup>

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## 1.2.12 External links

- WHO Rotavirus web page
- Rotavirus on Centers for Disease Control and Prevention (CDC) site
- Viralzone: Rotavirus
- Vaccine Resource Library: Rotavirus
- DefeatDD.org
- Centers for Disease Control and Prevention (2012). “Ch. 18: Rotavirus”. In Atkinson W, Wolfe S, Hamborsky J. *Epidemiology and Prevention of Vaccine-Preventable Diseases* (12th ed.). Washington DC: Public Health Foundation. pp. 263–274.
- 3D macromolecular structures of Rotaviruses from the EM Data Bank(EMDB)
- Ramig, RF (October 2004). “Pathogenesis of intestinal and systemic rotavirus infection”. *Journal of Virology* **78** (19): 10213–20. doi:10.1128/JVI.78.19.10213-10220.2004. PMC 516399. PMID 15367586.

## 1.3 Gastroenteritis

**Gastroenteritis** or **infectious diarrhea** is a medical condition from inflammation (“-itis”) of the gastrointestinal tract that involves both the stomach (“gastro”-) and the small intestine (“entero”-). It causes some combination of diarrhea, vomiting, and abdominal pain and cramping.<sup>[1]</sup> Dehydration may occur as a result. Gastroenteritis has been referred to as **gastro**, **stomach bug**, and **stomach virus**. Although unrelated to influenza, it has also been called **stomach flu** and **gastric flu**.

Globally, most cases in children are caused by rotavirus.<sup>[2]</sup> In adults, norovirus<sup>[3]</sup> and *Campylobacter*<sup>[4]</sup> are more common. Less common causes include other bacteria (or their toxins) and parasites. Transmission may occur due to consumption of improperly prepared foods or contaminated water or via close contact with individuals who are infectious. Prevention includes the use of fresh water, regular hand washing, and breast feeding especially in areas where sanitation is less good. The rotavirus vaccine is recommended for all children.

The key treatment is enough fluids. For mild or moderate cases, this can typically be achieved via **oral rehydration solution** (a combination of water, salts, and sugar). In those who are breast fed, continued breast feeding is recommended. For more severe cases, intravenous fluids from a healthcare centre may be needed. Antibiotics are generally not recommended. Gastroenteritis primarily affects children and those in the developing world. It results in about three to five billion cases and causes 1.4 million deaths a year.

### 1.3.1 Signs and symptoms

Gastroenteritis typically involves both diarrhea and vomiting,<sup>[5]</sup> or less commonly, presents with only one or the other.<sup>[1]</sup> Abdominal cramping may also be present.<sup>[1]</sup> Signs and symptoms usually begin 12–72 hours after contracting the infectious agent.<sup>[6]</sup> If due to a viral agent, the condition usually resolves within one week.<sup>[5]</sup> Some viral causes may also be associated with fever, fatigue, headache, and muscle pain.<sup>[5]</sup> If the stool is bloody, the cause is less likely to be viral<sup>[5]</sup> and more likely to be bacterial.<sup>[7]</sup> Some bacterial infections may be associated

## Bristol Stool Chart

Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on the surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges
Type 6		Fluffy pieces with ragged edges, a mushy stool
Type 7		Watery, no solid pieces. <b>Entirely Liquid</b>

Type 7 on the Bristol Stool Chart indicates diarrhea

with severe abdominal pain and may persist for several weeks.<sup>[7]</sup>

Children infected with rotavirus usually make a full recovery within three to eight days.<sup>[8]</sup> However, in poor countries treatment for severe infections is often out of reach and persistent diarrhea is common.<sup>[9]</sup> Dehydration is a common complication of diarrhea,<sup>[10]</sup> and a child with a significant degree of dehydration may have a prolonged capillary refill, poor skin turgor, and abnormal breathing.<sup>[11]</sup> Repeat infections are typically seen in areas with poor sanitation, and malnutrition,<sup>[6]</sup> stunted growth, and long-term cognitive delays can result.<sup>[12]</sup>

Reactive arthritis occurs in 1% of people following infections with *Campylobacter* species, and Guillain-Barre syndrome occurs in 0.1%.<sup>[7]</sup> Hemolytic uremic syndrome (HUS) may occur due to infection with Shiga toxin-producing *Escherichia coli* or *Shigella* species, causing low platelet counts, poor kidney function, and low red blood

cell count (due to their breakdown).<sup>[13]</sup> Children are more predisposed to getting HUS than adults.<sup>[12]</sup> Some viral infections may produce benign infantile seizures.<sup>[1]</sup>

### 1.3.2 Cause

Viruses (particularly rotavirus) and the bacteria *Escherichia coli* and *Campylobacter* species are the primary causes of gastroenteritis.<sup>[6][14]</sup> There are, however, many other infectious agents that can cause this syndrome.<sup>[12]</sup> Non-infectious causes are seen on occasion, but they are less likely than a viral or bacterial cause.<sup>[1]</sup> Risk of infection is higher in children due to their lack of immunity and relatively poor hygiene.<sup>[1]</sup>

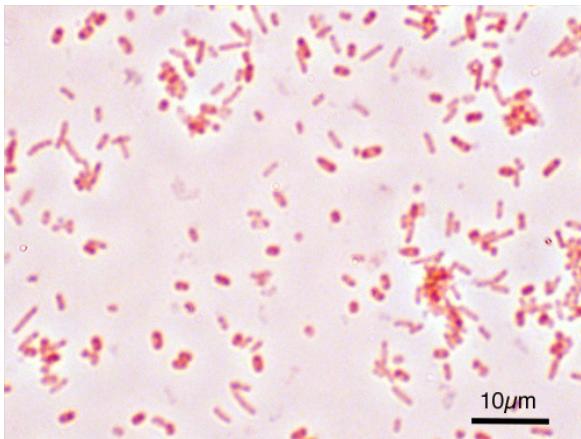
#### Viral

Rotavirus, norovirus, adenovirus, and astrovirus are known to cause viral gastroenteritis.<sup>[5][15]</sup> Rotavirus is the most common cause of gastroenteritis in children,<sup>[14]</sup> and produces similar rates in both the developed and developing world.<sup>[8]</sup> Viruses cause about 70% of episodes of infectious diarrhea in the pediatric age group.<sup>[16]</sup> Rotavirus is a less common cause in adults due to acquired immunity.<sup>[17]</sup> Norovirus is the cause in about 18% of all cases.<sup>[18]</sup>

Norovirus is the leading cause of gastroenteritis among adults in America, causing greater than 90% of outbreaks.<sup>[5]</sup> These localized epidemics typically occur when groups of people spend time in close physical proximity to each other, such as on cruise ships,<sup>[5]</sup> in hospitals, or in restaurants.<sup>[1]</sup> People may remain infectious even after their diarrhea has ended.<sup>[5]</sup> Norovirus is the cause of about 10% of cases in children.<sup>[1]</sup>

#### Bacterial

In the developed world *Campylobacter jejuni* is the primary cause of bacterial gastroenteritis, with half of these cases associated with exposure to poultry.<sup>[7]</sup> In children, bacteria are the cause in about 15% of cases, with the most common types being *Escherichia coli*, *Salmonella*, *Shigella*, and *Campylobacter* species.<sup>[16]</sup> If food becomes contaminated with bacteria and remains at room temperature for a pe-



*Salmonella enterica serovar Typhimurium (ATCC 14028)* as seen with a microscope at 1000 fold magnification and following Gram staining.

riod of several hours, the bacteria multiply and increase the risk of infection in those who consume the food.<sup>[12]</sup> Some foods commonly associated with illness include raw or undercooked meat, poultry, seafood, and eggs; raw sprouts; unpasteurized milk and soft cheeses; and fruit and vegetable juices.<sup>[19]</sup> In the developing world, especially sub-Saharan Africa and Asia, *cholera* is a common cause of gastroenteritis. This infection is usually transmitted by contaminated water or food.<sup>[20]</sup>

Toxigenic *Clostridium difficile* is an important cause of diarrhea that occurs more often in the elderly.<sup>[12]</sup> Infants can carry these bacteria without developing symptoms.<sup>[12]</sup> It is a common cause of diarrhea in those who are hospitalized and is frequently associated with antibiotic use.<sup>[21]</sup> *Staphylococcus aureus* infectious diarrhea may also occur in those who have used antibiotics.<sup>[22]</sup> "Traveler's diarrhea" is usually a type of bacterial gastroenteritis. Acid-suppressing medication appears to increase the risk of significant infection after exposure to a number of organisms, including *Clostridium difficile*, *Salmonella*, and *Campylobacter* species.<sup>[23]</sup> The risk is greater in those taking proton pump inhibitors than with H<sub>2</sub> antagonists.<sup>[23]</sup>

## Parasitic

A number of protozoans can cause gastroenteritis – most commonly *Giardia lamblia* – but *Entamoeba histolytica* and *Cryptosporidium* species have also been implicated.<sup>[16]</sup> As a group, these agents comprise about 10% of cases in children.<sup>[13]</sup> *Giardia* occurs more commonly in the developing world, but this etiologic agent causes this type of illness to some degree nearly everywhere.<sup>[24]</sup> It occurs more commonly in persons who have traveled to areas with high prevalence, children who attend day care, men who have sex with men, and following disasters.<sup>[24]</sup>

## Transmission

Transmission may occur via consumption of contaminated water, or when people share personal objects.<sup>[6]</sup> In places with wet and dry seasons, water quality typically worsens during the wet season, and this correlates with the time of outbreaks.<sup>[6]</sup> In areas of the world with four seasons, infections are more common in the winter.<sup>[12]</sup> Bottle-feeding of babies with improperly sanitized bottles is a significant cause on a global scale.<sup>[6]</sup> Transmission rates are also related to poor hygiene, especially among children,<sup>[5]</sup> in crowded households,<sup>[25]</sup> and in those with pre-existing poor nutritional status.<sup>[12]</sup> After developing tolerance, adults may carry certain organisms without exhibiting signs or symptoms, and thus act as natural reservoirs of contagion.<sup>[12]</sup> While some agents (such as *Shigella*) only occur in primates, others may occur in a wide variety of animals (such as *Giardia*).<sup>[12]</sup>

## Non-infectious

There are a number of non-infectious causes of inflammation of the gastrointestinal tract.<sup>[1]</sup> Some of the more common include medications (like NSAIDs), certain foods such as lactose (in those who are intolerant), and gluten (in those with celiac disease). Crohn's disease is also a non-infection source of (often severe) gastroenteritis.<sup>[1]</sup> Disease secondary to toxins may also occur. Some food related conditions associated with nausea, vomiting, and diarrhea include: ciguatera poisoning due to consumption of contaminated predatory fish, scombroid associated with the con-

sumption of certain types of spoiled fish, tetrodotoxin poisoning from the consumption of puffer fish among others, and botulism typically due to improperly preserved food.<sup>[26]</sup>

### 1.3.3 Pathophysiology

Gastroenteritis is defined as vomiting or diarrhea due to infection of the small or large bowel.<sup>[12]</sup> The changes in the small bowel are typically noninflammatory, while the ones in the large bowel are inflammatory.<sup>[12]</sup> The number of pathogens required to cause an infection varies from as few as one (for *Cryptosporidium*) to as many as  $10^8$  (for *Vibrio cholerae*).<sup>[12]</sup>

### 1.3.4 Diagnosis

Gastroenteritis is typically diagnosed clinically, based on a person's signs and symptoms.<sup>[5]</sup> Determining the exact cause is usually not needed as it does not alter management of the condition.<sup>[6]</sup> However, stool cultures should be performed in those with blood in the stool, those who might have been exposed to food poisoning, and those who have recently traveled to the developing world.<sup>[16]</sup> Diagnostic testing may also be done for surveillance.<sup>[5]</sup> As hypoglycemia occurs in approximately 10% of infants and young children, measuring serum glucose in this population is recommended.<sup>[11]</sup> Electrolytes and kidney function should also be checked when there is a concern about severe dehydration.<sup>[16]</sup>

### Dehydration

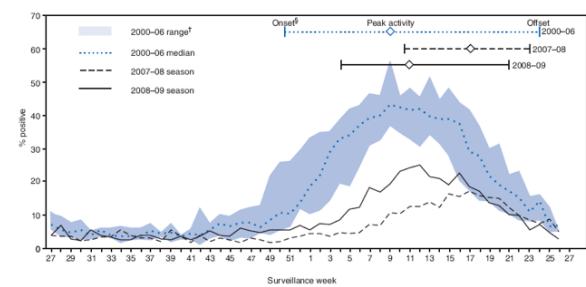
A determination of whether or not the person has dehydration is an important part of the assessment, with dehydration typically divided into mild (3–5%), moderate (6–9%), and severe ( $\geq 10\%$ ) cases.<sup>[1]</sup> In children, the most accurate signs of moderate or severe dehydration are a prolonged capillary refill, poor skin turgor, and abnormal breathing.<sup>[11][27]</sup> Other useful findings (when used in combination) include sunken eyes, decreased activity, a lack of tears, and a dry mouth.<sup>[1]</sup> A normal urinary output and oral fluid intake is reassuring.<sup>[11]</sup> Laboratory testing is of little clinical benefit in determining the degree of dehydration.<sup>[1]</sup>

### Differential diagnosis

Other potential causes of signs and symptoms that mimic those seen in gastroenteritis that need to be ruled out include appendicitis, volvulus, inflammatory bowel disease, urinary tract infections, and diabetes mellitus.<sup>[16]</sup> Pancreatic insufficiency, short bowel syndrome, Whipple's disease, coeliac disease, and laxative abuse should also be considered.<sup>[28]</sup> The differential diagnosis can be complicated somewhat if the person exhibits *only* vomiting or diarrhea (rather than both).<sup>[1]</sup>

Appendicitis may present with vomiting, abdominal pain, and a small amount of diarrhea in up to 33% of cases.<sup>[1]</sup> This is in contrast to the large amount of diarrhea that is typical of gastroenteritis.<sup>[1]</sup> Infections of the lungs or urinary tract in children may also cause vomiting or diarrhea.<sup>[1]</sup> Classical diabetic ketoacidosis (DKA) presents with abdominal pain, nausea, and vomiting, but without diarrhea.<sup>[1]</sup> One study found that 17% of children with DKA were initially diagnosed as having gastroenteritis.<sup>[1]</sup>

### 1.3.5 Prevention



Percentage of rotavirus tests with positive results, by surveillance week, United States, July 2000 – June 2009.

### Lifestyle

A supply of easily accessible uncontaminated water and good sanitation practices are important for reducing rates of infection and clinically significant gastroenteritis.<sup>[12]</sup> Personal measures (such as hand washing) have been found to decrease incidence and prevalence rates of gastroenteritis

in both the developing and developed world by as much as 30%.<sup>[11]</sup> Alcohol-based gels may also be effective.<sup>[11]</sup> Breastfeeding is important, especially in places with poor hygiene, as is improvement of hygiene generally.<sup>[6]</sup> Breast milk reduces both the frequency of infections and their duration.<sup>[11]</sup> Avoiding contaminated food or drink should also be effective.<sup>[29]</sup>

### Vaccination

Due to both its effectiveness and safety, in 2009 the World Health Organization recommended that the **rotavirus vaccine** be offered to all children globally.<sup>[14][30]</sup> Two commercial rotavirus vaccines exist and several more are in development.<sup>[30]</sup> In Africa and Asia these vaccines reduced severe disease among infants<sup>[30]</sup> and countries that have put in place national immunization programs have seen a decline in the rates and severity of disease.<sup>[31][32]</sup> This vaccine may also prevent illness in non-vaccinated children by reducing the number of circulating infections.<sup>[33]</sup> Since 2000, the implementation of a rotavirus vaccination program in the United States has substantially decreased the number of cases of diarrhea by as much as 80 percent.<sup>[34][35][36]</sup> The first dose of vaccine should be given to infants between 6 and 15 weeks of age.<sup>[14]</sup> The oral cholera vaccine has been found to be 50–60% effective over 2 years.<sup>[37]</sup>

### 1.3.6 Management

Gastroenteritis is usually an acute and self-limiting disease that does not require medication.<sup>[10]</sup> The preferred treatment in those with mild to moderate dehydration is **oral rehydration therapy (ORT)**.<sup>[13]</sup> Metoclopramide and/or **ondansetron**, however, may be helpful in some children,<sup>[38]</sup> and **butylscopolamine** is useful in treating abdominal pain.<sup>[39]</sup>

### Rehydration

The primary treatment of gastroenteritis in both children and adults is **rehydration**. This is preferably achieved by oral rehydration therapy, although intravenous delivery may be required if there is a decreased level of consciousness

or if dehydration is severe.<sup>[40][41]</sup> Oral replacement therapy products made with complex carbohydrates (i.e. those made from wheat or rice) may be superior to those based on simple sugars.<sup>[42]</sup> Drinks especially high in simple sugars, such as **soft drinks** and fruit juices, are not recommended in children under 5 years of age as they may *increase* diarrhea.<sup>[10]</sup> Plain water may be used if more specific and effective ORT preparations are unavailable or are not palatable.<sup>[10]</sup> A **nasogastric tube** can be used in young children to administer fluids if warranted.<sup>[16]</sup>

### Dietary

It is recommended that breast-fed infants continue to be nursed in the usual fashion, and that formula-fed infants continue their formula immediately after rehydration with ORT.<sup>[43]</sup> Lactose-free or lactose-reduced formulas usually are not necessary.<sup>[43]</sup> Children should continue their usual diet during episodes of diarrhea with the exception that foods high in **simple sugars** should be avoided.<sup>[43]</sup> The **BRAT diet** (bananas, rice, applesauce, toast and tea) is no longer recommended, as it contains insufficient nutrients and has no benefit over normal feeding.<sup>[43]</sup> Some **probiotics** have been shown to be beneficial in reducing both the duration of illness and the frequency of stools.<sup>[44]</sup> They may also be useful in preventing and treating **antibiotic associated diarrhea**.<sup>[45]</sup> Fermented milk products (such as **yogurt**) are similarly beneficial.<sup>[46]</sup> Zinc supplementation appears to be effective in both treating and preventing diarrhea among children in the developing world.<sup>[47]</sup>

### Antiemetics

Antiemetic medications may be helpful for treating vomiting in children. **Ondansetron** has some utility, with a single dose being associated with less need for intravenous fluids, fewer hospitalizations, and decreased vomiting.<sup>[48][49][50]</sup> **Metoclopramide** might also be helpful.<sup>[50]</sup> However, the use of ondansetron might possibly be linked to an increased rate of return to hospital in children.<sup>[51]</sup> The intravenous preparation of ondansetron may be given orally if clinical judgment warrants.<sup>[52]</sup> **Dimenhydrinate**, while reducing vomiting, does not appear to have a significant clinical benefit.<sup>[1]</sup>

## Antibiotics

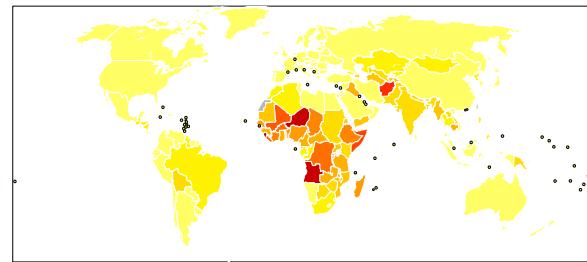
Antibiotics are not usually used for gastroenteritis, although they are sometimes recommended if symptoms are particularly severe<sup>[53]</sup> or if a susceptible bacterial cause is isolated or suspected.<sup>[54]</sup> If antibiotics are to be employed, a macrolide (such as azithromycin) is preferred over a fluoroquinolone due to higher rates of resistance to the latter.<sup>[7]</sup> Pseudomembranous colitis, usually caused by antibiotic use, is managed by discontinuing the causative agent and treating it with either metronidazole or vancomycin.<sup>[55]</sup> Bacteria and protozoans that are amenable to treatment include *Shigella*<sup>[56]</sup> *Salmonella typhi*,<sup>[57]</sup> and *Giardia* species.<sup>[24]</sup> In those with *Giardia* species or *Entamoeba histolytica*, tinidazole treatment is recommended and superior to metronidazole.<sup>[24][58]</sup> The World Health Organization (WHO) recommends the use of antibiotics in young children who have both bloody diarrhea and fever.<sup>[1]</sup>

## Antimotility agents

Antimotility medication has a theoretical risk of causing complications, and although clinical experience has shown this to be unlikely,<sup>[28]</sup> these drugs are discouraged in people with bloody diarrhea or diarrhea that is complicated by fever.<sup>[59]</sup> Loperamide, an opioid analogue, is commonly used for the symptomatic treatment of diarrhea.<sup>[60]</sup> Loperamide is not recommended in children, however, as it may cross the immature blood–brain barrier and cause toxicity. Bismuth subsalicylate, an insoluble complex of trivalent bismuth and salicylate, can be used in mild to moderate cases,<sup>[28]</sup> but salicylate toxicity is theoretically possible.<sup>[1]</sup>

### 1.3.7 Epidemiology

It is estimated that three to five billion cases of gastroenteritis resulting in 1.4 million deaths occur globally on an annual basis,<sup>[13][61]</sup> with children and those in the developing world being primarily affected.<sup>[6]</sup> As of 2011, in those less than five, there were about 1.7 billion cases resulting in 0.7 million deaths,<sup>[62]</sup> with most of these occurring in the world's poorest nations.<sup>[12]</sup> More than 450,000 of these fatalities are due to rotavirus in children under 5 years of age.<sup>[63][64]</sup> Cholera causes about three to five million



Disability-adjusted life year for diarrhea per 100,000 inhabitants in 2004.

cases of disease and kills approximately 100,000 people yearly.<sup>[20]</sup> In the developing world children less than two years of age frequently get six or more infections a year that result in clinically significant gastroenteritis.<sup>[12]</sup> It is less common in adults, partly due to the development of acquired immunity.<sup>[5]</sup>

In 1980, gastroenteritis from all causes caused 4.6 million deaths in children, with the majority occurring in the developing world.<sup>[55]</sup> Death rates were reduced significantly (to approximately 1.5 million deaths annually) by the year 2000, largely due to the introduction and widespread use of oral rehydration therapy.<sup>[65]</sup> In the US, infections causing gastroenteritis are the second most common infection (after the common cold), and they result in between 200 and 375 million cases of acute diarrhea<sup>[5][12]</sup> and approximately ten thousand deaths annually,<sup>[12]</sup> with 150 to 300 of these deaths in children less than five years of age.<sup>[1]</sup>

### 1.3.8 History

The first usage of “gastroenteritis” was in 1825.<sup>[66]</sup> Before this time it was more specifically known as typhoid fever or “cholera morbus”, among others, or less specifically as “gripping of the guts”, “surfeit”, “flux”, “colic”, “bowel complaint”, or any one of a number of other archaic names for acute diarrhea.<sup>[67]</sup>

### 1.3.9 Society and culture

Gastroenteritis is associated with many colloquial names, including "Montezuma's revenge", "Delhi belly", "la turista", and "back door sprint", among others.<sup>[12]</sup> It has played a role in many military campaigns and is believed to be the origin of the term "no guts no glory".<sup>[12]</sup>

Gastroenteritis is the main reason for 3.7 million visits to physicians a year in the United States<sup>[1]</sup> and 3 million visits in France.<sup>[68]</sup> In the United States gastroenteritis as a whole is believed to result in costs of 23 billion USD per year<sup>[69]</sup> with that due to rotavirus alone resulting in estimated costs of 1 billion USD a year.<sup>[1]</sup>

### 1.3.10 Research

There are a number of vaccines against gastroenteritis in development. For example, vaccines against *Shigella* and enterotoxigenic *Escherichia coli* (ETEC), two of the leading bacterial causes of gastroenteritis worldwide.<sup>[70][71]</sup>

### 1.3.11 Other animals

Many of the same agents cause gastroenteritis in cats and dogs as in humans. The most common organisms are *Campylobacter*, *Clostridium difficile*, *Clostridium perfringens*, and *Salmonella*.<sup>[72]</sup> A large number of toxic plants may also cause symptoms.<sup>[73]</sup>

Some agents are more specific to a certain species. Transmissible gastroenteritis coronavirus (TGEV) occurs in pigs resulting in vomiting, diarrhea, and dehydration.<sup>[74]</sup> It is believed to be introduced to pigs by wild birds and there is no specific treatment available.<sup>[75]</sup> It is not transmissible to humans.<sup>[76]</sup>

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#### 1.3.13 External links

- Gastroenteritis at DMOZ
- Diarrhoea and Vomiting Caused by Gastroenteritis: Diagnosis, Assessment and Management in Children Younger than 5 Years - NICE Clinical Guidelines, No. 84.

## 1.4 Rotaviral enteritis

**Rotavirus enteritis** is the most common cause of severe diarrhoea among infants and young children.<sup>[1]</sup> It is caused by **Rotavirus**, a genus of double-stranded RNA virus in the family *Reoviridae*. By the age of five, nearly every child in the world has been infected with rotavirus at least once.<sup>[2]</sup> However, with each infection, immunity develops, and subsequent infections are less severe; adults are rarely affected.<sup>[3]</sup> There are five species of this virus, referred to as A, B, C, D, and E.<sup>[4]</sup> Rotavirus A, the most common, causes more than 90% of infections in humans.

The virus is transmitted by the faecal-oral route. It infects and damages the **cells** that line the small intestine and causes **gastroenteritis** (which is often called “stomach flu” despite

having no relation to **influenza**). Although rotavirus was discovered in 1973<sup>[5]</sup> and accounts for up to 50% of hospitalisations for severe diarrhoea in infants and children,<sup>[6]</sup> its importance is still not widely known within the **public health** community, particularly in **developing countries**.<sup>[7]</sup> In addition to its impact on human health, rotavirus also infects animals, and is a pathogen of livestock.<sup>[8]</sup>

Rotavirus is usually an easily managed disease of childhood, but worldwide nearly 500,000 children under five years of age still die from rotavirus infection each year<sup>[9]</sup> and almost two million more become severely ill.<sup>[7]</sup> In the United States, before initiation of the rotavirus vaccination programme, rotavirus caused about 2.7 million cases of severe gastroenteritis in children, almost 60,000 hospitalisations, and around 37 deaths each year.<sup>[10]</sup> Public health campaigns to combat rotavirus focus on providing oral **rehydration therapy** for infected children and vaccination to prevent the disease.<sup>[11]</sup> The incidence and severity of rotavirus infections has declined significantly in countries that have added rotavirus vaccine to their routine childhood immunisation policies.<sup>[12][13]</sup>

#### 1.4.1 Signs and symptoms

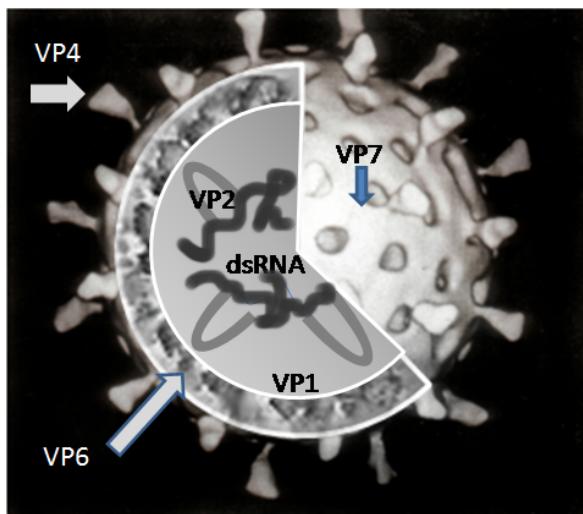
Rotavirus gastroenteritis is a mild to severe disease characterised by **vomiting**, watery **diarrhoea**, and low-grade **fever**. Once a child is infected by the virus, there is an **incubation period** of about two days before symptoms appear.<sup>[14]</sup> Symptoms often start with vomiting followed by four to eight days of profuse diarrhoea. **Dehydration** is more common in rotavirus infection than in most of those caused by bacterial pathogens, and is the most common cause of death related to rotavirus infection.<sup>[15]</sup>

Rotavirus A infections can occur throughout life: the first usually produces **symptoms**, but subsequent infections are typically mild or **asymptomatic**,<sup>[16][17]</sup> as the immune system provides some protection.<ref name="isbn0-471-49663-4"3>Offit PA (2001). *Gastroenteritis viruses*. New York: Wiley. pp. 106–124. ISBN 0-471-49663-4.</ref><sup>[18]</sup> Consequently, symptomatic infection rates are highest in children under two years of age and decrease progressively towards 45 years of age.<ref name="isbn0-89603-736-3"2>Ramsay M and Brown D (2000). Desselberger, U.; Gray, James, ed. *Rotaviruses: methods and*

protocols. Totowa, NJ: Humana Press. p. 217. ISBN 0-89603-736-3.</ref> Infection in newborn children, although common, is often associated with mild or asymptomatic disease;<sup>[3]</sup> the most severe symptoms tend to occur in children six months to two years of age, the elderly, and those with compromised or absent immune system functions. Due to immunity acquired in childhood, most adults are not susceptible to rotavirus; gastroenteritis in adults usually has a cause other than rotavirus, but asymptomatic infections in adults may maintain the transmission of infection in the community.<sup>[19]</sup>

### 1.4.2 Virology

Main article: Rotavirus



A simplified diagram of the location of rotavirus structural proteins

### Transmission

Rotavirus is transmitted by the faecal-oral route, via contact with contaminated hands, surfaces and objects,<sup>[20]</sup> and possibly by the respiratory route.<sup>[1]</sup> The faeces of an infected person can contain more than 10 trillion infectious particles per gram;<sup>[17]</sup> fewer than 100 of these are required to transmit infection to another person.<sup>[3]</sup>

Rotaviruses are stable in the environment and have been found in estuary samples at levels as high as 1–5 infectious particles per US gallon.<sup>[21]</sup> Sanitary measures adequate for eliminating bacteria and parasites seem to be ineffective in control of rotavirus, as the incidence of rotavirus infection in countries with high and low health standards is similar.<sup>[1]</sup>

### Types

There are five species of rotavirus, referred to as A, B, C, D and E. Humans are primarily infected by species A, B and C, most commonly by species A. All five species cause disease in other animals.<sup>[22]</sup> Within rotavirus A there are different strains, called serotypes.<sup>[23]</sup> As with influenza virus, a dual classification system is used based on two proteins on the surface of the virus. The glycoprotein VP7 defines the G serotypes and the protease-sensitive protein VP4 defines P serotypes.<sup>[24]</sup> Because the two genes that determine G-types and P-types can be passed on separately to progeny viruses, different combinations are found.<sup>[25]</sup>

### Replication

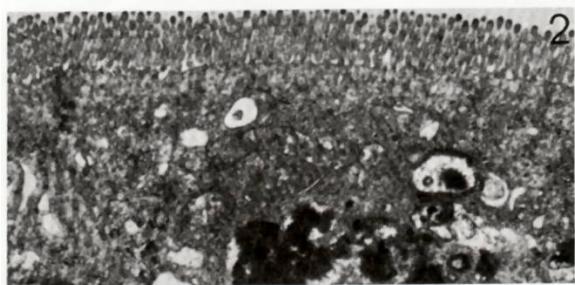
Rotaviruses replicate mainly in the gut,<sup>[26]</sup> and infect enterocytes of the villi of the small intestine, leading to structural and functional changes of the epithelium.<sup>[27]</sup> The triple protein coats make them resistant to the acidic pH of the stomach and the digestive enzymes in the gut.

The virus enters cells by receptor mediated endocytosis and forms a vesicle known as an endosome. Proteins in the third layer (VP7 and the VP4 spike) disrupt the membrane of the endosome, creating a difference in the calcium concentration. This causes the breakdown of VP7 trimers into single protein subunits, leaving the VP2 and VP6 protein coats around the viral dsRNA, forming a double-layered particle (DLP).<sup>[28]</sup>

The eleven dsRNA strands remain within the protection of the two protein shells and the viral RNA-dependent RNA polymerase creates mRNA transcripts of the double-stranded viral genome. By remaining in the core, the viral RNA evades innate host immune responses called RNA interference that are triggered by the presence of double-stranded RNA.

During the infection, rotavirus produces mRNA for both protein biosynthesis and gene replication. Most of the rotavirus proteins accumulate in viroplasm, where the RNA is replicated and the DLPs are assembled. Viroplasm is formed around the cell nucleus as early as two hours after virus infection, and consists of viral factories thought to be made by two viral nonstructural proteins: NSP5 and NSP2. Inhibition of NSP5 by RNA interference results in a sharp decrease in rotavirus replication. The DLPs migrate to the endoplasmic reticulum where they obtain their third, outer layer (formed by VP7 and VP4). The progeny viruses are released from the cell by lysis.<sup>[29][30][31]</sup>

#### 1.4.3 Pathophysiology



Electron micrograph of a rotavirus infected enterocyte (top) compared to an uninfected cell (bottom). The bar = approx. 500 nm

The diarrhoea is caused by multiple activities of the virus. Malabsorption occurs because of the destruction of gut cells called enterocytes. The toxic rotavirus protein NSP4 induces age- and calcium ion-dependent chloride secretion, disrupts SGLT1 transporter-mediated reabsorption of water, apparently reduces activity of brush-border mem-

brane disaccharidases, and possibly activates the calcium ion-dependent secretory reflexes of the enteric nervous system.<sup>[32]</sup> Healthy enterocytes secrete lactase into the small intestine; milk intolerance due to lactase deficiency is a symptom of rotavirus infection,<sup>[33]</sup> which can persist for weeks.<sup>[34]</sup> A recurrence of mild diarrhoea often follows the reintroduction of milk into the child's diet, due to bacterial fermentation of the disaccharide lactose in the gut.<sup>[35]</sup>

#### 1.4.4 Diagnosis

Diagnosis of infection with rotavirus normally follows diagnosis of gastroenteritis as the cause of severe diarrhoea. Most children admitted to hospital with gastroenteritis are tested for rotavirus A.<sup>[36][37]</sup> Specific diagnosis of infection with rotavirus A is made by finding the virus in the child's stool by enzyme immunoassay. There are several licensed test kits on the market which are sensitive, specific and detect all serotypes of rotavirus A.<sup>[38]</sup> Other methods, such as electron microscopy and PCR, are used in research laboratories.<sup>[39]</sup> Reverse transcription-polymerase chain reaction (RT-PCR) can detect and identify all species and serotypes of human rotavirus.<sup>[40]</sup>

#### 1.4.5 Prevention

Because improved sanitation does not decrease the prevalence of rotaviral disease, and the rate of hospitalisations remains high, despite the use of oral rehydrating medicines, the primary public health intervention is vaccination.<sup>[2]</sup> Two rotavirus vaccines against Rotavirus A infection are safe and effective in children:<sup>[13]</sup> Rotarix by GlaxoSmithKline<sup>[41]</sup> and RotaTeq by Merck.<sup>[42]</sup> Both are taken orally and contain attenuated live virus.<sup>[13]</sup>

Rotavirus vaccines are licensed in more than 100 countries, but only 17 countries have introduced routine rotavirus vaccination.<sup>[43]</sup> Following the introduction of routine rotavirus vaccination in the US in 2006, the health burden of rotavirus gastroenteritis "rapidly and dramatically reduced" despite lower coverage levels compared to other routine infant immunizations.<sup>[44]</sup> Clinical trials of the Rotarix rotavirus vaccine in South Africa and Malawi, found that the vaccine significantly reduced severe diarrhoea episodes caused by rotavirus, and that the infection

was preventable by vaccination.<sup>[45]</sup> A 2012 Cochrane review of 41 clinical trials that included 186,263 participants concluded Rotarix and RotaTeq are effective vaccines.<sup>[46]</sup> Additional rotavirus vaccines are under development.<sup>[47]</sup> The World Health Organization(WHO) recommends that rotavirus vaccine be included in all national immunisation programmes.<sup>[48]</sup> The incidence and severity of rotavirus infections has declined significantly in countries that have acted on this recommendation.<sup>[12][13]</sup>

The Rotavirus Vaccine Program is a collaboration between PATH, the (WHO), and the U.S. Centers for Disease Control and Prevention, and is funded by the GAVI Alliance. The Program aims to reduce child morbidity and mortality from diarrhoeal disease by making a vaccine against rotavirus available for use in developing countries.<sup>[49]</sup>

#### 1.4.6 Treatment and prognosis

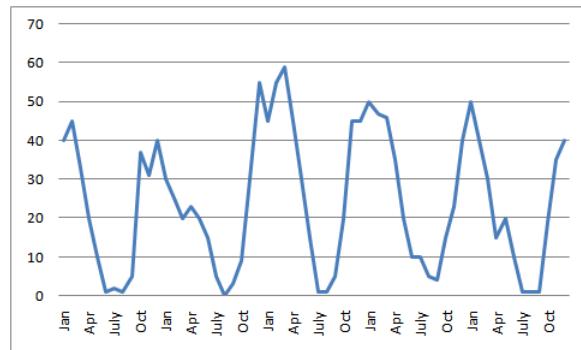
Treatment of acute rotavirus infection is nonspecific and involves management of symptoms and, most importantly, maintenance of hydration.<sup>[11]</sup> If untreated, children can die from the resulting severe dehydration.<sup>[50]</sup> Depending on the severity of diarrhea, treatment consists of oral rehydration, during which the child is given extra water to drink that contains small amounts of salt and sugar.<sup>[51]</sup> Some infections are serious enough to warrant hospitalisation where fluids are given by intravenous drip or nasogastric tube, and the child's electrolytes and blood sugar are monitored.<sup>[36]</sup> Antibiotics are not recommended.

Rotavirus infections rarely cause other complications and for a well managed child the prognosis is excellent.<sup>[52]</sup>

#### 1.4.7 Epidemiology

Rotavirus A, which accounts for more than 90% of rotavirus gastroenteritis in humans,<sup>[53]</sup> is endemic worldwide. Each year rotavirus causes millions of cases of diarrhea in developing countries, almost 2 million resulting in hospitalisation<sup>[7]</sup> and an estimated 453,000 resulting in the death of a child younger than five.<sup>[9]</sup> This is about 40 per cent of all hospital admissions related to diarrhea in children under five worldwide.<sup>[54]</sup>

In the United States alone—before initiation of the ro-



*The seasonal variation of rotavirus A infections in a region of England: rates of infection peak during the winter months.*

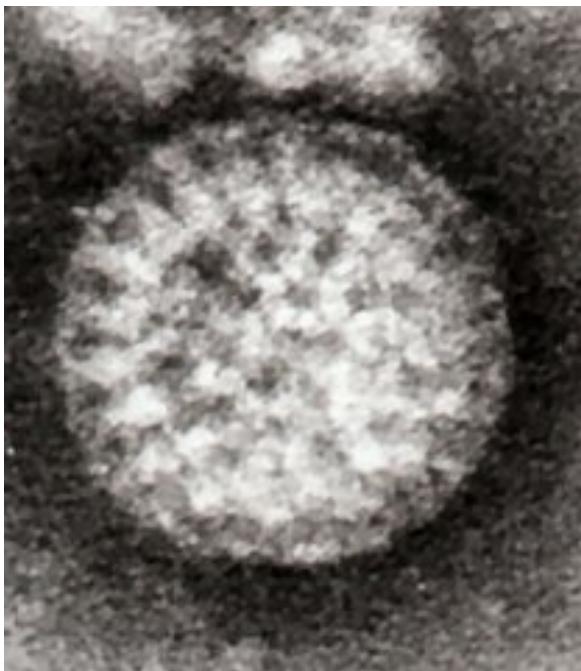
tavirus vaccination programme<sup>[55]</sup>—over 2.7 million cases of rotavirus gastroenteritis occurred annually, 60,000 children were hospitalised and around 37 died from the results of the infection.<sup>[10]</sup> The major role of rotavirus in causing diarrhea is not widely recognised within the public health community,<sup>[56]</sup> particularly in developing countries.<sup>[7]</sup> Almost every child has been infected with rotavirus by age five.<sup>[57]</sup> It is the leading single cause of severe diarrhea among infants and children, being responsible for about 20% of cases, and accounts for 50% of the cases requiring hospitalisation.<sup>[7]</sup> Rotavirus causes 37% of deaths attributable to diarrhea and 5% of all deaths in children younger than five.<sup>[9]</sup> Boys are twice as likely as girls to be admitted to hospital.<sup>[61][58]</sup> Rotavirus infections occur primarily during cool, dry seasons.<sup>[59]</sup> The number attributable to food contamination is unknown.<sup>[60]</sup>

Outbreaks of rotavirus A diarrhea are common among hospitalised infants, young children attending day care centres, and elderly people in nursing homes.<sup>[61]</sup> An outbreak caused by contaminated municipal water occurred in Colorado in 1981.<sup>[62]</sup> During 2005, the largest recorded epidemic of diarrhea occurred in Nicaragua. This unusually large and severe outbreak was associated with mutations in the rotavirus A genome, possibly helping the virus escape the prevalent immunity in the population.<sup>[63]</sup> A similar large outbreak occurred in Brazil in 1977.<sup>[64]</sup>

Rotavirus B, also called adult diarrhea rotavirus or ADRV, has caused major epidemics of severe diarrhea affecting thousands of people of all ages in China. These epidemics

occurred as a result of sewage contamination of drinking water.<sup>[65][66]</sup> Rotavirus B infections also occurred in India in 1998; the causative strain was named CAL. Unlike ADRV, the CAL strain is endemic.<sup>[67][68]</sup> To date, epidemics caused by rotavirus B have been confined to mainland China, and surveys indicate a lack of immunity to this species in the United States.<sup>[69]</sup>

#### 1.4.8 History



One of Flewett's original electron micrographs

In 1943, Jacob Light and Horace Hodes proved that a filterable agent in the faeces of children with infectious diarrhoea also caused scours (livestock diarrhoea) in cattle.<sup>[70]</sup> Three decades later, preserved samples of the agent were shown to be rotavirus.<sup>[71]</sup> In the intervening years, a virus in mice<sup>[72]</sup> was shown to be related to the virus causing scours.<sup>[73]</sup> In 1973, Ruth Bishop and colleagues described related viruses found in children with gastroenteritis.<sup>[5]</sup>

In 1974, Thomas Henry Flewett suggested the name *rotavirus* after observing that, when viewed through an

electron microscope, a rotavirus particle looks like a wheel (*rota* in Latin);<sup>[74][75]</sup> the name was officially recognised by the International Committee on Taxonomy of Viruses four years later.<sup>[76]</sup> In 1976, related viruses were described in several other species of animals.<sup>[73]</sup> These viruses, all causing acute gastroenteritis, were recognised as a collective pathogen affecting humans and animals worldwide.<sup>[74]</sup> Rotavirus serotypes were first described in 1980,<sup>[77]</sup> and in the following year, rotavirus from humans was first grown in cell cultures derived from monkey kidneys, by adding trypsin (an enzyme found in the duodenum of mammals and now known to be essential for rotavirus to replicate) to the culture medium.<sup>[78]</sup> The ability to grow rotavirus in culture accelerated the pace of research, and by the mid-1980s the first candidate vaccines were being evaluated.<sup>[79]</sup>

In 1998, a rotavirus vaccine was licensed for use in the United States. Clinical trials in the United States, Finland, and Venezuela had found it to be 80 to 100% effective at preventing severe diarrhoea caused by rotavirus A, and researchers had detected no statistically significant serious adverse effects.<sup>[80][81]</sup> The manufacturer, however, withdrew it from the market in 1999, after it was discovered that the vaccine may have contributed to an increased risk for intussusception, a type of bowel obstruction, in one of every 12,000 vaccinated infants.<sup>[82]</sup> The experience provoked intense debate about the relative risks and benefits of a rotavirus vaccine.<sup>[83]</sup> In 2006, two new vaccines against rotavirus A infection were shown to be safe and effective in children,<sup>[84]</sup> and in June 2009 the World Health Organization recommended that rotavirus vaccination be included in all national immunisation programmes to provide protection against this virus.<sup>[85]</sup>

#### 1.4.9 Other animals

Rotaviruses infect the young of many species of animals and they are a major cause of diarrhoea in wild and reared animals worldwide.<sup>[8]</sup> As a pathogen of livestock, notably in young calves and piglets, rotaviruses cause economic loss to farmers because of costs of treatment associated with high morbidity and mortality rates.<sup>[86]</sup> These rotaviruses are a potential reservoir for genetic exchange with human rotaviruses.<sup>[86]</sup> There is evidence that animal rotaviruses can infect humans, either by direct transmission of the

virus or by contributing one or several RNA segments to reassortants with human strains.<sup>[87][88]</sup>

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#### 1.4.11 External links

- WHO Rotavirus web page
- CDC About Rotavirus
- Viralzone: Rotavirus
- Rotavirus resource library

## 1.5 NSP1 (rotavirus)

**NSP1**, the product of **rotavirus** gene 5, is an **nonstructural RNA-binding protein** that contains a **cysteine-rich region** and is a component of early replication intermediates. RNA-folding predictions suggest that this region of the **NSP1 mRNA** can interact with itself, producing a stem-loop structure similar to that found near the 5'-terminus of the **NSP1 mRNA**.<sup>[1]</sup>

The carboxyl-half of the **rotavirus** nonstructural protein **NSP1** is not required for virus replication.<sup>[2]</sup>

**NSP1** could play a role in host range restriction.<sup>[3]</sup>

The cysteine-rich region of **NSP1** is not considered essential for genome segment reassortment with heterologous virus.<sup>[4]</sup>

**NSP1** interacts with **IRF3** in the infected cell. **NSP1** is an antagonist of the IFN-signaling pathway.<sup>[5]</sup>

Interferon regulatory factor 3 (**IRF3**) is a key transcription factor involved in the induction of interferon (IFN) in response to viral infection. **NSP1** binds to and targets **IRF3** for proteasome degradation early post-infection. **IRF3** degradation is dependent on the presence of **NSP1** and the integrity of the N-terminal zinc-binding domain, coupled with the regulated stability of **IRF3** and **NSP1** by the proteasome, collectively support the hypothesis that **NSP1** is an E3 ubiquitin ligase.<sup>[6]</sup>

**NSP1** could mediate the degradation of **IRF3**, **IRF5**, and **IRF7** by recognizing a common element of IRF proteins, thereby allowing **NSP1** to act as a broad-spectrum antagonist of IRF function.<sup>[7]</sup>

**NSP1** also inhibits activation of **NFκB**.<sup>[8]</sup>

**NSP1** inhibits cellular apoptosis by directly interacting p85 subunit of PI3K and thus activating PI3K/Akt survival pathway during early stages of rotavirus infection.<sup>[9][10]</sup>

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## 1.6 NSP2 (rotavirus)

**NSP2**, is a rotavirus nonstructural RNA-binding protein that accumulates in cytoplasmic inclusions (viroplasms) and is required for genome replication.<sup>[1]</sup> NSP2 is closely associated in vivo with the viral replicase.<sup>[2]</sup> The non-structural protein **NSP5** plays a role in the structure of viroplasms mediated by its interaction with **NSP2**.<sup>[3]</sup>

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## 1.7 NSP3 (rotavirus)

Rotavirus protein **NSP3** (NS34) is bound to the 3' end consensus sequence of viral mRNAs in infected cells.<sup>[1]</sup>

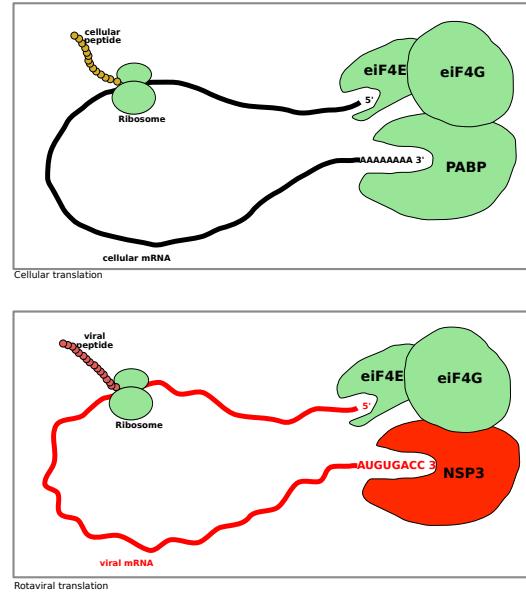
Four nucleotides are the minimal requirement for RNA recognition by rotavirus non-structural protein NSP3: using short oligoribonucleotides, it was established that the minimal RNA sequence required for binding of NSP3A is **GACC**.<sup>[2]</sup>

Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A)-binding protein from eIF4F. And NSP3A, by taking the place of PABP on eIF4GI, is responsible for the shut-off of cellular protein synthesis.<sup>[3]</sup>

Expression of NSP3 in mammalian cells allows the efficient translation of virus-like mRNA: NSP3 forms a link between viral mRNA and the cellular translation machinery and hence is a functional analogue of cellular poly(A)-binding protein.<sup>[4]</sup>

Site-directed mutagenesis and isothermal titration calorimetry documented that NSP3 and PABP use analogous eIF4G recognition strategies, despite marked differences in tertiary structure.<sup>[5]</sup>

Using the yeast two-hybrid assay, RoXan a novel cellular protein was found to bind NSP3. The interaction between NSP3 and RoXaN does not impair the interaction between NSP3 and eIF4GI, and a ternary complex made of NSP3, RoXaN, and eIF4G I can be detected in rotavirus-infected cells, implicating RoXaN in translation regulation.<sup>[6]</sup>



#### *Cellular vs Rotavirus Translation*

##### 1.7.1 References

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PMID 15047801.

## 1.8 NSP4 (rotavirus)

The rotavirus nonstructural protein **NSP4** was the first viral enterotoxin discovered. It induces diarrhea and causes  $\text{Ca}^{2+}$ -dependent transepithelial secretion.<sup>[1]</sup>

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## 1.9 NSP5 (rotavirus)

**NSP5** (nonstructural protein 5) encoded by genome segment 11 of group A rotaviruses. In virus-infected cells NSP5 accumulates in the viroplasms. NSP5 has been shown to be autophosphorylated.<sup>[1]</sup> Interaction of NSP5 with NSP2 was also demonstrated.<sup>[2]</sup> In rotavirus-infected cells, the non-structural proteins NSP5 and NSP2 localize in complexes called viroplasms, where replication and assembly occur and they can drive the formation of viroplasm-like structures in the absence of other rotaviral proteins and rotavirus replication.<sup>[3]</sup>

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## 1.10 NSP6 (rotavirus)

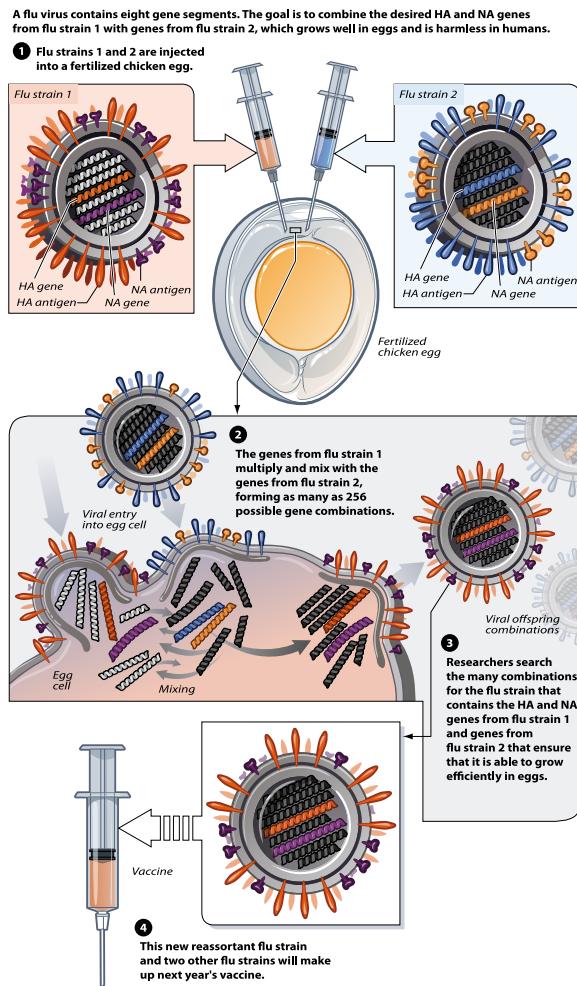
Gene 11 of Rotavirus encodes a nonstructural protein, NSP5 and also encodes NSP6, from an out of phase open reading frame.<sup>[1]</sup> In contrast to the other rotavirus non-structural proteins, NSP6 was found to have a high rate of turnover, being completely degraded within 2h of synthesis. NSP6 was found to be a sequence independent nucleic acid binding protein, with similar affinities for ssRNA and dsRNA.<sup>[2]</sup>

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## 1.11 Reassortment

**Reassortment** is the mixing of the genetic material of a species into new combinations in different individuals. Several different processes contribute to reassortment, including assortment of chromosomes, and chromosomal crossover.<sup>[1]</sup> It is particularly used when two similar viruses that are infecting the same cell exchange genetic material. In particular, reassortment occurs among influenza viruses, whose genomes consist of eight distinct segments of RNA. These segments act like mini-chromosomes, and each time a flu virus is assembled, it requires one copy of each segment.



The process of reassortment in biotechnology

If a single host (a human, a chicken, or other animal) is infected by two different strains of the influenza virus, then it is possible that new assembled viral particles will be created from segments whose origin is mixed, some coming from one strain and some coming from another. The new reassortant strain will share properties of both of its parental lineages.

Reassortment is responsible for some of the major genetic shifts in the history of the influenza virus. The 1957 and 1968 pandemic flu strains were caused by reassortment be-

tween an avian virus and a human virus, whereas the H1N1 virus responsible for the 2009 swine flu outbreak has an unusual mix of swine, avian and human influenza genetic sequences.<sup>[2]</sup>

### 1.11.1 See also

- Antigenic shift(antigenic drift)

### 1.11.2 References

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  - [2] “Deadly new flu virus in US and Mexico may go pandemic”. *New Scientist*. 2009-04-24. Retrieved 2009-04-26.
- History of April-2009 flu collected by Bionty.

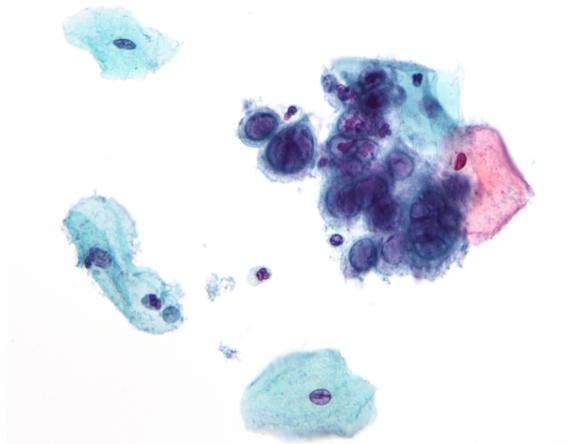
### 1.11.3 External links

- An animation from hhmi.org illustrating the process
- Hood E (February 2006). “Flu Vaccine Production Gets a Shot in the Arm”. *Environ Health Perspect* **114** (2): A108-11. doi:10.1289/ehp.114-a108. PMC 1367863. PMID 16451835.

## 1.12 Cytopathic effect

**Cytopathic effect** or **cytopathogenic effect** (abbreviated **CPE**) refers to damage to host cells during virus invasion. This damage is measurable by obtaining viral titers. Degenerative changes in **cells** can also be studied in **tissue culture**.

When in tissue culture the spread of virus is restricted by an overlay of **agar** or other suitable substance. This barrier means the cytopathic effect may lead to the formation of a **viral plaque**.<sup>[1]</sup> Thus identification of a viral infection can be made by examining the characteristic cytopathic effect produced on different cell sheets. This method however is relatively slow and not all viruses will grow on cell sheets.



HCoV-229E and HCoV-OC43, by an Indirect Immunoperoxidase Assay. SARS- and Other Coronaviruses : Laboratory Protocols. Series: Methods in Molecular Biology. Volume: 454. Pub. Date: May-01-2008. 93-102. DOI: 10.1007/978-1-59745-181-9\_8

*Micrograph showing the viral cytopathic effect of herpes simplex virus (multi-nucleation, ground glass chromatin). Pap test. Pap stain.*

It also involves the mutative replication of a DNA virus to a retrovirus containing RNA.

Cytopathic effects have been shown in conjunction with non-viral infections as well, such as those changes seen in fibroblasts during the early lesion of periodontal disease.<sup>[2]</sup>

Not all viruses cause a measurable cytopathic effect. These include two human coronaviruses HCoV-229E and HCoV-OC43. <sup>[3]</sup>

### 1.12.1 See also

- Viral culture
- Indirect immunoperoxidase assay

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- [3] Francine Lambert, Hélène Jacomy<sup>1</sup> Gabriel Marceau<sup>1</sup>, Pierre J Talbot<sup>\*1</sup> Titration of Human Coronaviruses,

# Chapter 2

## Rotaviral Mechanisms

### 2.1 Sodium-glucose transport proteins

Sodium-dependent glucose cotransporters (or sodium-glucose linked transporter, SGLT) are a family of glucose transporter found in the intestinal mucosa (enterocytes) of the small intestine (SGLT1) and the proximal tubule of the nephron (SGLT2 in PCT and SGLT1 in PST). They contribute to renal glucose reabsorption. In the kidneys, 100% of the filtered glucose in the glomerulus has to be reabsorbed along the nephron (98% in PCT, via SGLT2). In case of too high plasma glucose concentration (hyperglycemia), glucose is excreted in urine (glucosuria); because SGLT are saturated with the filtered monosaccharide. Glucose is never secreted by the nephron.

#### 2.1.1 Types

The two most well known members of SGLT family are SGLT1 and SGLT2, which are members of the SLC5A gene family.

Including SGLT1 and SGLT2, there are total seven members in the human protein family SLC5A, several of which may also be sodium-glucose transporters.<sup>[3]</sup>

#### 2.1.2 SGLT2 inhibitors for diabetes

Inhibition of SGLT2 leads to a reduction in blood glucose levels. Therefore, SGLT2 inhibitors have potential use in

the treatment of type II diabetes. Several drug candidates have been developed or are currently undergoing clinical trials, including:<sup>[4]</sup>

- Dapagliflozin, approval rejected in 2012 by Food and Drug Administration due to safety concerns however after resubmitting additional clinical data is under review with Dec 12, 2013 as PDUFA Date,<sup>[5]</sup> but marketed in Europe and Australia. Dapagliflozin was the first SGLT2 approved anywhere in the world in 2011 by the EU.
- Canagliflozin, approved in the United States and Canada<sup>[6]</sup>
- Ipragliflozin (ASP-1941), in Phase III clinical trials<sup>[7]</sup>
- Tofogliflozin, in Phase III clinical trials<sup>[7]</sup>
- Empagliflozin (BI-10773), in Phase III clinical trials<sup>[7]</sup>
- Sergliflozin etabonate, discontinued after Phase II trials
- Remoglitiflozin etabonate, in phase IIb trials

#### 2.1.3 Function

Firstly, the Na+/K+ ATPase pump on the basolateral membrane of the proximal tubule cell uses ATP to move 3 sodium ions outward into the blood, while bringing in 2 potassium ions. This creates a downhill sodium ion gradient inside the proximal tubule cell in comparison to both

the blood and the tubule. The SGLT proteins use the energy from this downhill sodium ion gradient created by the ATPase pump to transport glucose across the apical membrane against an uphill glucose gradient. Therefore, these co-transporters are an example of secondary active transport. (The GLUT uniports then transport the glucose across the basolateral membrane, into the peritubular capillaries.) Both SGLT1 and SGLT2 are known as symporters, since both sodium ions and glucose are transported in the same direction across the membrane.

### 2.1.4 Discovery of sodium-glucose cotransport

In August 1960, in Prague, Robert K. Crane presented for the first time his discovery of the sodium-glucose cotransport as the mechanism for intestinal glucose absorption.<sup>[8]</sup>

Crane's discovery of cotransport was the first-ever proposal of flux coupling in biology.<sup>[9][10]</sup>

### 2.1.5 See also

- Cotransport
- Cotransporter
- Glucose-galactose malabsorption
- Renal sodium reabsorption

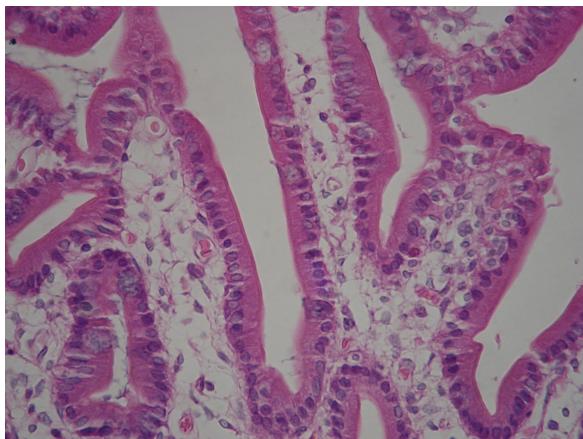
### 2.1.6 References

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- [10] Boyd CA (March 2008). "Facts, fantasies and fun in epithelial physiology". *Exp. Physiol.* **93** (3): 303–14. doi:10.1113/expphysiol.2007.037523. PMID 18192340. "p. 304. "the insight from this time that remains in all current text books is the notion of Robert Crane published originally as an appendix to a symposium paper published in 1960 (Crane et al. 1960). The key point here was 'flux coupling', the cotransport of sodium and glucose in the apical membrane of the small intestinal epithelial cell. Half a century later this idea has turned into one of the most studied of all transporter proteins (SGLT1), the sodium–glucose cotransporter."

### 2.1.7 External links

- Sodium-Glucose Transport Proteins at the US National Library of Medicine Medical Subject Headings (MeSH)

## 2.2 Brush border



Duodenum with brush border (microvillus)

A **brush border** (or **striated border** or **brush border membrane**) is the name for the microvilli-covered surface of **simple cuboidal epithelium** and **simple columnar epithelium** cells found in certain locations of the body. Microvilli are approximately 100 nanometers in diameter and their length varies from approximately 100 to 2,000 nanometers in length. Because individual microvilli are so small and are tightly packed in the brush border, individual microvilli can only be resolved using electron microscopes;<sup>[1]</sup> with a **light microscope** they can usually only be seen collectively as a fuzzy fringe at the surface of the epithelium. This fuzzy appearance gave rise to the term **brush border**, as early anatomists noted that this structure appeared very much like the bristles of a paintbrush.

**Brush border cells** are found in two main locations:

- The **small intestine tract**: This is where absorption takes place.<sup>[2][3][4]</sup> The brush borders of the intestinal lining are the site of terminal carbohydrate digestions. The microvilli that constitute the brush border have enzymes for this final part of digestion anchored into their apical plasma membrane as integral membrane proteins. These enzymes are found near to the transporters that will then allow absorption of the digested nutrients.

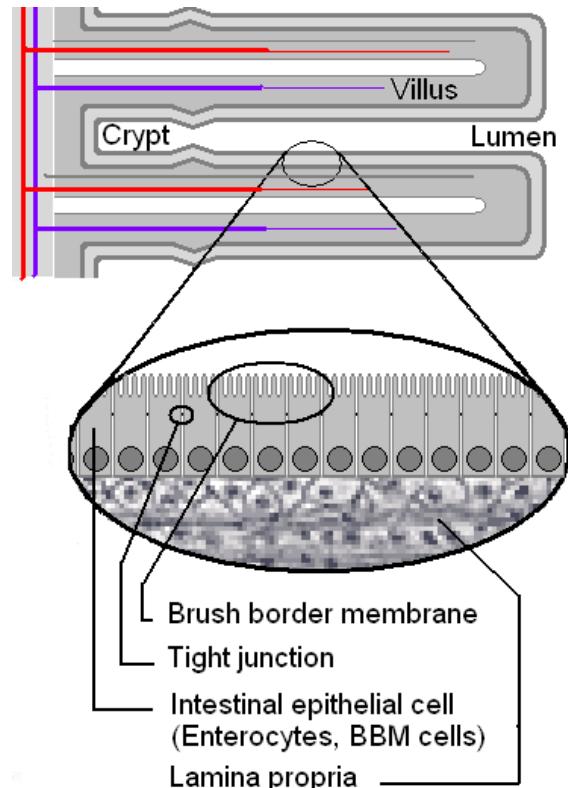


Illustration of the brush border membrane of small intestinal villi

- The **kidney**: Here the brush border is useful in distinguishing the **proximal tubule** (which possesses the brush border) from the **distal tubule** (which does not).<sup>[5][6]</sup>

The brush border morphology increases a cell's **surface area**, a trait which is especially useful in absorptive cells. Cells that absorb substances need a large surface area in contact with the substance to be efficient.<sup>[7]</sup>

In intestinal cells, the microvilli are referred to as **striated border**, while in the kidneys, microvilli are referred to as **brush border**.<sup>[8]</sup>

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- [3] Histology image:11703loa from Vaughan, Deborah (2002). *A Learning System in Histology: CD-ROM and Guide.* Oxford University Press. ISBN 978-0195151732.
- [4] Basic Histology - Intestinal Columnar Epithelium
- [5] Histology at OU 35\_19 - Kidney
- [6] Histology at KUMC *urinary-renal13* "Tubules"
- [7] Southern Illinois School of Medicine: Specialized GI Cells
- [8] Ross, Michael H. *Histology : a text and atlas / Michael H. Ross, Wojciech Pawlina., –5th ed.* p 102.

the Global Alliance for Vaccines and Immunization.<sup>[8]</sup> It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system.<sup>[9]</sup>

### 2.3.1 Rotarix

Rotarix is a monovalent, human, live attenuated rotavirus vaccine containing one rotavirus strain of G1P[8] specificity. ROTARIX is indicated for the prevention of rotavirus gastroenteritis caused by G1 and non-G1 types (G3, G4, and G9) when administered as a 2-dose series in infants and children.<sup>[10]</sup> On March 22, 2010, FDA provided an early communication regarding Rotarix, manufactured by GlaxoSmithKline Biologicals (GSK). At that time, FDA recommended that clinicians and public health professionals in the United States temporarily suspend the use of Rotarix while the agency and manufacturer investigated the finding of DNA from porcine circovirus-1 (PCV1) in the vaccine. Since that time, both FDA and GSK have confirmed the presence of PCV1 in the vaccine.<sup>[11]</sup> Although this contamination was thought to be benign, vaccines are supposed to be sterile. In May 2010 the suspension of the vaccine was lifted.<sup>[12]</sup>

### 2.3.2 RotaTeq

RotaTeq is a live, oral pentavalent vaccine that contains five rotavirus strains produced by reassortment. The rotavirus A parent strains of the reassortants were isolated from human and bovine hosts. Four reassortant rotaviruses express one of the outer capsid, VP7, proteins (serotypes G1, G2, G3, or G4) from the human rotavirus parent strain and the attachment protein VP4 (type P7) from the bovine rotavirus parent strain. The fifth reassortant virus expresses the attachment protein VP4, (type P1A), from the human rotavirus parent strain and the outer capsid protein VP7 (serotype G6) from the bovine rotavirus parent strain. In February 2006, the U.S. Food and Drug Administration approved RotaTeq for use in the United States. In August 2006, Health Canada approved RotaTeq for use in Canada.<sup>[13]</sup> Merck is working with a range of partners including governmental and non-governmental organisations to develop and implement mechanisms for providing access

## 2.3 Rotavirus vaccine

A **rotavirus vaccine** protects children from rotaviruses, which are the leading cause of severe diarrhea among infants and young children.<sup>[1]</sup> Each year an estimated 453,000 children die from diarrhoeal disease caused by rotavirus,<sup>[2]</sup> most of whom live in developing countries,<sup>[3]</sup> and another two million are hospitalised.<sup>[4]</sup> Rotavirus is highly contagious and resistant and, regardless of water quality and available sanitation, nearly every child in the world is at risk of infection.<sup>[5]</sup>

There are two effective rotavirus vaccines: Rotarix by GlaxoSmithKline and RotaTeq by Merck.<sup>[6]</sup> A vaccine has recently been developed in India. Phase III trials have been conducted for the vaccine.<sup>[7]</sup>

On June 5, 2009, the World Health Organization (WHO) recommended that rotavirus vaccine be included in all national immunization programs. The *Rotavirus Vaccine Program* and the Accelerated Vaccine Introduction initiative have worked to study rotavirus vaccines among developing-country populations to assist developing countries in introducing rotavirus vaccines into routine immunization programs. These partnerships are spearheaded by international non-governmental organization PATH, WHO, the U.S. Centers for Disease Control and Prevention, and



*H. Fred Clark and Paul Offit, the inventors of RotaTeq.*

to this vaccine in the developing world.<sup>[14]</sup>

### Temporary suspension

On March 22, 2010, the detection of DNA from porcine circovirus types 1 and 2 within RotaTeq and Rotarix prompted the FDA to suspend the use of rotavirus vaccines while conducting an investigation in collaboration with the 12 members of the Vaccines and Related Biological Products Advisory Committee (VRBPAC).<sup>[11]</sup> On May 6, 2010, the FDA announced its decision to revoke the suspension, asserting that porcine circovirus types 1 and 2 pose no safety risks in humans and concluded that health risks involved did not offset the benefits of the vaccination.<sup>[11]</sup>

### 2.3.3 Rotavac

An Indian-made rotavirus vaccine, Rotavac, was developed and announced in 2013. If approved by the Drugs Controller General of India, it would be available at Rs. 54 per dose, more affordable than the two vaccines now available costing more than Rs. 1,000 per dose.<sup>[15]</sup>

### 2.3.4 Vaccines in development

Other vaccines are undergoing clinical trials. These include: a human neonatal P[6]G3 strain, RV3, developed by Ruth Bishop and colleagues in Australia, a human bovine reassortant vaccine developed by Albert Kapikian and presently undergoing development and trials in different countries and a neonatal strain vaccine (G9P11) being developed by Bharat Biotech in India. This G9P(11) virus strain has the VP4 of bovine rotavirus origin, and all other segments of human rotavirus origin. Other approaches to the development of rotavirus vaccines are also being pursued. Rotavirus antigens for parenteral delivery have received some attention as virus-like particles prepared in baculovirus, expressed antigens, DNA vaccines, and killed virus. These novel approaches are being pursued using animal models.<sup>[16]</sup>

### 2.3.5 Effectiveness and cost

A 2009 review estimated that vaccination against rotavirus would prevent about 45% of deaths due to rotavirus gastroenteritis, or about 228,000 deaths annually worldwide. At \$5 per dose the estimated cost per life saved was \$3,015, \$9,951 and \$11,296 in low-, lower-middle-, and upper-middle-income countries, respectively.<sup>[17]</sup> The cost of rotavirus vaccination has fallen by 67 percent between 2006 and 2011 to USD 2.50 per dose,<sup>[18]</sup> as part of an offer made by a pharmaceutical company to the GAVI Alliance. However, the vaccine is still more expensive than most other childhood vaccines included in the WHO's Expanded Programme on Immunization.<sup>[19]</sup>

Safety and efficacy trials of Rotarix and RotaTeq in Africa and Asia found that the vaccines dramatically reduced severe disease among infants in developing countries, where a majority of rotavirus-related deaths occur.<sup>[20]</sup> A 2012 Cochrane review of 41 clinical trials that included 186,263 participants concluded Rotarix and RotaTeq are effective vaccines.<sup>[6]</sup> Additional rotavirus vaccines are under development.<sup>[21]</sup>

Rotavirus vaccines are licensed in more than 100 countries, but only 31<sup>[22]</sup> countries have introduced routine rotavirus vaccination as of 2011.<sup>[23]</sup> The incidence and severity of rotavirus infections has declined significantly in coun-

tries that have acted on the recommendation to introduce the rotavirus vaccine.<sup>[24]</sup> In Mexico, which in 2006 was among the first countries in the world to introduce rotavirus vaccine, the diarrheal disease death rates from rotavirus dropped by more than 65% among children age two and under during the 2009 rotavirus season.<sup>[25]</sup> In Nicaragua, which in 2006 became the first developing country to introduce the rotavirus vaccine, investigators recorded a substantial impact, with rotavirus vaccine preventing 60% of cases against severe rotavirus and cutting emergency room visits in half.<sup>[26]</sup> In the United States, vaccination has reduced rotavirus-related hospitalizations by as much as 86% since 2006. The vaccines may also prevent illness in non-vaccinated children by limiting exposure through the number of circulating infections.<sup>[5]</sup> In September 2013, the vaccine will be offered to all children in the UK, aged between two and three months, and it is expected to halve the cases of severe infection and reduce the number of children admitted to hospital because of the infection by 70 percent.<sup>[27]</sup>

### 2.3.6 History

In 1998, a rotavirus vaccine (RotaShield, by Wyeth) was licensed for use in the United States. Clinical trials in the United States, Finland, and Venezuela had found it to be 80 to 100% effective at preventing severe diarrhea caused by rotavirus A, and researchers had detected no statistically significant serious adverse effects. The manufacturer of the vaccine, however, withdrew it from the market in 1999, after it was discovered that the vaccine may have contributed to an increased risk for intussusception, or bowel obstruction, in one of every 12,000 vaccinated infants. There then followed 8 years delay until rival manufacturers were able to introduce new vaccines which due to different adjuvant were shown to be more safe and effective in children: Rotarix by GlaxoSmithKline<sup>[10]</sup> and RotaTeq by Merck.<sup>[28]</sup> Both are taken orally and contain disabled live virus.

The experience, however, provoked debate about the relative risks and benefits of a rotavirus vaccine. This is because rotavirus is responsible for 29% of deaths from diarrhea in children below 5 years worldwide, and thus 4.2 million deaths could be avoided during that 8 years in different parts of the world. Meanwhile, other countries such as Brazil and Mexico undertook their own independent

epidemiological studies which demonstrated that 4 deaths were attributable to vaccine, while it had prevented approximately 80,000 hospitalization and 1300 deaths from diarrhea each year in their countries.<sup>[29]</sup> This example shed light on the importance of careful country-specific epidemiology which enables rational balancing between benefit and risk.

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### 2.3.8 External links

- DefeatDD.org
- National Clearinghouse Guideline: *Prevention of rotavirus gastroenteritis among infants and children. Recommendations of the Advisory Committee on Immunization Practices (ACIP)*.
- PATH Rotavirus Vaccine Program
- Vaccine Information Statement from the US Centers for Disease Control and Prevention (as of December 1, 2007, available in English, Spanish, Somali, and Thai)

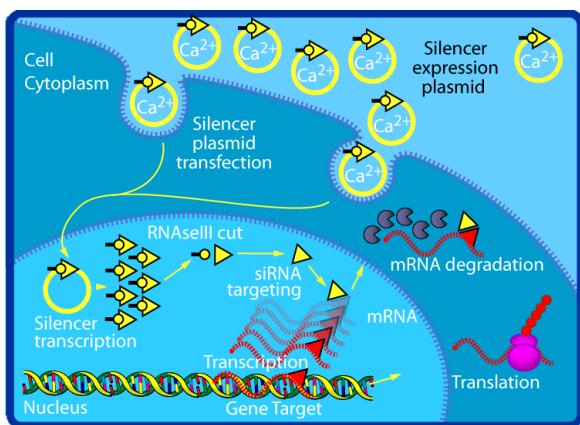
- Vaccine Resource Library: Rotavirus

# Chapter 3

## siRNAs

### 3.1 Small interfering RNA

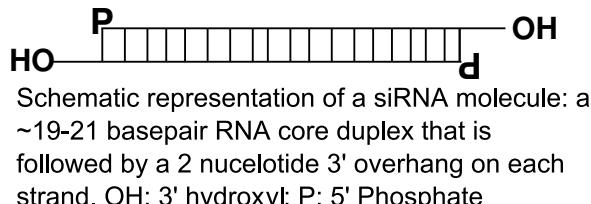
See also *RNA interference*



**Small interfering RNA (siRNA)**, sometimes known as **short interfering RNA or silencing RNA**, is a class of **double-stranded RNA molecules**, 20-25 base pairs in length. siRNA plays many roles, but it is most notable in the **RNA interference (RNAi) pathway**, where it interferes with the **expression of specific genes** with complementary nucleotide sequences. siRNA functions by causing mRNA to be broken down after transcription,<sup>[1]</sup> resulting in no translation. siRNA also acts in RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the **chromatin structure** of a genome. The complexity of these pathways is only now being elucidated.

siRNAs and their role in post-transcriptional gene silencing (PTGS) in plants were first discovered by David Baulcombe's group at the Sainsbury Laboratory in Norwich, England and reported in *Science* in 1999.<sup>[2]</sup> Thomas Tuschl and colleagues soon reported in *Nature* that synthetic siRNAs could induce RNAi in mammalian cells.<sup>[3]</sup> This discovery led to a surge in interest in harnessing RNAi for biomedical research and drug development.

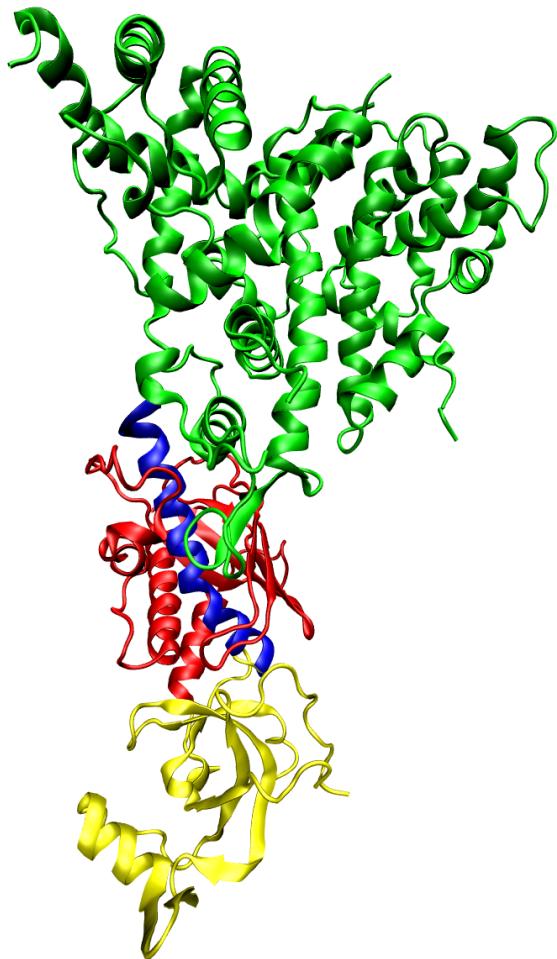
#### 3.1.1 Structure



Schematic representation of a siRNA molecule: a ~19-21 basepair RNA core duplex that is followed by a 2 nucleotide 3' overhang on each strand. OH: 3' hydroxyl; P: 5' Phosphate

siRNAs have a well-defined structure: a short (usually 20 to 24-bp) double-stranded RNA (dsRNA) with **phosphorylated 5' ends** and **hydroxylated 3' ends** with two overhanging nucleotides. The **Dicer enzyme** catalyzes production of siRNAs from long dsRNAs and small hairpin RNAs.<sup>[4]</sup> siRNAs can also be introduced into cells by **transfection**. Since in principle any gene can be **knocked down** by a synthetic siRNA with a complementary sequence, siRNAs are an important tool for validating gene function and drug targeting in the post-genomic era.

### 3.1.2 RNAi induction using siRNAs or their biosynthetic precursors



*Dicer protein colored by protein domain.*

Gene knockdown by transfection of exogenous siRNA is often unsatisfactory because the effect is only transient, especially in rapidly dividing cells. This may be overcome by creating an expression vector for the siRNA. The siRNA sequence is modified to introduce a short loop between the two strands. The resulting transcript is a short hairpin RNA (shRNA), which can be processed into a functional siRNA by Dicer in its usual fashion.. Typical transcription cassettes use an RNA polymerase III promoter (e.g., U6 or H1) to di-

rect the transcription of small nuclear RNAs (snRNAs) (U6 is involved in gene splicing; H1 is the RNase component of human RNase P). It is theorized that the resulting siRNA transcript is then processed by Dicer.

The gene knockdown efficiency can also be improved by using **Cell squeezing**, a high-throughput vector-free microfluidic platform for intracellular delivery developed at the Massachusetts Institute of Technology in the labs of Robert S. Langer.<sup>[5]</sup>

The activity of siRNAs in RNAi is largely dependent on its binding ability to the RNA-induced silencing complex (RISC). Binding of the duplex siRNA to RISC is followed by unwinding and cleavage of the sense strand with endonucleases. The remaining anti-sense strand-RISC complex can then bind to target mRNAs for initiating transcriptional silencing.<sup>[6]</sup>

### 3.1.3 RNA activation

It has recently been found that dsRNA can also activate gene expression, a mechanism that has been termed “small RNA-induced gene activation” or **RNAa**. It has been shown that dsRNAs targeting gene promoters induce potent transcriptional activation of associated genes. RNAa was demonstrated in human cells using synthetic dsRNAs, termed “small activating RNAs” (saRNAs). It is currently not known whether RNAa is conserved in other organisms.<sup>[7]</sup>

### 3.1.4 Challenges: avoiding nonspecific effects

Because RNAi intersects with a number of other pathways, it is not surprising that on occasion nonspecific effects are triggered by the experimental introduction of an siRNA. When a mammalian cell encounters a double-stranded RNA such as an siRNA, it may mistake it as a viral by-product and mount an immune response. Furthermore, because structurally related **microRNAs** modulate gene expression largely via incomplete complementarity base pair interactions with a target **mRNA**, the introduction of an siRNA may cause unintended off-targeting.

### Innate immunity

Introduction of too much siRNA can result in nonspecific events due to activation of innate immune responses.<sup>[8]</sup> Most evidence to date suggests that this is probably due to activation of the dsRNA sensor PKR, although retinoic acid-inducible gene I (RIG-I) may also be involved. The induction of cytokines via toll-like receptor 7 (TLR7) has also been described. One promising method of reducing the nonspecific effects is to convert the siRNA into a microRNA. MicroRNAs occur naturally, and by harnessing this endogenous pathway it should be possible to achieve similar gene knockdown at comparatively low concentrations of resulting siRNAs. This should minimize nonspecific effects.

### Off-targeting

Off-targeting is another challenge to the use of siRNAs as a gene knockdown tool. Here, genes with incomplete complementarity are inadvertently downregulated by the siRNA (in effect, the siRNA acts as a miRNA), leading to problems in data interpretation and potential toxicity. This, however, can be partly addressed by designing appropriate control experiments, and siRNA design algorithms are currently being developed to produce siRNAs free from off-targeting. Genome-wide expression analysis, e.g., by microarray technology, can then be used to verify this and further refine the algorithms. A 2006 paper from the laboratory of Dr. Khvorova implicates 6- or 7-basepair-long stretches from position 2 onward in the siRNA matching with 3'UTR regions in off-targeted genes.<sup>[9]</sup>

### 3.1.5 Therapeutic applications and challenges

Given the ability to knock down, in essence, any gene of interest, RNAi via siRNAs has generated a great deal of interest in both basic<sup>[10]</sup> and applied biology. There are an increasing number of large-scale RNAi screens that are designed to identify the important genes in various biological pathways. Because disease processes also depend on the activity of multiple genes, it is expected that in some situations turning off the activity of a gene with an siRNA could

produce a therapeutic benefit.

However, applying RNAi via siRNAs to living animals, especially humans, poses many challenges. Under experiments, siRNAs show different effectiveness in different cell types in a manner as yet poorly understood: Some cells respond well to siRNAs and show a robust knockdown, whereas others show no such knockdown (even despite efficient transfection).

Phase I results of the first two therapeutic RNAi trials (indicated for age-related macular degeneration, aka AMD) reported at the end of 2005 that siRNAs are well tolerated and have suitable pharmacokinetic properties.<sup>[11]</sup>

In a phase 1 clinical trial, 41 patients with advanced cancer metastasised to liver were administered with RNAi delivered through lipid nanoparticles. The RNAi targeted two genes encoding key proteins in the growth of the cancer cells, vascular endothelial growth factor, (VEGF), and kinesin spindle protein (KSP). The results showed clinical benefits, with the cancer either stabilized after six months or regression of metastasis in some of the patients. Pharmacodynamics analysis of biopsy samples from the patients revealed the presence of the RNAi constructs in the samples, proving that the molecules reached the intended target.<sup>[12][13]</sup>

Proof of concept trials have indicated that Ebola-targeted siRNAs may be effective as post-exposure prophylaxis in humans, with 100% of non-human primates surviving a lethal dose of Zaire Ebolavirus, the most lethal strain.<sup>[14]</sup>

### 3.1.6 See also

- Oligonucleotide synthesis
- EsiRNA
- NatsiRNA
- MicroRNA
- Viroid
- RNA interference

### 3.1.7 References

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### 3.1.8 Further reading

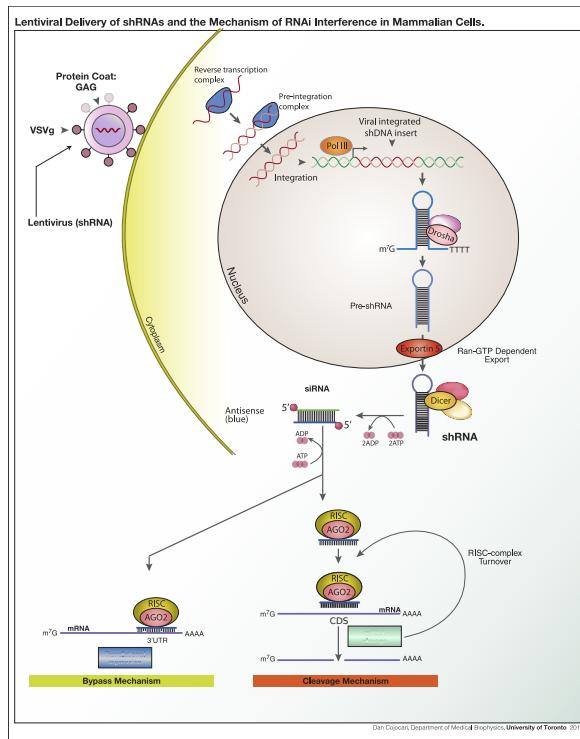
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### 3.1.9 External links

RNAiAtlas provides a siRNA oligonucleotide data from different sources and companies like Dharmacon (ThermoFisher), Qiagen, Ambion, esiRNA for human from Sigma Life Science, and visualize interactions between siRNA oligo and predicted off-target.

- RNAAtlas: a database of RNAi Libraries and their target analysis
- An animation of the mechanism of siRNA by Nature Reviews Genetics can be found [HERE](#)
- DesiRM: Designing of Complementary and Mismatch siRNAs for Silencing a Gene .

## 3.2 RNA interference



*Lentiviral delivery of designed shRNA's and the mechanism of RNA interference in mammalian cells.*

**RNA interference (RNAi)** is a biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules. Historically, it was known by other names, including *co-suppression*, *post transcriptional gene silencing* (PTGS), and *quelling*. Only after these apparently unrelated processes were fully understood did it become clear that they

all described the RNAi phenomenon. Andrew Fire and Craig C. Mello shared the 2006 Nobel Prize in Physiology or Medicine for their work on RNA interference in the nematode worm *Caenorhabditis elegans*, which they published in 1998.

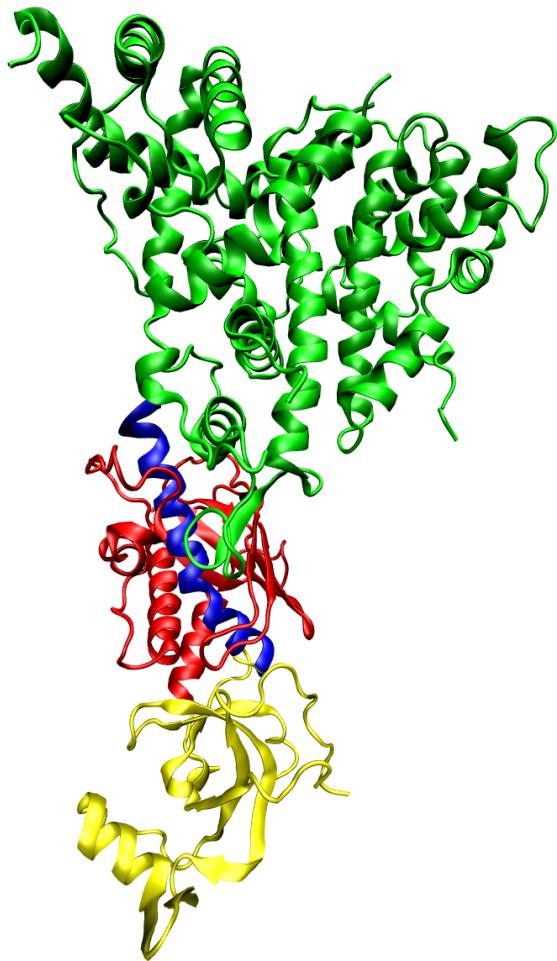
Two types of small ribonucleic acid (RNA) molecules – microRNA (miRNA) and small interfering RNA (siRNA) – are central to RNA interference. RNAs are the direct products of genes, and these small RNAs can bind to other specific messenger RNA (mRNA) molecules and either increase or decrease their activity, for example by preventing an mRNA from producing a protein. RNA interference has an important role in defending cells against parasitic nucleotide sequences – viruses and transposons. It also influences development.

The RNAi pathway is found in many eukaryotes including animals and is initiated by the enzyme Dicer, which cleaves long double-stranded RNA (dsRNA) molecules into short double stranded fragments of ~20 nucleotide siRNAs. Each siRNA is unwound into two single-stranded (ss) ssRNAs, respectively the passenger strand and the guide strand. The passenger strand is degraded and the guide strand is incorporated into the RNA-induced silencing complex (RISC). The most well-studied outcome is post-transcriptional gene silencing, which occurs when the guide strand pairs with a complementary sequence in a messenger RNA molecule and induces cleavage by Argonaute, the catalytic component of the RISC complex. In some organisms, this process spreads systemically, despite the initially limited molar concentrations of siRNA.

RNAi is a valuable research tool, both in cell culture and in living organisms, because synthetic dsRNA introduced into cells can selectively and robustly induce suppression of specific genes of interest. RNAi may be used for large-scale screens that systematically shut down each gene in the cell, which can help to identify the components necessary for a particular cellular process or an event such as cell division. The pathway is also used as a practical tool in biotechnology, medicine and insecticides.<sup>[1]</sup>

### 3.2.1 Cellular mechanism

RNAi is an RNA-dependent gene silencing process that is controlled by the RNA-induced silencing complex (RISC)



The dicer protein from *Giardia intestinalis*, which catalyzes the cleavage of dsRNA to siRNAs. The RNase domains are colored green, the PAZ domain yellow, the platform domain red, and the connector helix blue.<sup>[2]</sup>

and is initiated by short double-stranded RNA molecules in a cell's cytoplasm, where they interact with the catalytic RISC component argonaute.<sup>[3]</sup> When the dsRNA is exogenous (coming from infection by a virus with an RNA genome or laboratory manipulations), the RNA is imported directly into the cytoplasm and cleaved to short fragments by Dicer. The initiating dsRNA can also be endogenous (originating in the cell), as in pre-microRNAs expressed from RNA-coding genes in the genome. The primary tran-

scripts from such genes are first processed to form the characteristic stem-loop structure of pre-miRNA in the nucleus, then exported to the cytoplasm. Thus, the two dsRNA pathways, exogenous and endogenous, converge at the RISC complex.<sup>[4]</sup>

### dsRNA cleavage

Endogenous dsRNA initiates RNAi by activating the ribonuclease protein Dicer,<sup>[5]</sup> which binds and cleaves double-stranded RNAs (dsRNAs) to produce double-stranded fragments of 20–25 base pairs with a 2-nucleotide overhang at the 3' end.<sup>[6]</sup> Bioinformatics studies on the genomes of multiple organisms suggest this length maximizes target-gene specificity and minimizes non-specific effects.<sup>[7]</sup> These short double-stranded fragments are called small interfering RNAs (siRNAs). These siRNAs are then separated into single strands and integrated into an active RISC complex. After integration into the RISC, siRNAs base-pair to their target mRNA and cleave it, thereby preventing it from being used as a translation template.<sup>[8]</sup>

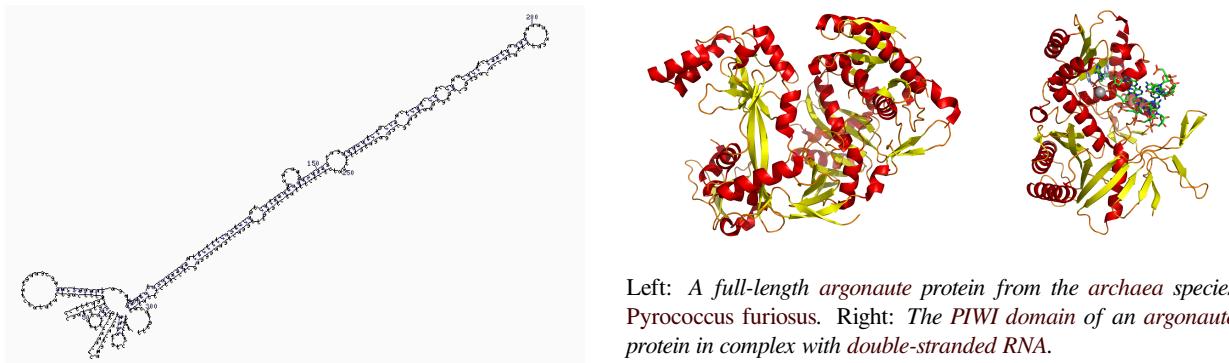
Exogenous dsRNA is detected and bound by an effector protein, known as RDE-4 in *C. elegans* and R2D2 in *Drosophila*, that stimulates dicer activity.<sup>[9]</sup> This protein only binds long dsRNAs, but the mechanism producing this length specificity is unknown.<sup>[9]</sup> This RNA-binding protein then facilitates the transfer of cleaved siRNAs to the RISC complex.<sup>[10]</sup>

In *C. elegans* this initiation response is amplified through the synthesis of a population of 'secondary' siRNAs during which the dicer-produced initiating or 'primary' siRNAs are used as templates.<sup>[11]</sup> These 'secondary' siRNAs are structurally distinct from dicer-produced siRNAs and appear to be produced by an RNA-dependent RNA polymerase (RdRP).<sup>[12][13]</sup>

### MicroRNA

Main article: MicroRNA

MicroRNAs (miRNAs) are genetically encoded non-coding RNAs that help regulate gene expression, particularly during development.<sup>[14]</sup> The phenomenon of RNA



The stem-loop secondary structure of a pre-microRNA from *Brassica oleracea*.

interference, broadly defined, includes the endogenously induced gene silencing effects of miRNAs as well as silencing triggered by foreign dsRNA. Mature miRNAs are structurally similar to siRNAs produced from exogenous dsRNA, but before reaching maturity, miRNAs must first undergo extensive post-transcriptional modification. A miRNA is expressed from a much longer RNA-coding gene as a primary transcript known as a *pri-miRNA* which is processed, in the cell nucleus, to a 70-nucleotide stem-loop structure called a *pre-miRNA* by the microprocessor complex. This complex consists of an RNase III enzyme called *Drosha* and a dsRNA-binding protein *DGCR8*. The dsRNA portion of this pre-miRNA is bound and cleaved by Dicer to produce the mature miRNA molecule that can be integrated into the RISC complex; thus, miRNA and siRNA share the same downstream cellular machinery.<sup>[15]</sup>

siRNAs derived from long dsRNA precursors differ from miRNAs in that miRNAs, especially those in animals, typically have incomplete base pairing to a target and inhibit the translation of many different mRNAs with similar sequences. In contrast, siRNAs typically base-pair perfectly and induce mRNA cleavage only in a single, specific target.<sup>[16]</sup> In *Drosophila* and *C. elegans*, miRNA and siRNA are processed by distinct argonaute proteins and dicer enzymes.<sup>[17][18]</sup>

Left: A full-length argonaute protein from the archaea species *Pyrococcus furiosus*. Right: The PIWI domain of an argonaute protein in complex with double-stranded RNA.

### RISC activation and catalysis

The active components of an RNA-induced silencing complex (RISC) are endonucleases called argonaute proteins, which cleave the target mRNA strand complementary to their bound siRNA.<sup>[3]</sup> As the fragments produced by dicer are double-stranded, they could each in theory produce a functional siRNA. However, only one of the two strands, which is known as the *guide strand*, binds the argonaute protein and directs gene silencing. The other *anti-guide strand* or *passenger strand* is degraded during RISC activation.<sup>[19]</sup> Although it was first believed that an ATP-dependent helicase separated these two strands,<sup>[20]</sup> the process proved to be ATP-independent and performed directly by the protein components of RISC.<sup>[21][22]</sup> However, an *in vitro* kinetic analysis of RNAi in the presence and absence of ATP showed that ATP may be required to unwind and remove the cleaved mRNA strand from the RISC complex after catalysis.<sup>[23]</sup> The guide strand tends to be the one whose 5' end is less stably paired to its complement,<sup>[24]</sup> but strand selection is unaffected by the direction in which dicer cleaves the dsRNA before RISC incorporation.<sup>[25]</sup> Instead, the R2D2 protein may serve as the differentiating factor by binding the more-stable 5' end of the passenger strand.<sup>[26]</sup>

The structural basis for binding of RNA to the argonaute protein was examined by X-ray crystallography of the binding domain of an RNA-bound argonaute protein. Here, the phosphorylated 5' end of the RNA strand enters a conserved basic surface pocket and makes contacts through a divalent cation (an atom with two positive charges) such as magnesium and by aromatic stacking (a process that allows more than one atom to share an electron by passing it

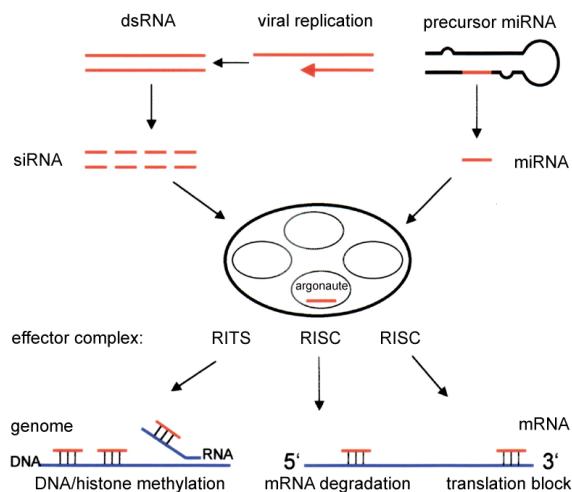
back and forth) between the 5' nucleotide in the siRNA and a conserved tyrosine residue. This site is thought to form a nucleation site for the binding of the siRNA to its mRNA target.<sup>[27]</sup> Analysis of the inhibitory effect of mismatches in either the 5' or 3' end of the guide strand has demonstrated that the 5' end of the guide strand is likely responsible for matching and binding the target mRNA, while the 3' end is responsible for physically arranging target mRNA into a cleavage-favorable RISC region.<sup>[23]</sup>

It is not understood how the activated RISC complex locates complementary mRNAs within the cell. Although the cleavage process has been proposed to be linked to translation, translation of the mRNA target is not essential for RNAi-mediated degradation.<sup>[28]</sup> Indeed, RNAi may be more effective against mRNA targets that are not translated.<sup>[29]</sup> Argonaute proteins are localized to specific regions in the cytoplasm called P-bodies (also cytoplasmic bodies or GW bodies), which are regions with high rates of mRNA decay;<sup>[30]</sup> miRNA activity is also clustered in P-bodies.<sup>[31]</sup> Disruption of P-bodies decreases the efficiency of RNA interference, suggesting that they are a critical site in the RNAi process.<sup>[32]</sup>

### Transcriptional silencing

Components of the RNAi pathway are used in many eukaryotes in the maintenance of the organization and structure of their genomes. Modification of histones and associated induction of heterochromatin formation serves to downregulate genes pre-transcriptionally;<sup>[34]</sup> this process is referred to as RNA-induced transcriptional silencing (RITS), and is carried out by a complex of proteins called the RITS complex. In fission yeast this complex contains argonaute, a chromodomain protein Chp1, and a protein called Tas3 of unknown function.<sup>[35]</sup> As a consequence, the induction and spread of heterochromatic regions requires the argonaute and RdRP proteins.<sup>[36]</sup> Indeed, deletion of these genes in the fission yeast *S. pombe* disrupts histone methylation and centromere formation,<sup>[37]</sup> causing slow or stalled anaphase during cell division.<sup>[38]</sup> In some cases, similar processes associated with histone modification have been observed to transcriptionally upregulate genes.<sup>[39]</sup>

The mechanism by which the RITS complex induces heterochromatin formation and organization is not well under-

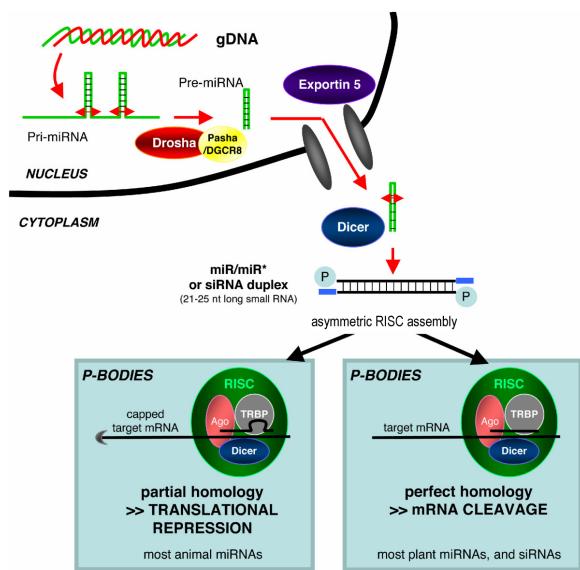


*The enzyme dicer trims double stranded RNA, to form small interfering RNA or microRNA. These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA to prevent translation.<sup>[33]</sup>*

stood. Most studies have focused on the mating-type region in fission yeast, which may not be representative of activities in other genomic regions/organisms. In maintenance of existing heterochromatin regions, RITS forms a complex with siRNAs complementary to the local genes and stably binds local methylated histones, acting co-transcriptionally to degrade any nascent pre-mRNA transcripts that are initiated by RNA polymerase. The formation of such a heterochromatin region, though not its maintenance, is dicer-dependent, presumably because dicer is required to generate the initial complement of siRNAs that target subsequent transcripts.<sup>[40]</sup> Heterochromatin maintenance has been suggested to function as a self-reinforcing feedback loop, as new siRNAs are formed from the occasional nascent transcripts by RdRP for incorporation into local RITS complexes.<sup>[41]</sup> The relevance of observations from fission yeast mating-type regions and centromeres to mammals is not clear, as heterochromatin maintenance in mammalian cells may be independent of the components of the RNAi pathway.<sup>[42]</sup>

### Crosstalk with RNA editing

The type of RNA editing that is most prevalent in higher eukaryotes converts adenosine nucleotides into inosine in dsRNAs via the enzyme adenosine deaminase (ADAR).<sup>[43]</sup> It was originally proposed in 2000 that the RNAi and A→I RNA editing pathways might compete for a common dsRNA substrate.<sup>[44]</sup> Some pre-miRNAs do undergo A→I RNA editing<sup>[45][46]</sup> and this mechanism may regulate the processing and expression of mature miRNAs.<sup>[46]</sup> Furthermore, at least one mammalian ADAR can sequester siRNAs from RNAi pathway components.<sup>[47]</sup> Further support for this model comes from studies on ADAR-null *C. elegans* strains indicating that A→I RNA editing may counteract RNAi silencing of endogenous genes and transgenes.<sup>[48]</sup>



*Illustration of the major differences between plant and animal gene silencing. Natively expressed microRNA or exogenous small interfering RNA is processed by dicer and integrated into the RISC complex, which mediates gene silencing.*<sup>[49]</sup>

### Variation among organisms

Organisms vary in their ability to take up foreign dsRNA and use it in the RNAi pathway. The effects of RNA interference can be both systemic and heritable in plants and *C.*

*elegans*, although not in *Drosophila* or mammals. In plants, RNAi is thought to propagate by the transfer of siRNAs between cells through plasmodesmata (channels in the cell walls that enable communication and transport).<sup>[20]</sup> Heritability comes from methylation of promoters targeted by RNAi; the new methylation pattern is copied in each new generation of the cell.<sup>[50]</sup> A broad general distinction between plants and animals lies in the targeting of endogenously produced miRNAs; in plants, miRNAs are usually perfectly or nearly perfectly complementary to their target genes and induce direct mRNA cleavage by RISC, while animals' miRNAs tend to be more divergent in sequence and induce translational repression.<sup>[49]</sup> This translational effect may be produced by inhibiting the interactions of translation initiation factors with the messenger RNA's polyadenine tail.<sup>[51]</sup>

Some eukaryotic protozoa such as *Leishmania major* and *Trypanosoma cruzi* lack the RNAi pathway entirely.<sup>[52][53]</sup> Most or all of the components are also missing in some fungi, most notably the model organism *Saccharomyces cerevisiae*.<sup>[54]</sup> The presence of RNAi in other budding yeast species such as *Saccharomyces castellii* and *Candida albicans*, further demonstrates that inducing two RNAi-related proteins from *S. castellii* facilitates RNAi in *S. cerevisiae*.<sup>[55]</sup> That certain ascomycetes and basidiomycetes are missing RNA interference pathways indicates that proteins required for RNA silencing have been lost independently from many fungal lineages, possibly due to the evolution of a novel pathway with similar function, or to the lack of selective advantage in certain niches.<sup>[56]</sup>

### Related prokaryotic systems

Gene expression in prokaryotes is influenced by an RNA-based system similar in some respects to RNAi. Here, RNA-encoding genes control mRNA abundance or translation by producing a complementary RNA that anneals to an mRNA. However these regulatory RNAs are not generally considered to be analogous to miRNAs because the dicer enzyme is not involved.<sup>[57]</sup> It has been suggested that CRISPR interference systems in prokaryotes are analogous to eukaryotic RNA interference systems, although none of the protein components are orthologous.<sup>[58]</sup>

### 3.2.2 Biological functions

#### Immunity

RNA interference is a vital part of the immune response to viruses and other foreign genetic material, especially in plants where it may also prevent the self-propagation of transposons.<sup>[59]</sup> Plants such as *Arabidopsis thaliana* express multiple dicer homologs that are specialized to react differently when the plant is exposed to different viruses.<sup>[60]</sup> Even before the RNAi pathway was fully understood, it was known that induced gene silencing in plants could spread throughout the plant in a systemic effect and could be transferred from stock to scion plants via grafting.<sup>[61]</sup> This phenomenon has since been recognized as a feature of the plant adaptive immune system and allows the entire plant to respond to a virus after an initial localized encounter.<sup>[62]</sup> In response, many plant viruses have evolved elaborate mechanisms to suppress the RNAi response.<sup>[63]</sup> These include viral proteins that bind short double-stranded RNA fragments with single-stranded overhang ends, such as those produced by dicer.<sup>[64]</sup> Some plant genomes also express endogenous siRNAs in response to infection by specific types of bacteria.<sup>[65]</sup> These effects may be part of a generalized response to pathogens that downregulates any metabolic process in the host that aids the infection process.<sup>[66]</sup>

Although animals generally express fewer variants of the dicer enzyme than plants, RNAi in some animals produces an antiviral response. In both juvenile and adult *Drosophila*, RNA interference is important in antiviral innate immunity and is active against pathogens such as *Drosophila X virus*.<sup>[67][68]</sup> A similar role in immunity may operate in *C. elegans*, as argonaute proteins are upregulated in response to viruses and worms that overexpress components of the RNAi pathway are resistant to viral infection.<sup>[69][70]</sup>

The role of RNA interference in mammalian innate immunity is poorly understood, and relatively little data is available. However, the existence of viruses that encode genes able to suppress the RNAi response in mammalian cells may be evidence in favour of an RNAi-dependent mammalian immune response,<sup>[71][72]</sup> although this hypothesis has been challenged as poorly substantiated.<sup>[73]</sup> Maillard et al.<sup>[74]</sup> and Li et al.<sup>[75]</sup> provide evidence for the existence of a functional antiviral RNAi pathway in mammalian cells. Other functions for RNAi in mammalian viruses also exist,

such as miRNAs expressed by the *herpes virus* that may act as heterochromatin organization triggers to mediate viral latency.<sup>[39]</sup>

#### Downregulation of genes

Endogenously expressed miRNAs, including both intronic and intergenic miRNAs, are most important in translational repression<sup>[49]</sup> and in the regulation of development, especially on the timing of morphogenesis and the maintenance of undifferentiated or incompletely differentiated cell types such as stem cells.<sup>[76]</sup> The role of endogenously expressed miRNA in downregulating gene expression was first described in *C. elegans* in 1993.<sup>[77]</sup> In plants this function was discovered when the “JAW microRNA” of *Arabidopsis* was shown to be involved in the regulation of several genes that control plant shape.<sup>[78]</sup> In plants, the majority of genes regulated by miRNAs are transcription factors;<sup>[79]</sup> thus miRNA activity is particularly wide-ranging and regulates entire gene networks during development by modulating the expression of key regulatory genes, including transcription factors as well as F-box proteins.<sup>[80]</sup> In many organisms, including humans, miRNAs are linked to the formation of tumors and dysregulation of the cell cycle. Here, miRNAs can function as both oncogenes and tumor suppressors.<sup>[81]</sup>

#### Upregulation of genes

RNA sequences (siRNA and miRNA) that are complementary to parts of a promoter can increase gene transcription, a phenomenon dubbed RNA activation. Part of the mechanism for how these RNA upregulate genes is known: dicer and argonaute are involved, possibly via histone demethylation.<sup>[82]</sup> miRNAs have been proposed to upregulate their target genes upon cell cycle arrest, via unknown mechanisms.<sup>[83]</sup>

### 3.2.3 Evolution

Based on parsimony-based phylogenetic analysis, the most recent common ancestor of all eukaryotes most likely already possessed an early RNA interference pathway; the absence of the pathway in certain eukaryotes is thought to be a derived characteristic.<sup>[84]</sup> This ancestral RNAi system

probably contained at least one dicer-like protein, one argonaute, one PIWI protein, and an RNA-dependent RNA polymerase that may also have played other cellular roles. A large-scale comparative genomics study likewise indicates that the eukaryotic crown group already possessed these components, which may then have had closer functional associations with generalized RNA degradation systems such as the exosome.<sup>[85]</sup> This study also suggests that the RNA-binding argonaute protein family, which is shared among eukaryotes, most archaea, and at least some bacteria (such as *Aquifex aeolicus*), is homologous to and originally evolved from components of the translation initiation system.

The ancestral function of the RNAi system is generally agreed to have been immune defense against exogenous genetic elements such as transposons and viral genomes.<sup>[84][86]</sup> Related functions such as histone modification may have already been present in the ancestor of modern eukaryotes, although other functions such as regulation of development by miRNA are thought to have evolved later.<sup>[84]</sup>

RNA interference genes, as components of the antiviral innate immune system in many eukaryotes, are involved in an evolutionary arms race with viral genes. Some viruses have evolved mechanisms for suppressing the RNAi response in their host cells, particularly for plant viruses.<sup>[63]</sup> Studies of evolutionary rates in *Drosophila* have shown that genes in the RNAi pathway are subject to strong directional selection and are among the fastest-evolving genes in the *Drosophila* genome.<sup>[87]</sup>

### 3.2.4 Applications

#### Gene knockdown

The RNA interference pathway is often exploited in experimental biology to study the function of genes in cell culture and *in vivo* in model organisms.<sup>[3]</sup> Double-stranded RNA is synthesized with a sequence complementary to a gene of interest and introduced into a cell or organism, where it is recognized as exogenous genetic material and activates the RNAi pathway. Using this mechanism, researchers can cause a drastic decrease in the expression of a targeted gene. Studying the effects of this decrease

can show the physiological role of the gene product. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a "knockdown", to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated.<sup>[88]</sup>

Extensive efforts in computational biology have been directed toward the design of successful dsRNA reagents that maximize gene knockdown but minimize "off-target" effects. Off-target effects arise when an introduced RNA has a base sequence that can pair with and thus reduce the expression of multiple genes. Such problems occur more frequently when the dsRNA contains repetitive sequences. It has been estimated from studying the genomes of humans, *C. elegans* and *S. pombe* that about 10% of possible siRNAs have substantial off-target effects.<sup>[7]</sup> A multitude of software tools have been developed implementing algorithms for the design of general<sup>[89][90]</sup> mammal-specific,<sup>[91]</sup> and virus-specific<sup>[92]</sup> siRNAs that are automatically checked for possible cross-reactivity.

Depending on the organism and experimental system, the exogenous RNA may be a long strand designed to be cleaved by dicer, or short RNAs designed to serve as siRNA substrates. In most mammalian cells, shorter RNAs are used because long double-stranded RNA molecules induce the mammalian interferon response, a form of innate immunity that reacts nonspecifically to foreign genetic material.<sup>[93]</sup> Mouse oocytes and cells from early mouse embryos lack this reaction to exogenous dsRNA and are therefore a common model system for studying mammalian gene-knockdown effects.<sup>[94]</sup> Specialized laboratory techniques have also been developed to improve the utility of RNAi in mammalian systems by avoiding the direct introduction of siRNA, for example, by stable transfection with a plasmid encoding the appropriate sequence from which siRNAs can be transcribed,<sup>[95]</sup> or by more elaborate lentiviral vector systems allowing the inducible activation or deactivation of transcription, known as *conditional RNAi*.<sup>[96][97]</sup>

#### Functional genomics

Most functional genomics applications of RNAi in animals have used *C. elegans*<sup>[98]</sup> and *Drosophila*,<sup>[99]</sup> as these are the common model organisms in which RNAi is most effec-



A normal adult *Drosophila* fly, a common model organism used in RNAi experiments.

tive. *C. elegans* is particularly useful for RNAi research for two reasons: firstly, the effects of gene silencing are generally heritable, and secondly because delivery of the dsRNA is extremely simple. Through a mechanism whose details are poorly understood, bacteria such as *E. coli* that carry the desired dsRNA can be fed to the worms and will transfer their RNA payload to the worm via the intestinal tract. This “delivery by feeding” is just as effective at inducing gene silencing as more costly and time-consuming delivery methods, such as soaking the worms in dsRNA solution and injecting dsRNA into the gonads.<sup>[100]</sup> Although delivery is more difficult in most other organisms, efforts are also underway to undertake large-scale genomic screening applications in cell culture with mammalian cells.<sup>[101]</sup>

Approaches to the design of genome-wide RNAi libraries can require more sophistication than the design of a single siRNA for a defined set of experimental conditions. Artificial neural networks are frequently used to design siRNA libraries<sup>[102]</sup> and to predict their likely efficiency at gene knockdown.<sup>[103]</sup> Mass genomic screening is widely seen as a promising method for genome annotation and has triggered the development of high-throughput screening methods based on microarrays.<sup>[104][105]</sup> However, the utility of these screens and the ability of techniques developed on model organisms to generalize to even closely related species has been questioned, for example from *C. elegans*.

to related parasitic nematodes.<sup>[106][107]</sup>

Functional genomics using RNAi is a particularly attractive technique for genomic mapping and annotation in plants because many plants are polyploid, which presents substantial challenges for more traditional genetic engineering methods. For example, RNAi has been successfully used for functional genomics studies in bread wheat (which is hexaploid)<sup>[108]</sup> as well as more common plant model systems *Arabidopsis* and maize.<sup>[109]</sup>

### Medicine



An adult *C. elegans* worm, grown under RNAi suppression of a nuclear hormone receptor involved in desaturase regulation. These worms have abnormal fatty acid metabolism but are viable and fertile.<sup>[110]</sup>

It may be possible to exploit RNA interference in therapy. Although it is difficult to introduce long dsRNA strands into mammalian cells due to the interferon response, the use of short interfering RNA has been more successful.<sup>[111]</sup> Among the first applications to reach clinical trials were in the treatment of macular degeneration and respiratory syncytial virus.<sup>[112]</sup> RNAi has also been shown to be effective in reversing induced liver failure in mouse models.<sup>[113]</sup>

**Antiviral** Potential antiviral therapies include topical microbicide treatments that use RNAi to treat infection (at Harvard Medical School; in mice, so far) by herpes simplex virus type 2 and the inhibition of viral gene expression in cancerous cells,<sup>[114]</sup> knockdown of host receptors and coreceptors for HIV,<sup>[115]</sup> the silencing of hepatitis A<sup>[116]</sup> and hepatitis B genes,<sup>[117]</sup> silencing of influenza gene expression,<sup>[39]</sup> and inhibition of measles viral replication.<sup>[118]</sup> Potential treatments for neurodegenerative diseases have also been proposed, with

particular attention to polyglutamine diseases such as Huntington's disease.<sup>[119]</sup>

RNA interference-based applications are being developed to target persistent HIV-1 infection. Viruses like HIV-1 are particularly difficult targets for RNAi-attack because they are escape-prone, which requires combinatorial RNAi strategies to prevent viral escape.<sup>[120]</sup>

**Cancer** RNA interference is also a promising way to treat cancers by silencing genes differentially upregulated in tumor cells or genes involved in cell division.<sup>[121][122]</sup> A key area of research in the use of RNAi for clinical applications is the development of a safe delivery method, which to date has involved mainly viral vector systems similar to those suggested for gene therapy.<sup>[123][124]</sup>

Due to safety concerns with viral vectors, nonviral delivery methods, typically employing lipid-based<sup>[125]</sup> or polymeric<sup>[126]</sup> vectors, are also promising candidates. Computational modeling of nonviral siRNA delivery paired with *in vitro* and *in vivo* gene knockdown studies elucidated the temporal behavior of RNAi in these systems. The model used an input bolus dose of siRNA and computationally and experimentally showed that knockdown duration was dependent mainly on the doubling time of the cells to which siRNA was delivered, while peak knockdown depended primarily on the delivered dose. Kinetic considerations of RNAi are imperative to safe and effective dosing schedules as nonviral methods of inducing RNAi continue to be developed.<sup>[127]</sup>

**Safety** Despite the proliferation of promising cell culture studies for RNAi-based drugs, some concern has been raised regarding the safety of RNA interference, especially the potential for "off-target" effects in which a gene with a coincidentally similar sequence to the targeted gene is also repressed.<sup>[128]</sup> A computational genomics study estimated that the error rate of off-target interactions is about 10%.<sup>[7]</sup> One major study of liver disease in mice reported that 23 out of 49 distinct RNAi treatment protocols resulted in death.<sup>[129]</sup> Researchers hypothesized this alarmingly high rate to be the result of "oversaturation" of the dsRNA pathway,<sup>[130]</sup> due to the use of shRNAs that have to be processed in the nucleus and exported to the cytoplasm using an active mechanism. Such considerations are under

active investigation, to reduce their impact in the potential therapeutic applications.

RNAi *in vivo* delivery to tissues still eludes science—especially to tissues deep within the body. RNAi delivery is only easily accessible to surface tissues such as the eye and respiratory tract. In these instances, siRNA has been used in direct contact with the tissue for transport. The resulting RNAi successfully focused on target genes. When delivering siRNA to deep tissues, the siRNA must be protected from nucleases, but targeting specific areas becomes the main difficulty. This difficulty has been combatuated with high dosage levels of siRNA to ensure the tissues have been reached, however in these cases hepatotoxicity was reported.<sup>[130]</sup>

## Biotechnology

RNA interference has been used for applications in biotechnology and is nearing commercialization in others.

**Foods** RNAi has been used to genetically engineer plants to produce lower levels of natural plant toxins. Such techniques take advantage of the stable and heritable RNAi phenotype in plant stocks. Cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption. RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is itself important in preventing damage from plant pests.<sup>[131]</sup> Similar efforts have been directed toward the reduction of the cyanogenic natural product linamarin in cassava plants.<sup>[132]</sup>

No plant products that use RNAi-based genetic engineering have yet exited the experimental stage. Development efforts have successfully reduced the levels of allergens in tomato plants<sup>[133]</sup> and fortification of plants such as tomatoes with dietary antioxidants.<sup>[134]</sup> Previous commercial products, including the Flavr Savr tomato and two cultivars of ringspot-resistant papaya, were originally developed using antisense technology but likely exploited the RNAi pathway.<sup>[135][136]</sup>

**Other crops** Another effort decreased the precursors of likely carcinogens in tobacco plants.<sup>[137]</sup> Other plant traits that have been engineered in the laboratory include the production of non-narcotic natural products by the opium poppy<sup>[138]</sup> and resistance to common plant viruses.<sup>[139]</sup>

**Insecticide** RNAi is under development as an insecticide, employing multiple approaches, including genetic engineering and topical application. Cells in the midgut of many larvae take up the molecules and help spread the signal throughout the insect's body.<sup>[1]</sup>

RNAi has varying effects in different species of Lepidoptera (butterflies and moths).<sup>[140]</sup> Possibly because their saliva is better at breaking down RNA, the cotton bollworm, the beet armyworm and the Asiatic rice borer have so far not been proven susceptible to RNAi by feeding.<sup>[1]</sup>

To develop resistance to RNAi, the western corn rootworm would have to change the genetic sequence of its Snf7 gene at multiple sites. Combining multiple strategies, such as engineering the protein Cry, derived from a bacterium called *Bacillus thuringiensis* (Bt), and RNAi in one plant delay the onset of resistance.<sup>[1]</sup>

One unconfirmed 2012 paper detected small RNAs from food plants in the blood of mice and humans. The consequences of RNA insecticides in the human bloodstream have not been investigated. Biological barriers—including saliva and blood enzymes and stomach acids may break down any ingested RNA. Critics charge that the human equivalent of the mouse diet in the study would be 33 kilograms of cooked rice a day. Two 2013 studies failed to detect RNAs in humans. Athletes consuming a diet of apples and bananas and monkeys consuming a fruit shake both appeared to be RNA-free.<sup>[1]</sup>

**Transgenic plants** Transgenic crops have been made to express small bits of RNA, carefully chosen to silence crucial genes in target pests. RNAs exist that affect only insects that have specific genetic sequences. In 2009 a study showed RNAs that could kill any one of four fruit fly species while not harming the other three.<sup>[1]</sup>

In 2012 Syngenta bought Belgian RNAi firm Devgen for \$522 million and Monsanto paid \$29.2 million for the exclusive rights to intellectual property from Alnylam Phar-

maceuticals. The International Potato Center in Lima, Peru is looking for genes to target in the sweet potato weevil, a beetle whose larvae ravage sweet potatoes globally. Other researchers are trying to silence genes in ants, caterpillars and pollen beetles. Monsanto will likely be first to market, with a transgenic corn seed that expresses dsRNA based on gene Snf7 from the western corn rootworm, a beetle whose larvae annually cause one billion dollars in damage in the United States alone. A 2012 paper showed that silencing Snf7 stunts larval growth, killing them within days. In 2013 the same team showed that the RNA affects very few other species.<sup>[1]</sup>

**Topical** Alternatively dsRNA can be supplied without genetic engineering. One approach is to add them to irrigation water. The molecules are absorbed into the plants' vascular system and poison insects feeding on them. Another approach involves spraying RNA like a conventional pesticide. This would allow faster adaptation to resistance. Such approaches would require low cost sources of RNAs that do not currently exist.<sup>[1]</sup>

### Genome-scale screening

Genome-scale RNAi research relies on high-throughput screening (HTS) technology. RNAi HTS technology allows genome-wide loss-of-function screening and is broadly used in the identification of genes associated with specific phenotypes. This technology has been hailed as the second genomics wave, following the first genomics wave of gene expression microarray and single nucleotide polymorphism discovery platforms.<sup>[141]</sup> One major advantage of genome-scale RNAi screening is its ability to simultaneously interrogate thousands of genes. With the ability to generate a large amount of data per experiment, genome-scale RNAi screening has led to an explosion in data generation rates. Exploiting such large data sets is a fundamental challenge, requiring suitable statistics/bioinformatics methods. The basic process of cell-based RNAi screening includes the choice of an RNAi library, robust and stable cell types, transfection with RNAi agents, treatment/incubation, signal detection, analysis and identification of important genes or therapeutic targets.<sup>[142]</sup>

### 3.2.5 History



Example *petunia* plants in which genes for pigmentation are silenced by RNAi. The left plant is wild-type; the right plants contain transgenes that induce suppression of both transgene and endogenous gene expression, giving rise to the unpigmented white areas of the flower.<sup>[143]</sup>

The discovery of RNAi was preceded first by observations of transcriptional inhibition by antisense RNA expressed in transgenic plants,<sup>[144]</sup> and more directly by reports of unexpected outcomes in experiments performed by plant scientists in the United States and the Netherlands in the early 1990s.<sup>[145]</sup> In an attempt to alter flower colors in *petunias*, researchers introduced additional copies of a gene encoding chalcone synthase, a key enzyme for flower pigmentation into *petunia* plants of normally pink or violet flower color. The overexpressed gene was expected to result in darker flowers, but instead produced less pigmented, fully or partially white flowers, indicating that the activity of chalcone synthase had been substantially decreased; in fact, both the endogenous genes and the transgenes were downregulated in the white flowers. Soon after, a related event termed *quelling* was noted in the fungus *Neurospora crassa*,<sup>[146]</sup> although it was not immediately recognized as related. Further investigation of the phenomenon in plants indicated that the downregulation was due to post-transcriptional inhibition of gene expression via an increased rate of mRNA degradation.<sup>[147]</sup> This phenomenon was called *co-suppression of gene expression*, but the molecular mechanism remained unknown.<sup>[148]</sup>

Not long after, plant virologists working on improving plant resistance to viral diseases observed a similar unexpected phenomenon. While it was known that plants expressing virus-specific proteins showed enhanced tolerance or resistance to viral infection, it was not expected that plants carrying only short, non-coding regions of viral RNA sequences would show similar levels of protection. Researchers believed that viral RNA produced by transgenes

could also inhibit viral replication.<sup>[149]</sup> The reverse experiment, in which short sequences of plant genes were introduced into viruses, showed that the targeted gene was suppressed in an infected plant. This phenomenon was labeled “virus-induced gene silencing” (VIGS), and the set of such phenomena were collectively called *post transcriptional gene silencing*.<sup>[150]</sup>

After these initial observations in plants, laboratories searched for this phenomenon in other organisms.<sup>[151][152]</sup> Craig C. Mello and Andrew Fire's 1998 *Nature* paper reported a potent gene silencing effect after injecting double stranded RNA into *C. elegans*.<sup>[153]</sup> In investigating the regulation of muscle protein production, they observed that neither mRNA nor antisense RNA injections had an effect on protein production, but double-stranded RNA successfully silenced the targeted gene. As a result of this work, they coined the term *RNAi*. This discovery represented the first identification of the causative agent for the phenomenon. Fire and Mello were awarded the 2006 Nobel Prize in Physiology or Medicine.<sup>[3]</sup>

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### 3.2.7 Further reading

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### 3.2.8 External links

- RNAi Atlas: a database of RNAi libraries and their target analysis results in Homo sapiens
- Overview of the RNAi process, from *Cambridge University's The Naked Scientists*
- Animation of the RNAi process, from *Nature*
- NOVA scienceNOW explains RNAi – A 15 minute video of the *Nova* broadcast that aired on PBS, July 26, 2005
- “Planting the Seeds of a New Paradigm” at *PLoS — Public Library of Science*
- Silencing Genomes RNA interference (RNAi) experiments and bioinformatics in *C. elegans* for education. From the Dolan DNA Learning Center of Cold Spring Harbor Laboratory.



- RNAi screens in *C. elegans* in a 96-well liquid format and their application to the systematic identification of genetic interactions (a protocol)
- 2 American ‘Worm People’ Win Nobel for RNA Work, from NY Times
- *Molecular Therapy* web focus: “The development of RNAi as a therapeutic strategy”, a collection of free articles about RNAi as a therapeutic strategy.
- GenomeRNAi: a database of phenotypes from RNA interference screening experiments in *Drosophila melanogaster* and *Homo sapiens*

# Chapter 4

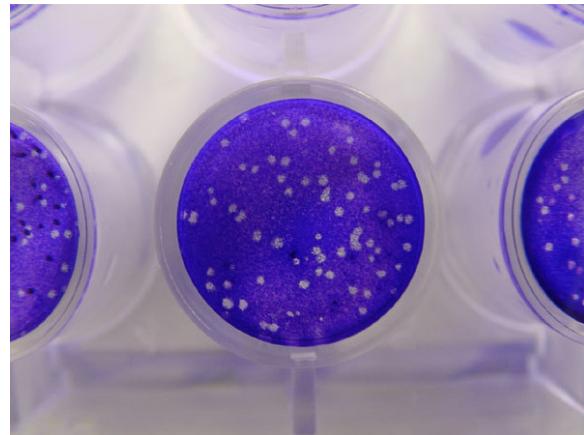
## Viral Quantification

### 4.1 Virus quantification

Virus quantification involves counting the number of viruses in a specific volume to determine the virus concentration. It is utilized in both research and development (R&D) in commercial and academic laboratories as well as production situations where the quantity of virus at various steps is an important variable. For example, the production of viral vaccines, recombinant proteins using viral vectors and viral antigens all require **virus quantification** to continually adapt and monitor the process in order to optimize production yields and respond to ever changing demands and applications. Examples of specific instances where known viruses need to be quantified include clone screening, **multiplicity of infection (MOI)** optimization and adaptation of methods to cell culture. This page discusses various techniques currently used to quantify viruses in liquid samples. These methods are separated into two categories, traditional vs. modern methods. Traditional methods are industry-standard methods that have been used for decades but are generally slow and labor-intensive. Modern methods are relatively new commercially available products and kits that greatly reduce quantification time. This is not meant to be an exhaustive review of all potential methods, but rather a representative cross-section of traditional methods and new, commercially available methods. While other published methods may exist for virus quantification, non-commercial methods are not discussed here.

#### 4.1.1 Traditional methods

##### Plaque assay



*Viral Plaques of Herpes Simplex Virus*

Plaque-based assays are the standard method used to determine virus concentration in terms of infectious dose. **Viral plaque** assays determine the number of plaque forming units (pfu) in a virus sample, which is one measure of virus quantity. This assay is based on a microbiological method conducted in **petri dishes** or multi-well plates. Specifically, a confluent monolayer of **host cells** is infected with the virus at varying dilutions and covered with a semi-solid medium, such as **agar** or **carboxymethyl cellulose**, to prevent the virus infection from spreading indiscriminately. A viral plaque is formed when a virus infects a cell within the fixed cell monolayer.<sup>[1]</sup> The virus infected cell will lyse and spread the infection to adjacent cells where the infection-to-lysis cycle is repeated. The infected cell

area will create a plaque (an area of infection surrounded by uninfected cells) which can be seen visually or with an optical microscope. Plaque formation can take 3 – 14 days, depending on the virus being analyzed. Plaques are generally counted manually and the results, in combination with the dilution factor used to prepare the plate, are used to calculate the number of plaque forming units per sample unit volume (pfu/mL). The pfu/mL result represents the number of infective particles within the sample and is based on the assumption that each plaque formed is representative of one infective virus particle.<sup>[2]</sup>

**Focus Forming Assay (FFA)** The focus forming assay (FFA) is a variation of the plaque assay, but instead of relying on cell lysis in order to detect plaque formation, the FFA employs immunostaining techniques using fluorescently labeled antibodies specific for a viral antigen to detect infected host cells and infectious virus particles before an actual plaque is formed. The FFA is particularly useful for quantifying classes of viruses that do not lyse the cell membranes, as these viruses would not be amenable to the plaque assay. Like the plaque assay, host cell monolayers are infected with various dilutions of the virus sample and allowed to incubate for a relatively brief incubation period (e.g., 24–72 hours) under a semisolid overlay medium that restricts the spread of infectious virus, creating localized clusters (foci) of infected cells. Plates are subsequently probed with fluorescently labeled antibodies against a viral antigen, and fluorescence microscopy is used to count and quantify the number of foci. The FFA method typically yields results in less time than plaque or TCID<sub>50</sub> assays, but it can be more expensive in terms of required reagents and equipment. Assay completion time is also dependent on the size of area that the user is counting. A larger area will require more time but can provide a more accurate representation of the sample. Results of the FFA are expressed as focus forming units per milliliter, or FFU/mL.<sup>[3]</sup>

### 50% Tissue Culture Infective Dose (TCID<sub>50</sub>)

TCID<sub>50</sub> is the measure of infectious virus titer. This endpoint dilution assay quantifies the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells. This as-

say may be more common in clinical research applications where the lethal dose of virus must be determined or if the virus does not form plaques. When used in the context of tissue culture, host cells are plated and serial dilutions of the virus are added. After incubation, the percentage of cell death (i.e. infected cells) is manually observed and recorded for each virus dilution, and results are used to mathematically calculate a TCID<sub>50</sub> result.<sup>[3][4]</sup> Due to distinct differences in assay methods and principles, TCID<sub>50</sub> and pfu/mL or other infectivity assay results are not equivalent. This method can take up to a week due to cell infectivity time.<sup>[5]</sup>

Two methods commonly used to calculate TCID<sub>50</sub> (can also be used to calculate other types of 50% endpoints such EC<sub>50</sub>, IC<sub>50</sub>, and LD<sub>50</sub>) are:

- Spearman-Karber
- Reed-Muench method

The *theoretical* relationship between TCID<sub>50</sub> and PFU is approximately 1 PFU = 0.69 TCID<sub>50</sub> based on the Poisson distribution,<sup>[6]</sup> a probability distribution which describes how many random events (virus particles) occurring at a known average rate (virus titer) are likely to occur in a fixed space (the amount of virus medium in a well). However it must be emphasized that in practice, this relationship may not hold even for the same virus + cell combination, as the two types of assay are set up differently and virus infectivity is very sensitive to various factors such as cell age, overlay media, etc.

### Protein assays

There are several variations of protein-based virus quantification assays. In general, these methods quantify either the amount of all protein or the amount of a specific virus protein in the sample rather than the number of infected cells or virus particles. Quantification most commonly relies on fluorescence detection. Some assay variations quantify protein directly in a sample while other variations require host cell infection and incubation to allow virus growth prior to protein quantification. The variation used depends primarily on the amount of protein (i.e. virus) in the initial sample and the sensitivity of the assay itself. If incubation and

virus growth are required, cell and/or virus lysis/digestion are often conducted prior to analysis. Most protein-based methods are relatively fast and sensitive but require quality standards for accurate calibration, and quantify protein, not actual virus particle concentrations. Below are specific examples of widely used protein-based assays.

**Hemagglutination assay** Main article: [Hemagglutination assay](#)

The hemagglutination assay (HA) is a common non-fluorescence protein quantification assay specific for influenza. It relies on the fact that **hemagglutinin**, a surface protein of influenza viruses, agglutinates red blood cells (i.e. causes red blood cells to clump together). In this assay, dilutions of an influenza sample are incubated with a 1% **erythrocyte** solution for one hour and the virus dilution at which agglutination first occurs is visually determined. The assay produces a result of hemagglutination units (HAU), with typical pfu to HAU ratios in the  $10^6$  range.<sup>[7][8][9]</sup> This assay takes ~1–2 hours to complete and results can differ widely based on the technical expertise of the operator.

The hemagglutination inhibition assay is a common variation of the HA assay used to measure flu-specific antibody levels in blood serum. In this variation, serum antibodies to the influenza virus will interfere with the virus attachment to red blood cells. Therefore hemagglutination is inhibited when antibodies are present at a sufficient concentration.<sup>[10]</sup>

**Bicinchoninic acid assay** Main article: [Bicinchoninic acid assay](#)

The **bicinchoninic acid** assay (BCA) is based on a simple colorimetric measurement and is the most common protein quantification assay. BCA is similar to the **Lowry** or **Bradford** protein assays and was first made commercially available by Pierce, which is now owned by **Thermo Fisher Scientific**. In the BCA assay, a protein's peptide bonds quantitatively reduce  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$ , which produces a light blue color. BCA **chelates**  $\text{Cu}^{1+}$  at a 2:1 ratio resulting in a more intensely colored species that absorbs at 562 nm. **Absorbance** of a sample at 562 nm is used to determine the bulk protein concentration in the sample. Assay results are

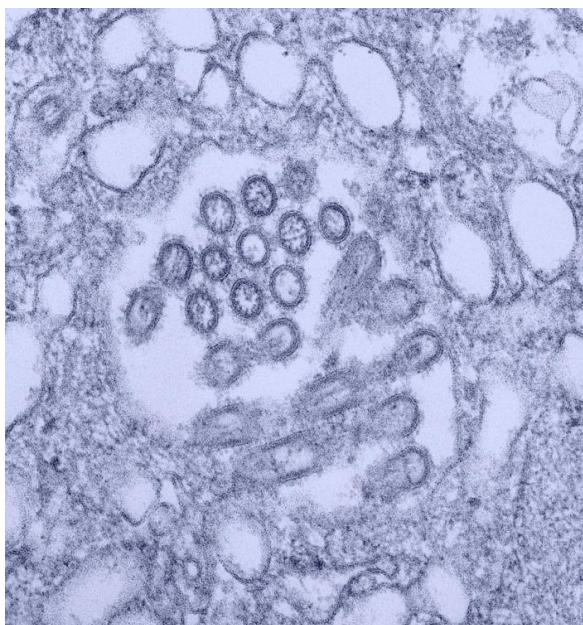
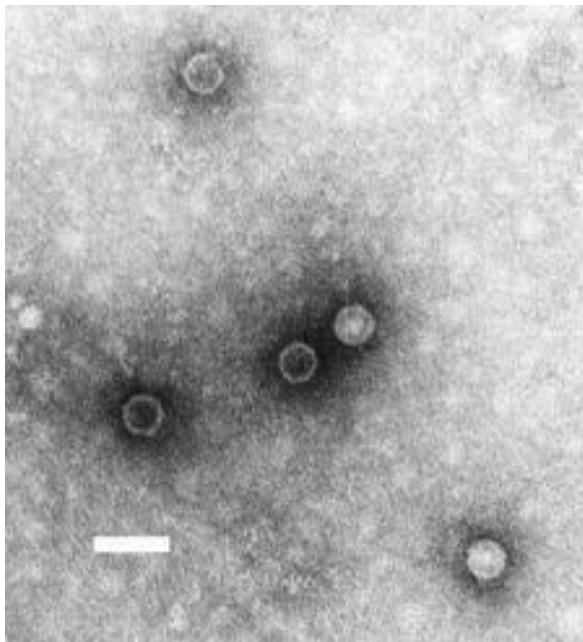
compared with known standard curves after analysis with a spectrophotometer or plate reader.<sup>[11]</sup> Total assay time is 30 minutes to one hour. While this assay is ubiquitous and fast, it lacks specificity since it counts all protein, the virus preparation to be quantified must contain very low levels host cell proteins.

**Single radial immunodiffusion assay** Single radial immunodiffusion assay (SRID), also known as the Mancini method, is a protein assay that detects the amount of specific viral antigen by immunodiffusion in a semi-solid medium (e.g. agar). The medium contains **antiserum** specific to the antigen of interest and the antigen is placed in the center of the disc. As the antigen diffuses into the medium it creates a precipitate ring that grows until equilibrium is reached. Assay time can range from 10 hours to days depending on equilibration time of the antigen and antibody. The zone diameter from the ring is linearly related to the log of protein concentration and is compared to zone diameters for known protein standards for quantification.<sup>[12]</sup> There are kits and serums commercially available for this assay (e.g. The Binding Site Inc.).

**Transmission Electron Microscopy (TEM)**

Negative Stain TEM of Polio Virus, Bar = 50 nm  
Tissue Embedded Section of Novel H1N1 Virions

TEM is a specialized type of microscopy that utilizes a beam of electrons focused with a magnetic field to image a sample. TEM provides imaging with 1000x greater spatial resolution than a light microscope (resolution down to 0.2 nm).<sup>[13]</sup> An ultrathin, **negatively stained** sample is required. Sample preparations involve depositing specimens onto a coated TEM grid and negative staining with an electron-opaque liquid.<sup>[14]</sup> Tissue embedded samples can also be examined if thinly sectioned. Sample preparations vary depending on protocol and user but generally require hours to complete. TEM images can show individual virus particles and quantitative **image analysis** can be used to determine virus concentrations. These high resolution images also provide particle morphology information that most other methods cannot. Quantitative TEM results will often be greater than results from other assays as all particles, re-



Regardless of infectivity, are quantified in the reported virus-like particles per mL (vlp/mL) result. Quantitative TEM

generally works well for virus concentrations greater than  $10^6$  particles/mL. Because of high instrument cost and the amount of space and support facilities needed, TEM equipment is available in a limited number of facilities.

#### 4.1.2 Modern methods

##### Tunable Resistive Pulse Sensing (TRPS)

Main article: Tunable resistive pulse sensing

Tunable Resistive Pulse Sensing (TRPS) is a method that allows high-throughput single particle measurements of individual virus particles, as they are driven through a size-tunable nanopore, one at a time.<sup>[15]</sup> The technique has the advantage of simultaneously determining the size and concentration, of virus particles in solution with high resolution. This can be used in assessing sample stability and the contribution of aggregates, as well as total viral particle concentration (vp/mL).<sup>[16]</sup>

TRPS-based measurement occurs in an ionic buffer, and no pre-staining of samples is required prior to analysis, thus the technique is more rapid than those which require pre-treatment with fluorescent dyes, with a total preparation and measurement time of less than 10 minutes per sample. TRPS-based virus analysis is commercially available through qViro-X systems, which have the ability to be de-contaminated chemically by autoclaving after measurement has occurred.

##### Flow cytometry



*Virus counter*

While most flow cytometers do not have sufficient sensitiv-

ity, there are a few commercially available flow cytometers that can be used for virus quantification. A virus counter quantifies the number of intact virus particles in a sample using fluorescence to detect colocalized proteins and nucleic acids. Samples are stained with two dyes, one specific for proteins and one specific for nucleic acids, and analyzed as they flow through a laser beam. The quantity of particles producing simultaneous events on each of the two distinct fluorescence channels is determined, along with the measured sample flow rate, to calculate a concentration of virus particles (vp/mL).<sup>[17]</sup> The results are generally similar in absolute quantity to a TEM result. The assay has a linear working range of  $10^5$ – $10^9$  vp/mL and an analysis time of  $\approx$ 10 min with a short sample preparation time.

### Quantitative Polymerase Chain Reaction (qPCR)

Main article: Quantitative PCR

Quantitative PCR utilizes polymerase chain reaction chemistry to amplify viral DNA or RNA to produce high enough concentrations for detection and quantification by fluorescence. In general, quantification by qPCR relies on serial dilutions of standards of known concentration being analyzed in parallel with the unknown samples for calibration and reference. Quantitative detection can be achieved using a wide variety of fluorescence detection strategies, including sequence specific probes or universal probes such as SYBR Green dye.<sup>[18]</sup> Sequence specific probes, such as TaqMan (i.e. Applied Biosystems), Molecular Beacons, or Scorpion, bind only to the copied, or cDNA of the appropriate sequence produced during the reaction. SYBR Green dye binds to all double-stranded DNA<sup>[19]</sup> produced during the reaction. While SYBR Green is easy to use, its lack of specificity and lower sensitivity lead most labs to use probe-based qPCR detection schemes. There are many variations of qPCR including the comparative threshold method, which allows relative quantification through comparison of Ct values (PCR cycles that show statistically significant increases in the product) from multiple samples that include an internal standard.<sup>[20]</sup> Since PCR amplifies all target nucleic acid, whether from an intact virion or free nucleic acids in solution, qPCR results (expressed in terms of genome copies/mL) are likely to be higher in quantity than TEM results. For viral quantification, the ratio of whole virions to copies of nucleic acid is seldom one to one due

to the fact that during viral replication, viral assembly in the cytoplasm requires the production of proteins (structural and non-structural) and nucleic acid which are the precursors needed to assemble a whole virion. In the example of foot-and-mouth disease virus, the ratio of whole virions to RNA copies within an actively replicating host cell is approximately 1:1000.<sup>[21]</sup> Commercially available products for qPCR are available through numerous companies such as Invitrogen, Roche and Qiagen just to name a few. Real-time qPCR takes around 1–4 hours and can provide quantitative results containing too few viruses to be analyzed by other methods.

### Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a more modern variation of a protein assay that utilizes a specific antibody linked to an enzyme to detect the presence of an unknown amount of antigen (i.e. virus) in a sample. The antibody-antigen binding event is detected and/or quantified through the enzyme's ability to convert a reagent to a detectable signal that can be used to calculate the concentration of the antigen in the sample.<sup>[22]</sup> Horseradish peroxidase (HRP) is a common enzyme utilized in ELISA schemes due to its ability to amplify signal and increase assay sensitivity. There are many variations, or types of ELISA assays but they can generally be classified as either indirect, competitive, sandwich or reverse.<sup>[23]</sup> ELISA kits are commercially available from numerous companies and quantification generally occurs via chromogenic reporters or fluorescence (e.g. Invitrogen, Santa Cruz Biotechnology Inc.). This technique is much less labor-intensive than the traditional methods and can take anywhere from 4 to 24 hours based on antibody incubation time.

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## 4.2 Polymerase chain reaction

"PCR" redirects here. For other uses, see PCR (disambiguation).

The **polymerase chain reaction (PCR)** is a biochemical technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Developed in 1983 by Kary Mullis,<sup>[1][2]</sup> PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.<sup>[3][4]</sup>

These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.<sup>[5]</sup>

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps.

In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

### 4.2.1 PCR principles and procedure

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of between 0.1 and 10 kilo base pairs (kb),

although some techniques allow for amplification of fragments up to 40 kb in size.<sup>[6]</sup> The amount of amplified product is determined by the available substrates in the reaction, which become limiting as the reaction progresses.<sup>[7]</sup>

A basic PCR set up requires several components and reagents.<sup>[8]</sup> These components include:

- *DNA template* that contains the DNA region (target) to be amplified.
- Two *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.
- *Taq polymerase* or another DNA polymerase with a temperature optimum at around 70 °C.
- *Deoxynucleoside triphosphates* (dNTPs, sometimes called “deoxynucleotide triphosphates”; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- *Buffer solution*, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- *Bivalent cations*, magnesium or manganese ions; generally Mg<sup>2+</sup> is used, but Mn<sup>2+</sup> can be utilized for PCR-mediated DNA mutagenesis, as higher Mn<sup>2+</sup> concentration increases the error rate during DNA synthesis<sup>[9]</sup>
- *Monovalent cation potassium ions*.

The PCR is commonly carried out in a reaction volume of 10–200 µl in small reaction tubes (0.2–0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction (see below). Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require

a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

### Procedure

Typically, PCR consists of a series of 20-40 repeated temperature changes, called cycles, with each cycle commonly consisting of 2-3 discrete temperature steps, usually three (Figure below). The cycling is often preceded by a single temperature step at a high temperature ( $>90\text{ }^{\circ}\text{C}$ ), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature ( $T_m$ ) of the primers.<sup>[10]</sup>

- **Initialization step:** This step consists of heating the reaction to a temperature of 94–96 °C (or 98 °C if extremely thermostable polymerases are used), which is held for 1–9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.<sup>[11]</sup>
- **Denaturation step:** This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.
- **Annealing step:** The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3–5 °C below the  $T_m$  of the primers used. Stable DNA–DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.
- **Extension/elongation step:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C,<sup>[12][13]</sup> and commonly a temperature of 72 °C

is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

- **Final elongation:** This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.
- **Final hold:** This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplicon or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products (see Fig. 3).

### 4.2.2 PCR stages

The PCR process can be divided into three stages:

**Exponential amplification:** At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). The reaction is very sensitive: only minute quantities of DNA need to be present.<sup>[14]</sup>

**Leveling off stage:** The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers causes them to become limiting.

**Plateau:** No more product accumulates due to exhaustion of reagents and enzyme.

### PCR optimization

Main article: [PCR optimization](#)

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions.<sup>[15][16]</sup> Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants.<sup>[8]</sup> This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plasticware, and thoroughly cleaning the work surface between reaction setups. Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA. Addition of reagents, such as formamide, in buffer systems may increase the specificity and yield of PCR.<sup>[17]</sup> Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design.<sup>[18]</sup>

### 4.2.3 Application of PCR

Main article: [Applications of PCR](#)

#### Selective DNA isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many methods, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very

small amounts of starting material.

Other applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid or the genetic material of another organism. Bacterial colonies (*E. coli*) can be rapidly screened by PCR for correct DNA vector constructs.<sup>[19]</sup> PCR may also be used for genetic fingerprinting; a forensic technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.

Some PCR 'fingerprints' methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing (Fig. 4). This technique may also be used to determine evolutionary relationships among organisms.

#### Amplification and quantification of DNA

Because PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ADNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar and the body of English king Richard III.<sup>[20]</sup>

Quantitative PCR methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Quantitative PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

See also: [Use of DNA in forensic entomology](#)

### PCR in diagnosis of diseases

PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000 fold higher than that of other methods.

PCR allows for rapid and highly specific diagnosis of infectious diseases, including those caused by bacteria or viruses.<sup>[21]</sup> PCR also permits identification of non-cultivable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria, or viruses from tissue culture assays and animal models. The basis for PCR diagnostic applications in microbiology is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.<sup>[21][22]</sup>

Viral DNA can likewise be detected by PCR. The primers used need to be specific to the targeted sequences in the DNA of a virus, and the PCR can be used for diagnostic analyses or DNA sequencing of the viral genome. The high sensitivity of PCR permits virus detection soon after infection and even before the onset of disease.<sup>[21]</sup> Such early detection may give physicians a significant lead time in treatment. The amount of virus ("viral load") in a patient can also be quantified by PCR-based DNA quantitation techniques (see below).

#### 4.2.4 Variations on the basic PCR technique

Main article: Variants of PCR

- *Allele-specific PCR*: a diagnostic or cloning technique based on single-nucleotide variations (SNVs not to be confused with SNPs) (single-base differences in a patient). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNV (base pair buffer around SNV usually incorporated). PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an

SNP-specific primer signals presence of the specific SNP in a sequence.<sup>[23]</sup> See [SNP genotyping](#) for more information.

- *Assembly PCR* or *Polymerase Cycling Assembly (PCA)*: artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments, thereby selectively producing the final long DNA product.<sup>[24]</sup>
- *Asymmetric PCR*: preferentially amplifies one DNA strand in a double-stranded DNA template. It is used in [sequencing](#) and hybridization probing where amplification of only one of the two complementary strands is required. PCR is carried out as usual, but with a great excess of the primer for the strand targeted for amplification. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required.<sup>[25]</sup> A recent modification on this process, known as *Linear-After-The-Exponential-PCR (LATE-PCR)*, uses a limiting primer with a higher melting temperature ( $T_m$ ) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.<sup>[26]</sup>
- *Dial-out PCR*: a highly parallel method for retrieving accurate DNA molecules for gene synthesis. A complex library of DNA molecules is modified with unique flanking tags before massively parallel sequencing. Tag-directed primers then enable the retrieval of molecules with desired sequences by PCR.<sup>[27]</sup>
- *Digital PCR (dPCR)*: used to measure the quantity of a target DNA sequence in a DNA sample. The DNA sample is highly diluted so that after running many PCRs in parallel, some of them will not receive a single molecule of the target DNA. The target DNA concentration is calculated using the proportion of negative outcomes. Hence the name 'digital PCR'.
- *Helicase-dependent amplification*: similar to traditional PCR, but uses a constant temperature

rather than cycling through denaturation and annealing/extension cycles. DNA helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.<sup>[28]</sup>

- **Hot start PCR:** a technique that reduces non-specific amplification during the initial set up stages of the PCR. It may be performed manually by heating the reaction components to the denaturation temperature (e.g., 95 °C) before adding the polymerase.<sup>[29]</sup> Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody<sup>[11][30]</sup> or by the presence of covalently bound inhibitors that dissociate only after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.
- **In silico PCR** (digital PCR, virtual PCR, electronic PCR, e-PCR) refers to computational tools used to calculate theoretical polymerase chain reaction results using a given set of primers (probes) to amplify DNA sequences from a sequenced genome or transcriptome. In silico PCR was proposed as an educational tool for molecular biology.<sup>[31]</sup>
- **Intersequence-specific PCR (ISSR):** a PCR method for DNA fingerprinting that amplifies regions between simple sequence repeats to produce a unique fingerprint of amplified fragment lengths.<sup>[32]</sup>
- **Inverse PCR:** is commonly used to identify the flanking sequences around genomic inserts. It involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence.<sup>[33]</sup>
- **Ligation-mediated PCR:** uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking, and DNA footprinting.<sup>[34]</sup>
- **Methylation-specific PCR (MSP):** developed by Stephen Baylin and Jim Herman at the Johns Hopkins School of Medicine,<sup>[35]</sup> and is used to detect methylation of CpG islands in genomic DNA. DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. Two PCRs are then carried out on the modified DNA, using primer sets identical except at any CpG islands within the primer sequences. At these points, one primer set recognizes DNA with cytosines to amplify methylated DNA, and one set recognizes DNA with uracil or thymine to amplify unmethylated DNA. MSP using qPCR can also be performed to obtain quantitative rather than qualitative information about methylation.
- **Miniprimer PCR:** uses a thermostable polymerase (S-Tbr) that can extend from short primers ("smalligos") as short as 9 or 10 nucleotides. This method permits PCR targeting to smaller primer binding regions, and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene.<sup>[36]</sup>
- **Multiplex Ligation-dependent Probe Amplification (MLPA):** permits multiple targets to be amplified with only a single primer pair, thus avoiding the resolution limitations of multiplex PCR (see below).
- **Multiplex-PCR:** consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test-run that otherwise would require several times the reagents and more time to perform. Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction, and amplicon sizes. That is, their base pair length should be different enough to form distinct bands when visualized by gel electrophoresis.
- **Nanoparticle-Assisted PCR (nanoPCR):** In recent years, it has been reported that some nanoparticles (NPs) can enhance the efficiency of PCR (thus being called nanoPCR), and some even perform better than the original PCR enhancers. It was also found that quantum dots (QDs) can improve PCR specificity and efficiency. Single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) are efficient in enhancing the amplification of long PCR. Carbon nanopowder (CNP) was reported be

able to improve the efficiency of repeated PCR and long PCR. ZnO, TiO<sub>2</sub>, and Ag NPs were also found to increase PCR yield. Importantly, already known data has indicated that non-metallic NPs retained acceptable amplification fidelity. Given that many NPs are capable of enhancing PCR efficiency, it is clear that there is likely to be great potential for nanoPCR technology improvements and product development.<sup>[37][38]</sup>

- **Nested PCR:** increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA. Two sets of primers are used in two successive PCRs. In the first reaction, one pair of primers is used to generate DNA products, which besides the intended target, may still consist of non-specifically amplified DNA fragments. The product(s) are then used in a second PCR with a set of primers whose binding sites are completely or partially different from and located 3' of each of the primers used in the first reaction. Nested PCR is often more successful in specifically amplifying long DNA fragments than conventional PCR, but it requires more detailed knowledge of the target sequences.
- **Overlap-extension PCR** or *Splicing by overlap extension (SOEing)*: a genetic engineering technique that is used to splice together two or more DNA fragments that contain complementary sequences. It is used to join DNA pieces containing genes, regulatory sequences, or mutations; the technique enables creation of specific and long DNA constructs. It can also introduce deletions, insertions or point mutations into a DNA sequence.<sup>[39][40]</sup>
- **PAN-AC:** uses isothermal conditions for amplification, and may be used in living cells.<sup>[41][42]</sup>
- **quantitative PCR** (qPCR): used to measure the quantity of a target sequence (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA, or RNA. quantitative PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative PCR has a very high degree of precision. Quantitative PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount

of amplified product in real time. It is also sometimes abbreviated to **RT-PCR** (*real-time* PCR) but this abbreviation should be used only for reverse transcription PCR. qPCR is the appropriate contractions for quantitative PCR (real-time PCR).

- **Reverse Transcription PCR (RT-PCR):** for amplifying DNA from RNA. Reverse transcriptase reverse transcribes RNA into cDNA, which is then amplified by PCR. RT-PCR is widely used in expression profiling, to determine the expression of a gene or to identify the sequence of an RNA transcript, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5' end of a gene (corresponding to the transcription start site) is typically identified by **RACE-PCR** (*Rapid Amplification of cDNA Ends*).
- **Solid Phase PCR:** encompasses multiple meanings, including Polony Amplification (where PCR colonies are derived in a gel matrix, for example), Bridge PCR<sup>[43]</sup> (primers are covalently linked to a solid-support surface), conventional Solid Phase PCR (where Asymmetric PCR is applied in the presence of solid support bearing primer with sequence matching one of the aqueous primers) and Enhanced Solid Phase PCR<sup>[44]</sup> (where conventional Solid Phase PCR can be improved by employing high Tm and nested solid support primer with optional application of a thermal 'step' to favour solid support priming).
- **Suicide PCR:** typically used in paleogenetics or other studies where avoiding false positives and ensuring the specificity of the amplified fragment is the highest priority. It was originally described in a study to verify the presence of the microbe *Yersinia pestis* in dental samples obtained from 14th Century graves of people supposedly killed by plague during the medieval Black Death epidemic.<sup>[45]</sup> The method prescribes the use of any primer combination only once in a PCR (hence the term "suicide"), which should never have been used in any positive control PCR reaction, and the primers should always target a genomic region never amplified before in the lab using this or any other set of primers. This ensures that no contaminating DNA from previ-

ous PCR reactions is present in the lab, which could otherwise generate false positives.

- *Thermal asymmetric interlaced PCR (TAIL-PCR)*: for isolation of an unknown sequence flanking a known sequence. Within the known sequence, TAIL-PCR uses a nested pair of primers with differing annealing temperatures; a degenerate primer is used to amplify in the other direction from the unknown sequence.<sup>[46]</sup>
- *Touchdown PCR (Step-down PCR)*: a variant of PCR that aims to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually a few degrees (3-5 °C) above the  $T_m$  of the primers used, while at the later cycles, it is a few degrees (3-5 °C) below the primer  $T_m$ . The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles.<sup>[47]</sup>
- *Universal Fast Walking*: for genome walking and genetic fingerprinting using a more specific 'two-sided' PCR than conventional 'one-sided' approaches (using only one gene-specific primer and one general primer — which can lead to artefactual 'noise')<sup>[48]</sup> by virtue of a mechanism involving lariat structure formation. Streamlined derivatives of UFW are LaNe RAGE (lariat-dependent nested PCR for rapid amplification of genomic DNA ends),<sup>[49]</sup> 5'RACE LaNe<sup>[50]</sup> and 3'RACE LaNe.<sup>[51]</sup>

## 4.2.5 History

Main article: [History of polymerase chain reaction](#)

A 1971 paper in the *Journal of Molecular Biology* by Kleppe and co-workers first described a method using an enzymatic assay to replicate a short DNA template with primers *in vitro*.<sup>[52]</sup> However, this early manifestation of the basic PCR principle did not receive much attention, and the invention of the polymerase chain reaction in 1983 is generally credited to Kary Mullis.<sup>[53]</sup>

When Mullis developed the PCR in 1983, he was working in Emeryville, California for Cetus Corporation, one of the first biotechnology companies. There, he was responsible for synthesizing short chains of DNA. Mullis has written that he conceived of PCR while cruising along the Pacific Coast Highway one night in his car.<sup>[54]</sup> He was playing in his mind with a new way of analyzing changes (mutations) in DNA when he realized that he had instead invented a method of amplifying any DNA region through repeated cycles of duplication driven by DNA polymerase. In *Scientific American*, Mullis summarized the procedure: "Beginning with a single molecule of the genetic material DNA, the PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat."<sup>[55]</sup> He was awarded the Nobel Prize in Chemistry in 1993 for his invention,<sup>[5]</sup> seven years after he and his colleagues at Cetus first put his proposal to practice. However, some controversies have remained about the intellectual and practical contributions of other scientists to Mullis' work, and whether he had been the sole inventor of the PCR principle (see below).

At the core of the PCR method is the use of a suitable DNA polymerase able to withstand the high temperatures of >90 °C (194 °F) required for separation of the two DNA strands in the DNA double helix after each replication cycle. The DNA polymerases initially employed for *in vitro* experiments presaging PCR were unable to withstand these high temperatures.<sup>[3]</sup> So the early procedures for DNA replication were very inefficient and time consuming, and required large amounts of DNA polymerase and continuous handling throughout the process.

The discovery in 1976 of Taq polymerase — a DNA polymerase purified from the thermophilic bacterium, *Thermus aquaticus*, which naturally lives in hot (50 to 80 °C (122 to 176 °F)) environments<sup>[12]</sup> such as hot springs — paved the way for dramatic improvements of the PCR method. The DNA polymerase isolated from *T. aquaticus* is stable at high temperatures remaining active even after DNA denaturation,<sup>[13]</sup> thus obviating the need to add new DNA polymerase after each cycle.<sup>[4]</sup> This allowed an automated thermocycler-based process for DNA amplification.

### Patent disputes

The PCR technique was patented by Kary Mullis and assigned to Cetus Corporation, where Mullis worked when he invented the technique in 1983. The *Taq* polymerase enzyme was also covered by patents. There have been several high-profile lawsuits related to the technique, including an unsuccessful lawsuit brought by DuPont. The pharmaceutical company Hoffmann-La Roche purchased the rights to the patents in 1992 and currently holds those that are still protected.

A related patent battle over the *Taq* polymerase enzyme is still ongoing in several jurisdictions around the world between Roche and Promega. The legal arguments have extended beyond the lives of the original PCR and *Taq* polymerase patents, which expired on March 28, 2005.<sup>[56]</sup>

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a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence to quantify messenger RNA (mRNA) and non-coding RNA in cells or tissues.

#### 4.2.7 External links

- A Guide to PCR Technologies SelectScience
- OpenPCR Open-source PCR thermalcycler project
- US Patent for PCR
- Step-through animation of PCR - Cold Spring Harbor Laboratory
- OpenWetWare
- What is PCR plateau effect? YouTube tutorial video
- History of the Polymerase Chain Reaction from the Smithsonian Institution Archives
- 3d models of PCR equipment for 3D printing on thin-gverse.com
- Computer exercise. Design of PCR and PCR-RFLP experiments

The MIQE guidelines propose that the abbreviation qPCR be used for quantitative real-time PCR and that RT-qPCR be used for reverse transcription–qPCR . The acronym “RT-PCR” commonly denotes reverse transcription polymerase chain reaction and not real-time PCR, but not all authors adhere to this convention.<sup>[1]</sup>

#### 4.3.1 Background

Cells in all organisms regulate gene expression by turnover of gene transcripts (messenger RNA, abbreviated to mRNA): The amount of an expressed gene in a cell can be measured by the number of copies of an mRNA transcript of that gene present in a sample. In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction (PCR) is a common method for amplifying DNA; for mRNA-based PCR the RNA sample is first reverse-transcribed to cDNA with reverse transcriptase.

In order to amplify small amounts of DNA, the same methodology is used as in conventional PCR using a DNA template, at least one pair of specific primers, deoxyribonucleotides, a suitable buffer solution and a thermo-stable DNA polymerase. A substance marked with a fluorophore is added to this mixture in a thermal cycler that contains sensors for measuring the fluorescence of the fluorophore after it has been excited at the required wavelength allowing the generation rate to be measured for one or more specific products. This allows the rate of generation of the amplified product to be measured at each PCR cycle. The data thus generated can be analysed by computer software to calculate relative gene expression (or mRNA copy number) in several samples. Quantitative PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples.<sup>[2]</sup> This measurement is made after each amplification cycle, and this is the reason why this method is called real time PCR (that is, immediate or simultaneous PCR). In the case of RNA quantitation,

### 4.3 Real-time polymerase chain reaction

For reverse transcription polymerase chain reaction (RT-PCR), see reverse transcription polymerase chain reaction.

A **real-time polymerase chain reaction** is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR), which is used to amplify and simultaneously detect or quantify a targeted DNA molecule.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in “real time”. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for the detection of products in quantitative PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labelled with

the template is complementary DNA (cDNA), which is obtained by reverse transcription of ribonucleic acid (RNA). In this instance the technique used is quantitative RT-PCR or Q-RT-PCR.

Quantitative PCR and DNA microarray are modern methodologies for studying gene expression. Older methods were used to measure mRNA abundance: Differential display, RNase protection assay and Northern blot. Northern blotting is often used to estimate the expression level of a gene by visualizing the abundance of its mRNA transcript in a sample. In this method, purified RNA is separated by agarose gel electrophoresis, transferred to a solid matrix (such as a nylon membrane), and probed with a specific DNA or RNA probe that is complementary to the gene of interest. Although this technique is still used to assess gene expression, it requires relatively large amounts of RNA and provides only qualitative or semi quantitative information of mRNA levels.<sup>[3]</sup> Estimation errors arising from variations in the quantification method can be the result of DNA integrity, enzyme efficiency and many other factors. For this reason a number of standardization systems have been developed. Some have been developed for quantifying total gene expression, but the most common are aimed at quantifying the specific gene being studied in relation to another gene called a normalizing gene, which is selected for its almost constant rate of expression. These genes are also called housekeeping genes as they are usually involved in the functions related to basic cellular survival, which normally implies constitutive gene expression.<sup>[4][5]</sup> This enables researchers to report a ratio for the expression of the genes of interest divided by the expression of the selected normalizer, thereby allowing comparison of the former without actually knowing its absolute level of expression.

The most commonly used normalizing genes are those that code for the following molecules: tubulin, glyceraldehyde-3-phosphate dehydrogenase, albumin, cyclophilin, and ribosomal RNAs.<sup>[3]</sup>

### 4.3.2 Basic principles

Main article: Polymerase chain reaction

Quantitative PCR is carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and DNA polymerase.

The PCR process generally consists of a series of temperature changes that are repeated 25 – 40 times. These cycles normally consist of three stages: the first, at around 95 °C, allows the separation of the nucleic acid's double chain; the second, at a temperature of around 50-60 °C, allows the binding of the primers with the DNA template;<sup>[6]</sup> the third, at between 68 - 72 °C, facilitates the polymerization carried out by the DNA polymerase. Due to the small size of the fragments the last step is usually omitted in this type of PCR as the enzyme is able to increase their number during the change between the alignment stage and the denaturing stage. In addition, some thermal cyclers add another short temperature phase lasting only a few seconds to each cycle, with a temperature of, for example, 80 °C, in order to reduce the noise caused by the presence of primer dimers when a non-specific dye is used. The temperatures and the timings used for each cycle depend on a wide variety of parameters, such as: the enzyme used to synthesize the DNA, the concentration of divalent ions and deoxyribonucleotides (dNTPs) in the reaction and the bonding temperature of the primers.<sup>[7]</sup>

### 4.3.3 Classification

The type of quantitative PCR technique used depends on the DNA sequence in the samples, the technique can either use non-specific fluorochromes or hybridization probes.

#### Quantitative PCR with double-stranded DNA-binding dyes as reporters

A DNA-binding dye binds to all double-stranded (ds) DNA in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, dsDNA dyes such as SYBR Green will bind to all dsDNA

PCR products, including nonspecific PCR products (such as **Primer dimer**). This can potentially interfere with, or prevent, accurate quantification of the intended target sequence. The SYBR Green is excited using blue light ( $\lambda_{\text{max}} = 488 \text{ nm}$ ) and it emits green light ( $\lambda_{\text{max}} = 522 \text{ nm}$ ).<sup>[8]</sup>

1. The reaction is prepared as usual, with the addition of fluorescent dsDNA dye.
2. The reaction is run in a quantitative PCR instrument, and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

This method has the advantage of only needing a pair of primers to carry out the amplification, which keeps costs down; however, it is only possible to amplify a product using a chain reaction.

Like other quantitative PCR methods, the values obtained do not have absolute units associated with them (i.e., mRNA copies/cell). As described above, a comparison of a measured DNA/RNA sample to a standard dilution will only give a fraction or ratio of the sample relative to the standard, allowing only relative comparisons between different tissues or experimental conditions. To ensure accuracy in the quantification, it is usually necessary to normalize expression of a target gene to a stably expressed gene (see below). This can correct possible differences in RNA quantity or quality across experimental samples.

#### Fluorescent reporter probe method

Fluorescent reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. Fluorescent probes can be used in multiplex assays—for detection of several genes in the same reaction—based on specific probes with different-coloured labels, provided that all targeted genes are amplified with similar efficiency. The specificity of fluorescent reporter probes also prevents interference of measurements caused by **primer dimers**,

which are undesirable potential by-products in PCR. However, fluorescent reporter probes do not prevent the inhibitory effect of the primer dimers, which may depress accumulation of the desired products in the reaction.

The method relies on a DNA-based probe with a fluorescent reporter at one end and a **quencher** of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of the **Taq polymerase** breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after **excitation** with a laser. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.

1. The PCR is prepared as usual (see **PCR**), and the reporter probe is added.
2. As the reaction commences, during the annealing stage of the PCR both probe and primers anneal to the DNA target.
3. Polymerisation of a new DNA strand is initiated from the primers, and once the polymerase reaches the probe, its 5'-3'-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence.
4. Fluorescence is detected and measured in a real-time PCR machine, and its geometric increase corresponding to exponential increase of the product is used to determine the quantification cycle ( $C_q$ ) in each reaction.

#### 4.3.4 Fusion temperature analysis

Q-PCR permits the identification of specific, amplified DNA fragments using analysis of their **melting temperature** (also called  $T_m$  value, from *melting temperature*). The method used is usually PCR with double-stranded DNA-binding dyes as reporters and the dye used is usually SYBR Green. The DNA melting temperature is specific to the amplified fragment. The results of this technique are obtained

by comparing the dissociation curves of the analysed DNA samples.<sup>[10]</sup>

Unlike conventional PCR, this method avoids the previous use of **electrophoresis** techniques to demonstrate the results of all the samples. This is because, despite being a kinetic technique, quantitative PCR is usually evaluated at a distinct end point. The technique therefore usually provides more rapid results and / or uses fewer reactants than electrophoresis. If subsequent electrophoresis is required it is only necessary to test those samples that real time PCR has shown to be doubtful and / or to ratify the results for samples that have tested positive for a specific determinant.

### Quantification of gene expression

Quantifying gene expression by traditional DNA detection methods is unreliable. Detection of **mRNA** on a **Northern blot** or PCR products on a **gel** or **Southern blot** does not allow precise quantification.<sup>[11]</sup> For example, over the 20-40 cycles of a typical PCR, the amount of DNA product reaches a **plateau** that is not directly correlated with the amount of target DNA in the initial PCR.

Quantitative PCR can be used to quantify **nucleic acids** by two common methods: relative quantification and absolute quantification.<sup>[12]</sup> Absolute quantification gives the exact number of target DNA molecules by comparison with DNA standards using a **calibration curve**. It is therefore essential that the PCR of the sample and the standard have the same **amplification efficiency**. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. The quantification is expressed as the change in expression levels of mRNA interpreted as **complementary DNA** (cDNA, generated by **reverse transcription** of mRNA). Relative quantification is easier to carry out as it does not require a calibration curve as the amount of the studied gene is compared to the amount of a control housekeeping gene.

As the units used to express the results of relative quantification are unimportant the results can be compared across a number of different RT-Q-PCR. The reason for using one or more housekeeping genes is to correct non-specific variation, such as the differences in the quantity and quality of RNA used, which can affect the efficiency of reverse transcription and therefore that of the whole PCR process.

However, the most crucial aspect of the process is that the reference gene must be stable.<sup>[13]</sup>

The selection of these reference genes was traditionally carried out in molecular biology using qualitative or semi-quantitative studies such as the visual examination of RNA gels, Northern blot **densitometry** or semi-quantitative PCR (PCR mimics). Now, in the **genome** era, it is possible to carry out a more detailed estimate for many organisms using **DNA microarrays**.<sup>[14]</sup> However, research has shown that amplification of the majority of reference genes used in quantifying the expression of mRNA varies according to experimental conditions.<sup>[15][16][17]</sup> It is therefore necessary to carry out an initial statistically sound methodological study in order to select the most suitable reference gene.

A number of statistical algorithms have been developed that can detect which gene or genes are most suitable for use under given conditions. Those like **geNORM** or **BestKeeper** can compare pairs or **geometric means** for a matrix of different reference genes and tissues.<sup>[18][19]</sup>

### Modeling

Unlike end point PCR (conventional PCR) real time PCR allows quantification of the desired product at any point in the amplification process by measuring fluorescence (in reality, measurement is made of its level over a given threshold). A commonly employed method of DNA quantification by quantitative PCR relies on plotting fluorescence against the number of cycles on a **logarithmic scale**. A threshold for detection of DNA-based fluorescence is set slightly above background. The number of cycles at which the fluorescence exceeds the threshold is called the **threshold cycle** ( $C_t$ ) or, according to the MIQE guidelines, **quantification cycle** ( $C_q$ ).<sup>[20]</sup>

During the exponential amplification phase, the quantity of the target DNA template (amplicon) doubles every cycle. For example, a DNA sample whose  $C_q$  precedes that of another sample by 3 cycles contained  $2^3 = 8$  times more template. However, the efficiency of amplification is often variable among primers and templates. Therefore, the efficiency of a primer-template combination is assessed in a **titration experiment** with serial dilutions of DNA template to create a **standard curve** of the change in  $C_q$  with each dilution. The **slope** of the **linear regression** is then used to

determine the efficiency of amplification, which is 100% if a dilution of 1:2 results in a  $C_q$  difference of 1. The cycle threshold method makes several assumptions of reaction mechanism and has a reliance on data from low signal-to-noise regions of the amplification profile that can introduce substantial variance during the data analysis.<sup>[21]</sup>

To quantify gene expression, the  $C_q$  for an RNA or DNA from the gene of interest is subtracted from the  $C_q$  of RNA/DNA from a housekeeping gene in the same sample to normalize for variation in the amount and quality of RNA between different samples. This normalization procedure is commonly called the  $\Delta C_t$ -method<sup>[22]</sup> and permits comparison of expression of a gene of interest among different samples. However, for such comparison, expression of the normalizing reference gene needs to be very similar across all the samples. Choosing a reference gene fulfilling this criterion is therefore of high importance, and often challenging, because only very few genes show equal levels of expression across a range of different conditions or tissues.<sup>[23][24]</sup> Although cycle threshold analysis is integrated with many commercial software systems, there are more accurate and reliable methods of analysing amplification profile data that should be considered in cases where reproducibility is a concern.<sup>[21]</sup>

Mechanism-based qPCR quantification methods have also been suggested, and have the advantage that they do not require a standard curve for quantification. Methods such as MAK2<sup>[25]</sup> have been shown to have equal or better quantitative performance to standard curve methods. These mechanism-based methods use knowledge about the polymerase amplification process to generate estimates of the original sample concentration. An extension of this approach includes an accurate model of the entire PCR reaction profile, which allows for the use of high signal-to-noise data and the ability to validate data quality prior to analysis.<sup>[21]</sup>

### 4.3.5 Applications

There are numerous applications for quantitative polymerase chain reaction in the laboratory. It is commonly used for both diagnostic and basic research. Uses of the technique in industry include the quantification of microbial load in foods or on vegetable matter, the detection of

GMOs (Genetically modified organisms) and the quantification and genotyping of human viral pathogens.

#### Diagnostic uses

Diagnostic quantitative PCR is applied to rapidly detect nucleic acids that are diagnostic of, for example, infectious diseases, cancer and genetic abnormalities. The introduction of quantitative PCR assays to the clinical microbiology laboratory has significantly improved the diagnosis of infectious diseases,<sup>[26]</sup> and is deployed as a tool to detect newly emerging diseases, such as new strains of flu, in diagnostic tests.<sup>[27]</sup>

#### Microbiological uses

Quantitative PCR is also used by microbiologists working in the fields of food safety, food spoilage and fermentation and for the microbial risk assessment of water quality (drinking and recreational waters) and in public health protection.<sup>[28]</sup>

The antibacterial assay Virtual Colony Count<sup>[29]</sup> utilizes a data quantification technique called Quantitative Growth Kinetics (QGK) that is mathematically identical to QPCR, except bacterial cells, rather than copies of a PCR product, increase exponentially. The QGK equivalent of the threshold cycle is referred to as the “threshold time”.

#### Uses in research

In research settings, quantitative PCR is mainly used to provide quantitative measurements of gene transcription. The technology may be used in determining how the genetic expression of a particular gene changes over time, such as in the response of tissue and cell cultures to an administration of a pharmacological agent, progression of cell differentiation, or in response to changes in environmental conditions. It is also used for the determination of zygosity of transgenic animals used in research.

#### Detection of phytopathogens

The agricultural industry is constantly striving to produce plant propagules or seedlings that are free of pathogens

in order to prevent economic losses and safeguard health. Systems have been developed that allow detection of small amounts of the DNA of *Phytophthora ramorum*, an oomycete that kills Oaks and other species, mixed in with the DNA of the host plant. Discrimination between the DNA of the pathogen and the plant is based on the amplification of ITS sequences, spacers located in ribosomal RNA gene's coding area, which are characteristic for each taxon.<sup>[30]</sup> Field-based versions of this technique have also been developed for identifying the same pathogen.<sup>[31]</sup>

### Detection of genetically modified organisms

qPCR using reverse transcription (RT-qPCR) can be used to detect GMOs given its sensitivity and dynamic range in detecting DNA. Alternatives such as DNA or protein analysis are usually less sensitive. Specific primers are used that amplify not the transgene but the promoter, terminator or even intermediate sequences used during the process of engineering the vector. As the process of creating a transgenic plant normally leads to the insertion of more than one copy of the transgene its quantity is also commonly assessed. This is often carried out by relative quantification using a control gene from the treated species that is only present as a single copy.<sup>[32][33]</sup>

### Clinical quantification and genotyping

Viruses can be present in humans due to direct infection or co-infections. This makes diagnosis difficult using classical techniques and can result in an incorrect prognosis and treatment. The use of qPCR allows both the quantification and genotyping (characterization of the strain, carried out using melting curves) of a virus such as the Hepatitis B virus.<sup>[34]</sup> The degree of infection, quantified as the copies of the viral genome per unit of the patient's tissue, is relevant in many cases; for example, the probability that the type 1 herpes simplex virus reactivates is related to the number of infected neurons in the ganglia.<sup>[35]</sup> This quantification is carried out either with reverse transcription or without it, as occurs if the virus becomes integrated in the human genome at any point in its cycle, such as happens in the case of HPV (human papillomavirus), where some of its variants are associated with the appearance of cervical cancer.<sup>[36]</sup>

### 4.3.6 References

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### 4.3.8 External links

- Beginners Guide to Real Time PCR by Primerdesign
- The Reference in Q-PCR Academic & Industrial Information Platform
- Real Time PCR Tutorial by Dr Margaret Hunt, University of South Carolina, September 5, 2006

- openwetware
- RefGenes Open Access online tool to identify tissue specific reference genes for RT-qPCR
- Realtime PCR user experiences
- Articles about Real Time Pcr

## 4.4 Plaque-forming unit

In virology, a **plaque-forming unit (PFU)** is a measure of the number of particles capable of forming plaques per unit volume, such as **virus** particles. It is a functional measurement rather than a measurement of the absolute quantity of particles: viral particles that are defective or which fail to infect their target cell will not produce a plaque and thus will not be counted. For example, a solution of **tick-borne encephalitis virus** with a concentration of 1,000 PFU/ $\mu\text{l}$  indicates that 1  $\mu\text{l}$  of the solution contains enough virus particles to produce 1000 infectious plaques in a cell monolayer, but no inference can be made about the relationship of pfu to number of virus particles.

### 4.4.1 External links

- Definition from Biology-Online.org

## 4.5 Multiplicity of infection

In virology, the **multiplicity of infection** or **MOI** is the ratio of agents (e.g. **phage** or **virus**) to infection targets (e.g. **cell**). For example, when referring to a group of cells inoculated with virus particles, the multiplicity of infection or MOI is the ratio of the number of virus particles to the number of target cells present in a defined space.

### 4.5.1 Interpretation

The actual number of phages or viruses that will enter any given cell is a **statistical process**: some cells may absorb more than one virus particle while others may not absorb

any. The probability that a cell will absorb  $n$  virus particles when inoculated with an MOI of  $m$  can be calculated for a given population using a **Poisson distribution**.

$$P(n) = \frac{m^n \cdot e^{-m}}{n!}$$

where  $m$  is the multiplicity of infection or MOI,  $n$  is the number of infectious agents that enter the infection target, and  $P(n)$  is the probability that an infection target (a cell) will get infected by  $n$  infectious agents.

In fact the infectivity of the virus in question will alter this relationship. One way around this is to use a functional definition of infectious particles rather than a strict count, such as a **plaque forming unit**.

For example, when an MOI of 1 (1 viral particle per cell) is used to infect a population of cells, the probability that a cell will not get infected is  $P(0) = 36.79\%$ , and the probability that it be infected by a single particle is  $P(1) = 36.79\%$ , by two particles is  $P(2) = 18.39\%$ , by three particles is  $P(3) = 6.13\%$ , and so on.

The average percentage of cells that will become infected as a result of inoculation with a given MOI can be obtained by realizing that it is simply  $P(n > 0) = 1 - P(0)$ . Hence, the average fraction of cells that will become infected following an inoculation with an MOI of  $m$  is given by:

$$P(n > 0) = 1 - P(n = 0) = 1 - \frac{m^0 \cdot e^{-m}}{0!} = 1 - e^{-m}$$

which is approximately equal to  $m$  for small values of  $m \ll 1$ .

### MOI Examples

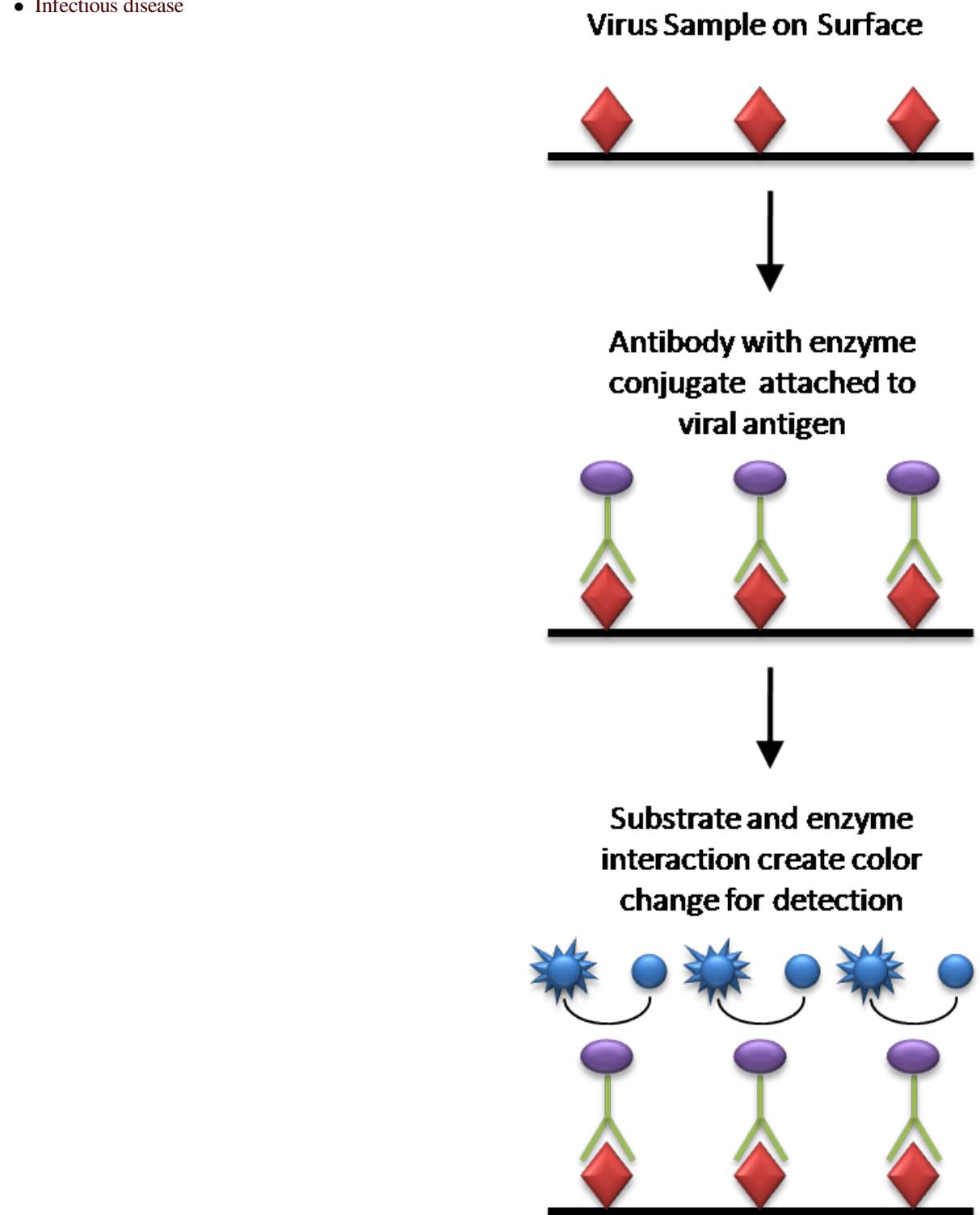
As the MOI increases, the percentages of cells infected with at least one viral particle also increases.

Fields' virology, Part 1 By Bernard N. Fields,David Mahan Knipe,Peter M. Howley,Diane E. Griffin

### 4.5.2 See also

- LD<sub>50</sub>

- Infectious disease



ELISA Diagram



*Figure 1a:* A thermal cycler for PCR

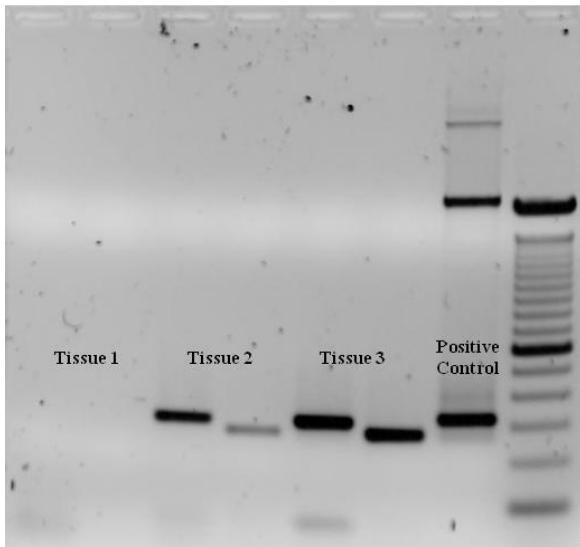
A strip of eight PCR tubes, each containing a 100  $\mu$ l reaction mixture



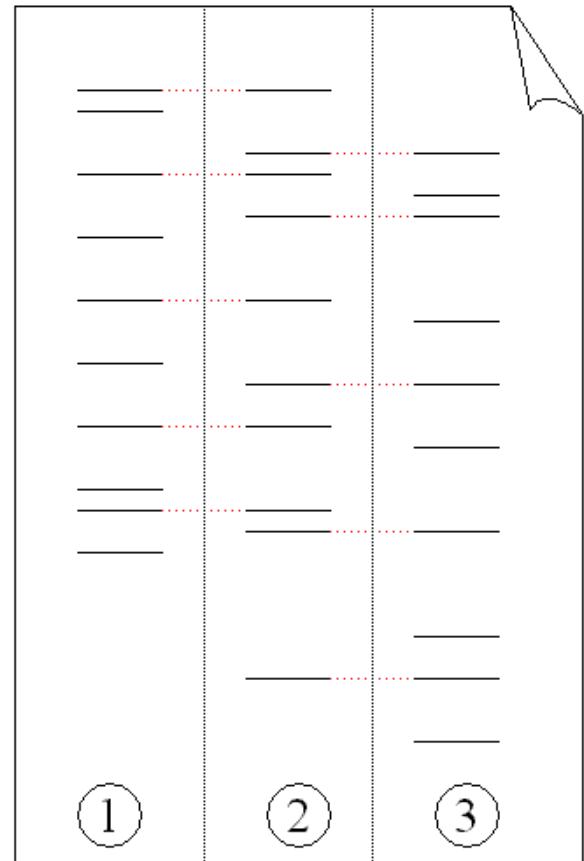
Placing a strip of eight PCR tubes, each containing a 100  $\mu$ l reaction mixture, into the thermal cycler



*Figure 1b:* An older model three-temperature thermal cycler for PCR



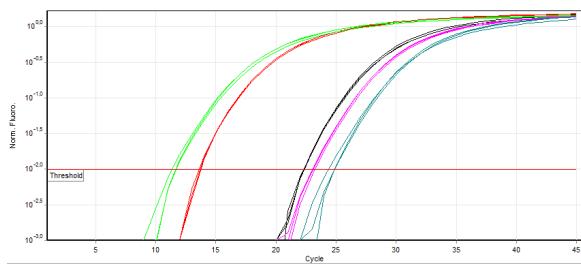
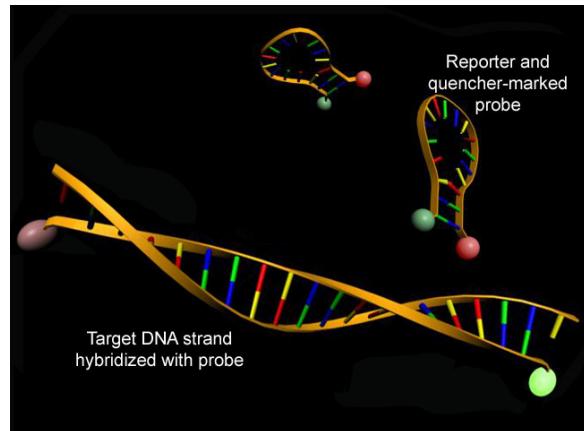
**Figure 3:** Ethidium bromide-stained PCR products after gel electrophoresis. Two sets of primers were used to amplify a target sequence from three different tissue samples. No amplification is present in sample #1; DNA bands in sample #2 and #3 indicate successful amplification of the target sequence. The gel also shows a positive control, and a DNA ladder containing DNA fragments of defined length for sizing the bands in the experimental PCRs.



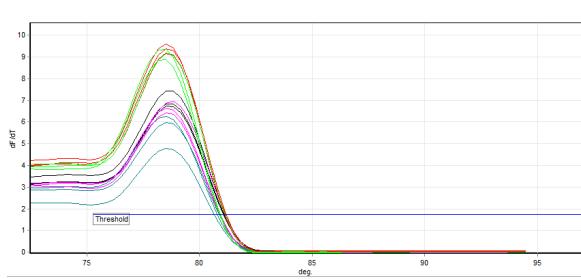
**Figure 4:** Electrophoresis of PCR-amplified DNA fragments. (1) Father. (2) Child. (3) Mother. The child has inherited some, but not all of the fingerprint of each of its parents, giving it a new, unique fingerprint.



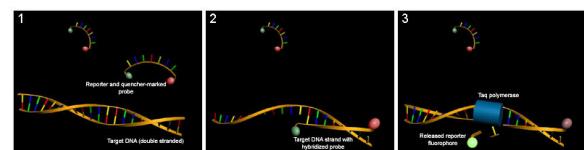
*“Baby Blue”, a 1986 prototype machine for doing PCR*



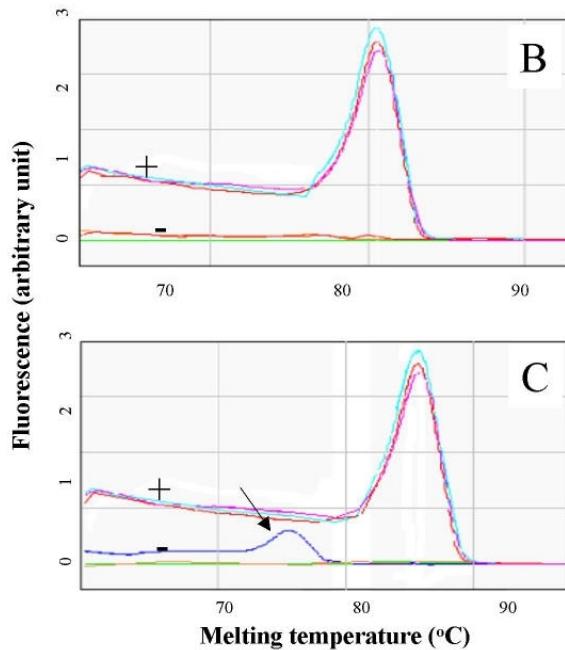
*SYBR Green fluorescence chart for five samples, each having three replicates, which is a result of quantitative PCR (qPCR).*



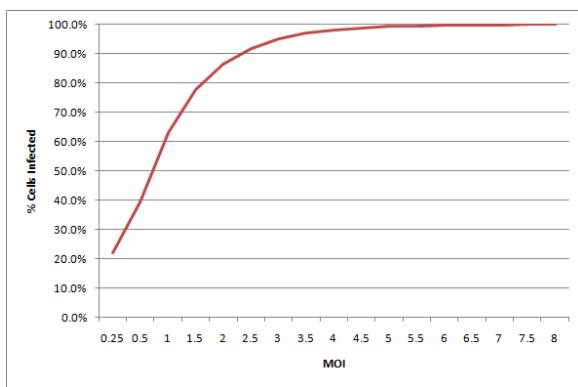
*Melting curve for five samples, three replicates each, which is a result of melting temperature analysis of quantitative PCR results (qPCR).*



(1) In intact probes, reporter fluorescence is quenched. (2) Probes and the complementary DNA strand are hybridized and reporter fluorescence is still quenched. (3) During PCR, the probe is degraded by the Taq polymerase and the fluorescent reporter released.



*Distinct fusion curves for a number of PCR products (showing distinct colours). Amplification reactions can be seen for a specific product (pink, blue) and others with a negative result (green, orange). The fusion peak indicated with an arrow shows the peak caused by primer dimers, which is different from the expected amplification product.<sup>[9]</sup>*



*Percentage of cells infected based on MOI.*

# Chapter 5

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### 5.1 Text

- **Double-stranded RNA viruses** *Source:* [http://en.wikipedia.org/wiki/Double-stranded\\_RNA\\_viruses?oldid=607146236](http://en.wikipedia.org/wiki/Double-stranded_RNA_viruses?oldid=607146236) *Contributors:* The Anome, Michael Hardy, Gabbe, Gadfium, Rich Farmbrough, Guettarda, Arcadian, Woohookitty, Rjwilmsi, Leptictidium, Martin.Budden, Nick Warren, RDBrown, Maksim-bot, ChazYork, Ruslik0, Narayanese, Magioladitis, Read-write-services, Mikael Häggström, Speciate, DrMicro, Hehkuviini, GrahamColm, Touchstone42, TableManners, Unused0026, Nobody of Consequence, DumZiBoT, XLinkBot, Addbot, Yobot, Citation bot, ArcadianOnUnsecuredLoc, RibotBOT, LucienBOT, Molitorppd22, EmausBot, CatPath, ComfyKem, Kevhack and Anonymous: 11
- **Rotavirus** *Source:* <http://en.wikipedia.org/wiki/Rotavirus?oldid=620894666> *Contributors:* AxelBoldt, Magnus Manske, Bryan Derksen, The Anome, Josh Grosse, Rmhermen, William Avery, Azhyd, Michael Hardy, Gabbe, Julesd, Raven in Orbit, Wik, Floydian, Wilke, SpellBott, Giftlite, Zigger, Mboverload, Chowbok, Lesgles, Karl-Henner, Davidfraser, Discospinster, Rich Farmbrough, Bobo192, Davidruben, Arcadian, Orangemarlin, Wouterstomp, Honeydew, Suruena, Tony Sidaway, Gene Nygaard, Mosesofmason, Stemonitis, Bobrayner, KaurJmeb, PeterJohnson, Hovea, Dysepsion, FreplySpang, Grammarbot, Rjwilmsi, Monocyte, RobertG, Salvadorjo, Stevenfruitsmaak, BradBeattie, Visor, Rsrikanth05, Thane, Draeco, NawlinWiki, -OOPSIE-, IlyaV, Ravedave, Tony1, Oliverdl, Calvin08, 21655, Lt-wiki-bot, Colin, Paul Erik, Jkpjkp, SmackBot, Espresso Addict, Unyoysga, ProveIt, HalfShadow, Rst20xx, RDBrown, SchfiftyThree, Uthbrian, Snowmanradio, Roncalli, EVula, G716, DMacks, The undertow, DOI11.10, Euchiasmus, LordFenix, NovumTestamentum, Ben Moore, Bless sins, Smith609, Stralle, Sandy-Georgia, Jetsfan196, Nreddyk, Blehfu, Dlohcierekim, Fvasconcellos, CWY2190, Ruslik0, Skoch3, HalJor, RelentlessRecusant, Narayanese, GuardsRedCoupe, Kozuch, DocLightning, Thijs!bot, Epbr123, Headbomb, Silver Edge, Yupik, Luna Santin, TimVickers, Darklilac, Spencer, MER-C, Magioladitis, WolfmanSF, Bongwarrior, Meredyth, Pinkstarmaci, AuburnPilot, Dhmuch, Zenomax, Recurring dreams, Ciar, Cool Nerd, Read-write-services, Yatta!, Yobol, MartinBot, R'n'B, J.delanoy, Silverxxx, Keesiewonder, Mikael Häggström, Plindenbaum, Mike V, Funandtrvl, DrMicro, AlnoktaBOT, Kakoui, DoorsAjar, GimmeBot, Una Smith, Iwhowley, Hannes Röst, Eubulides, Wikineer, Invtemp, Insanity Incarnate, Skarz, Jmh649, Pijuwy, SieBot, GrahamColm, Scarian, Gerakibot, Phe-bot, Dawn Bard, Flyer22, Nrvilla, Oxymoron83, Lightmouse, Mike2vil, Maralia, FV alternate, Kanonkas, Touchstone42, Elassint, ClueBot, The Thing That Should Not Be, Mild Bill Hiccup, Dosei2, Lartoven, Rhododendrites, Medos2, Dana boomer, Deldot on a public computer, Kruusamägi, Avoided, Zodon, Sgpsaros, Crankarm, Addbot, Ben10027, DOI bot, Jojbutton, Blechnic, Porkadelia, Diptanshu.D, LinkFA-Bot, Vasil', Teles, আর্দ্র মটনাগৰ, Luckas-bot, Yobot, ChildSurvival, Beniscool555, Viralzone, Tempodivalse, Rtyq2, Citation bot, Schieße, Xqbot, Michalisphyl, Capricorn42, ArcadianOnUnsecuredLoc, Bobby1012034, Useknowledge, J04n, GrouchoBot, Dheeraj, RibotBOT, Philippe Le Mercier, Some standardized rigour, VI, Citation bot 1, Chrisdwiki, CousinJohn, Jonkerz, Lotje, LawBot, Lzman0506, Slon02, EmausBot, Mythic Dawn Agent, Tommy2010, Pikachu4170, ZéroBot, Kgsbot, ChuispastonBot, Spicemix, ClueBot NG, Kendrawar, Champion98, Snotbot, Widr, Guptan99, Mikeds csu, CitationCleaner-Bot, Unaproachable, Biosthmors, BugDocCanuck, Editfromwithout, BrightStarSky, Br'er Rabbit, ComfyKem, Electronsaregreen, February 1514, Syedisa, RS10421, Seppi333, LittleAviator, Monkbot and Anonymous: 232
- **Gastroenteritis** *Source:* <http://en.wikipedia.org/wiki/Gastroenteritis?oldid=618081133> *Contributors:* Manning Bartlett, BlckKnght, Alex.tan, Michael Hardy, Menchi, Axrosen, Ahoerstemeier, Theresa knott, Ekips, Robbot, Nurg, Somercet, Giftlite, Zigger, Jfdwolff, Antandrus, Mza-

jac, Mvc, PFHLai, Tubedogg, JeffreyN, Rlcantwell, M1ss1ontomars2k4, SimonEast, Diablo SV, Discospinster, Rich Farmbrough, Pie4all88, Bender235, BjarteSorensen, Art LaPella, RoyBoy, Jonathan Drain, Spalding, Davidruben, Arcadian, La goutte de pluie, Sam Korn, Drphilngood, Alansohn, Wouterstomp, Hu, Noosphere, ReyBrujo, Stephan Leeds, Axeman89, Kenyon, Ron Ritzman, Stemonitis, Angr, 2004-12-29T22:45Z, Linas, Scjessey, Rickjpelleg, Tabletop, Preisler, GraemeLeggett, Graham87, BD2412, Kbdank71, Miq, Solace098, Rjwilmsi, Craig Sunderland, Bubba73, Gilesmorant, JohnElder, RexNL, Borgx, Hede2000, Hydrargyrum, Rsrikanth05, NawlinWiki, Wiki alf, Phileo, Zwobot, Barjazz, Rwalker, Blowdart, Nescio, Zzuuzz, MathsIsFun, Badgetrg, Kaicarver, Mdwyer, Zvika, Nick Michael, SmackBot, Espresso Addict, Michael Dorosh, Kslays, BiT, Edgar181, Shai-kun, Ohnoitsjamie, Eug, Quinsareth, RDBrown, Thumperward, Craig t moore, Can't sleep, clown will eat me, Yorick8080, Addshore, Krich, Bigturtle, BTerran, DMacks, NIRVANA2764, The undertow, LtPowers, Purplepumpkins, Goodnight-mush, Jcr13, Beetstra, Eridani, Xionbox, Walton One, GDallimore, Thewebdruid, Audiosmurf, IanOfNorwich, Radiant chains, BFD1, Contusio cordis, Nayeryoukim, JFreeman, CorpX, Valoc, DumbBOT, Thijs!bot, Barticus88, Dark Enigma, SvenAERTS, AntiVandalBot, Yupik, K.O.T., TimVickers, Voyages, JAnDbot, Ph.eyes, Vituperex, Charlene.fic, .anacondabot, TheChard, Blackicehorizon, Brusegadi, Jabaker75, Curdeius, WhatamIdoing, ZackTheJack, Ashnik, Edward321, Scottalter, Yobol, NAHID, Anaxial, J.delanoy, AltiusBimm, J intela, Jreferee, Ryohki98, Naniwako, Mikael Häggström, Trumps1337, Xenonice, Tfagrell, VolkovBot, Su-steve, DarthGator, JBazuzi, TXiKiBoT, Crohnie, Collegiate21, Una Smith, Jimmy87nottingham, Andymccullough27, Tim.mcintyre, Sbakka, Vanished user lkdfj39u3mfk4, 613 The Evil, Jmh649, Telemachus Claudius Rhade, SieBot, GrahamColm, Sakkura, Proterometra, Diego Grez, Shinedown89, Tatterfly, TheCatalyst31, ClueBot, Mizz Moo, DawnOfDark, Mattgirling, Drmies, Mild Bill Hiccup, SuperHamster, Kitty53, Cirt, Excirial, Socrates2008, SpikeToronto, Dtpong, Editor510, Noxia, Howfar, Dana boomer, DumZiBoT, 98dblachr, Peptastic, Doc9871, Airplaneman, Addbot, DOI bot, Yiiman, Fieldday-sunday, DylmnMcC, D0762, Orinoco-w, Diptanshu.D, Cst17, MrOllie, Moot0000, DubaiTerminator, 77755, Katkooot, MuZemike, Ettrig, Ben Ben, Beeswaxcandle, ChildSurvival, Lordmw, AnomieBOT, Citation bot, Jmarchn, ArthurBot, Xqbot, Drilnoth, Dr honest, Useknowledge, Tomball-guy, SURF24.7, Bigdaddyeor, Doulos Christos, Killdec, FrescoBot, Tanner566, HamburgerRadio, Citation bot 1, Bondjfhdas, Jsrgls, Bdbaby, Tomcat7, Corinne68, Tim1357, TobeBot, Pooman11111, Reaper Eternal, Angelito7, RjwilmsiBot, J36miles, EmausBot, WikitanvirBot, GA bot, Wong1133, Wham Bam Rock II, Mauro Tozzi, Uploadavirus, ZéroBot, ShabBot, Ox14c3t13n, Hazard-SJ, Fender112390, AzurisCaine, L Kensington, DASHBotAV, Petrb, ClueBot NG, Toast6578, Fprmad, Oaklandgiants, Jk2q3jrklse, Helpful Pixie Bot, Lowercase sigmabot, Nanook8badger, Drmaxwellfish, Megpori, Jonadin93, BattyBot, Biosthmors, Yeokesh, YFdyh-bot, KatieLikespotatoes, BrightStarSky, Ildiko Santana, Ponyy, Anrusna, Monkbot and Anonymous: 401

- **Rotaviral enteritis** *Source:* [http://en.wikipedia.org/wiki/Rotaviral\\_enteritis?oldid=618449562](http://en.wikipedia.org/wiki/Rotaviral_enteritis?oldid=618449562) *Contributors:* Gabbe, Rjwilmsi, RDBrown, Jmh649, Addbot, Diptanshu.D, FrescoBot, EmausBot, Bamyers99, Snotbot, Rtyho usa, BattyBot, ComfyKem, Monkbot and Anonymous: 1
- **NSP1 (rotavirus)** *Source:* [http://en.wikipedia.org/wiki/NSP1\\_\(rotavirus\)?oldid=604889242](http://en.wikipedia.org/wiki/NSP1_(rotavirus)?oldid=604889242) *Contributors:* The Anome, Arcadian, Chris Capoccia, ProveIt, Eastlaw, Skier Dude, Plindenbaum, Zodon, DOI bot, Citation bot, Citation bot 1, RjwilmsiBot, Parikshit44, BattyBot, Pbagchi, Monkbot and Anonymous: 3
- **NSP2 (rotavirus)** *Source:* [http://en.wikipedia.org/wiki/NSP2\\_\(rotavirus\)?oldid=592676003](http://en.wikipedia.org/wiki/NSP2_(rotavirus)?oldid=592676003) *Contributors:* Arcadian, Chris Capoccia, Smack-Bot, ProveIt, Yupik, Plindenbaum, DOI bot, Citation bot 1, ChrisGualtieri and Monkbot
- **NSP3 (rotavirus)** *Source:* [http://en.wikipedia.org/wiki/NSP3\\_\(rotavirus\)?oldid=561345880](http://en.wikipedia.org/wiki/NSP3_(rotavirus)?oldid=561345880) *Contributors:* Rjwilmsi, RussBot, Chris Capoccia, ProveIt, Emeraude, Skier Dude, Plindenbaum, Zodon, DOI bot, Dawynn, Citation bot, Citation bot 1, Tzu Zha Men and Anonymous: 1
- **NSP4 (rotavirus)** *Source:* [http://en.wikipedia.org/wiki/NSP4\\_\(rotavirus\)?oldid=545016841](http://en.wikipedia.org/wiki/NSP4_(rotavirus)?oldid=545016841) *Contributors:* Arcadian, RussBot, Chris Capoccia, ProveIt, Skier Dude, Plindenbaum, Butseriouslyfolks, Jack the Stripper, CorenSearchBot, Zodon, Addbot, DOI bot, Citation bot, Citation bot 1 and ZéroBot
- **NSP5 (rotavirus)** *Source:* [http://en.wikipedia.org/wiki/NSP5\\_\(rotavirus\)?oldid=592676015](http://en.wikipedia.org/wiki/NSP5_(rotavirus)?oldid=592676015) *Contributors:* The Anome, Arcadian, RussBot, Chris Capoccia, ProveIt, Skier Dude, Plindenbaum, Zodon, DOI bot, Citation bot, DrilBot and Monkbot
- **NSP6 (rotavirus)** *Source:* [http://en.wikipedia.org/wiki/NSP6\\_\(rotavirus\)?oldid=592676031](http://en.wikipedia.org/wiki/NSP6_(rotavirus)?oldid=592676031) *Contributors:* Arcadian, RussBot, Chris Capoccia, ProveIt, Skier Dude, Plindenbaum, Zodon, Addbot, DOI bot, Citation bot, Citation bot 1, DrilBot, ZéroBot and Monkbot
- **Reassortment** *Source:* <http://en.wikipedia.org/wiki/Reassortment?oldid=620819480> *Contributors:* Alan Liefing, Inkling, Icarins, Thorwald, DanielCD, Rich Farmbrough, JustinWick, Arcadian, Wipfeln, AySz88, JamesEG, WAS 4.250, EncycloPetey, Bluebot, RDBrown, Baronnet, Snowmanradio, JohnM4402, Kencf0618, Thijs!bot, Gustaw, Emw, Sabedon, Genometer, Nadiatalent, Addbot, AkhtaBot, Ettrig, Citation bot, Almabot, Mouagip, Pinethicket, Broager, ZéroBot, Vibrelm, Clarkdn, Helpful Pixie Bot, KLBot2 and Anonymous: 4
- **Cytopathic effect** *Source:* [http://en.wikipedia.org/wiki/Cytopathic\\_effect?oldid=611653626](http://en.wikipedia.org/wiki/Cytopathic_effect?oldid=611653626) *Contributors:* Nina, Skysmith, Alan Liefing, RJFJR, ^demon, Graham87, Nihiltres, Nephron, DRosenbach, SmackBot, CmdrObot, Kauczuk, Sabedon, Fences and windows, Theanatomica, Royissick, BotanyBot, The Stickler, Huku-chan, Addbot, Tohd8BohaihthuGh1, Amirobot, FrescoBot, SW3 5DL, Derild4921, ZéroBot, Earthbee24, Makecat-bot, Hirumeshi and Anonymous: 17
- **Sodium-glucose transport proteins** *Source:* [http://en.wikipedia.org/wiki/Sodium-glucose\\_transport\\_proteins?oldid=618156630](http://en.wikipedia.org/wiki/Sodium-glucose_transport_proteins?oldid=618156630) *Contributors:* Robbot, Enz1, Arcadian, Drphilharmonic, Dikteren, Vihsadas, Boghog, Rod57, Mikael Häggström, TXiKiBoT, Cmcnicoll, DumZiBoT, Addbot, Divedeep, Luckas-bot, Anypodetos, Armando Navarro, FrescoBot, RjwilmsiBot, Uanfala, Factman51, 1234r00t, Nrsmoll, Frietjes, BG19bot, Pbates 67, Jimw338, Monkbot and Anonymous: 19

- **Brush border** *Source:* [http://en.wikipedia.org/wiki/Brush\\_border?oldid=608766304](http://en.wikipedia.org/wiki/Brush_border?oldid=608766304) *Contributors:* Arcadian, FlaBot, The Rambling Man, Wavelength, IceCreamAntisocial, Reyk, Garion96, Crystallina, Bluebot, Clicketyclack, Was a bee, Joehall45, Pdeitiker, Wbensmith, Happy B., Bruinleth, DumZiBoT, Addbot, Aboctok, John of Reading, Dragoninferno3343 and Anonymous: 9
- **Rotavirus vaccine** *Source:* [http://en.wikipedia.org/wiki/Rotavirus\\_vaccine?oldid=621320945](http://en.wikipedia.org/wiki/Rotavirus_vaccine?oldid=621320945) *Contributors:* Rmhermen, Gabbe, Nina, Chowbok, Andycjp, Rich Farmbrough, Tripper, Arcadian, Mosesofmason, Ceyockey, Mike Dillon, Colin, Zvika, Jkpjkp, SmackBot, RDBrown, Cybercobra, Ruslik0, Yupik, MastCell, JPG-GR, Adacus12, Cool Nerd, Mschiffler, Yobol, J.delanoy, Ebulides, Jmh649, GrahamColm, Maralia, Gor n bein, SilvonenBot, Zodon, Crankarm, Addbot, Diptanshu.D, CheMoBot, ChildSurvival, Citation bot, Rezarj, Citation bot 1, Natisto, Fuzbaby, BogBot, ZéroBot, EmersonWhite, The chemists, ClueBot NG, BG19bot, MrBill3, BugDocCanuck, Editfromwithout, Singaramdoc, Monkbot, Formerly 98 and Anonymous: 23
- **Small interfering RNA** *Source:* [http://en.wikipedia.org/wiki/Small\\_interfering\\_RNA?oldid=619514139](http://en.wikipedia.org/wiki/Small_interfering_RNA?oldid=619514139) *Contributors:* AxelBoldt, Bryan Derksen, AdamRetchless, Lexor, Llull, Joseaperez, Diberri, Rich Farmbrough, Cacycle, ESkog, Perfecto, Rasbak, TheGoblin, Rjwilmsi, Klortho, FlaBot, Firebox, Kerowyn, Daycd, Chobot, Reo On, Icarus3, Daniel Mietchen, Kkmurray, Mike Serfas, Boogiezbass, Banus, Eykanal, SmackBot, Mitteldorf, Hydkat, Ohnoitsjamie, Bluebot, The Moose, Scray, Chlewbot, Drphilharmonic, Scientizzle, Thenothing, Seb951, Ben Moore, Beetstra, RBJ, MTSbot, JoeBot, Bioinformin, Dirk haussecker, CmdrObot, ShelfSkewed, Requestion, Pgr94, Ppgardne, Narayanese, Opabinia regalis, Speedyboy, David D., Smartse, .anacondabot, Dekimasu, WLW, Ggrimes, Anaxial, Wiki-producer, Skier Dude, FJPB, Lalvers, GrahamHardy, Speciate, Clarince63, McM.bot, Triesault, Cow109girl, Kosigrim, ViennaUK, 9eyedeeel, Drsean1, Wernervb, Sisalgs, Lascorz, Chriff, Forluvoft, Touchstone42, Opcassio, PixelBot, Sirnaomics, Allikendr1, Addbot, DOI bot, Madguitar07, Lightbot, Fried-peach, Aspeak, Luckas-bot, B.Mothes, TaBOT-zerem, Imtechchd, Materialscientist, Citation bot, Tomaschwutz, Websurferben, GrouchoBot, Richard.decal, Howard McCay, Chemist234, Sixtywell, RedBot, Jeangabin, CaylaInvivoGen, Transfectionista, Sven Jähnichen, Krishnenduroyut, Dancojocari, Skwerl masta, WikitanvirBot, Ronk01, Twilkinson99, ChuispastonBot, ClueBot NG, Helpful Pixie Bot, Vnatik, Hallows AG, Bavykinas, Ginger Maine Coon, ChrisGualtieri, Saltwolf, Szeherezadess, Wikitikitum, Eyesnore, Aaron.aude, YickChongLam, Giancarlobasile, AKS.9955, Demi lion, TimTechnical, Ankit.sqz and Anonymous: 112
- **RNA interference** *Source:* [http://en.wikipedia.org/wiki/RNA\\_interference?oldid=621357421](http://en.wikipedia.org/wiki/RNA_interference?oldid=621357421) *Contributors:* AxelBoldt, Bryan Derksen, LA2, PierreAbbat, SimonP, AdamRetchless, Erik Zachte, Zashaw, Lexor, Lqlilter, JWSchmidt, Quizkajer, Taxman, Bevo, Xuanwu, Peak, Ojigiri, Gidonb, Dp, Everyking, Jfdwolff, Pascal666, Mr d logan, Sonjaaa, PDH, Rdsmith4, PFHLai, Creidieki, Sam, A-giau, Rich Farmbrough, Nina Gerlach, Kbh3rd, La goutte de pluie, Pschemp, PasswOrd, Beyondthislife, Alansohn, Gary, Hipocrite, ClockworkSoul, TenOfAllTrades, Xmort, Reinoutr, Duncan.france, Kanenas, MarcoTolo, Magister Mathematicae, GoldRingChip, Dwaipayanc, Rjwilmsi, Westcairo, Brighterorange, TBHecht, Klortho, FlaBot, RobertG, AAMiller, Gurch, Daycd, Smithbrenon, Bgwhite, YurikBot, Wavelength, RussBot, Hyad, NawlinWiki, SuperMallen, Gaodifan, Aleichem, Lipothymia, Aaron Schulz, Bota47, Kkmurray, Tingo, ConnectChina, Paul White, WikiFew, SmackBot, TestPilot, Od Mishehu, Vald, Neptunius, Nickst, Kjaergaard, Bluebot, Tito4000, Goldfinger820, Miguel Andrade, Ribrob, Scray, Chlewbot, Karthik.raman, Stevenmitchell, TedE, Wirbelwind, Akriasas, Nishkid64, DO11.10, Scientizzle, JohnI, Tillalb, Mgiganteus1, Seb951, Ckatz, Smith609, SandyGeorgia, DabMachine, Andrew Davidson, Bioinformin, Fvasconcellos, Insanephantom, Outrigr, Ppgardne, Mato, Was a bee, Ve ri tas, Comcc, Narayanese, Thijstbot, Opabinia regalis, Mlg, Speedyboy, Z10x, Orlando52, David D., AntiVandalBot, Seaphoto, TimVickers, Smartse, Salgueiro, Dougher, Lfstevens, JAnDbot, Sangak, WolfmanSF, Skyzefawlun, Praedor, Silentaria, NSuizu, NescioNomen, Ggrimes, Swalot, R'n'B, CommonsDelinker, Nono64, VirtualDelight, Fconaway, J.delanoy, Alex68677, Benjamin Weiss, Lantonov, Bdekker, Skier Dude, MetsFan76, Treisijs, King Toadsworth, Million Moments, Volvox777, VolkovBot, CarolineFThornPhD, TXiKiBoT, Malljaja, Broadbot, Hannes Röst, Nlwsus, Ceranthon, HansHermans, GrahamColm, Petrgrk, Ipodams, SiameseTurtle, Gtadoc, Spartan-James, Dabomb87, Wernervb, Mberriman, Lascorz, Forluvoft, SallyForth123, Touchstone42, ClueBot, Alexbot, NuclearWarfare, GeneticsPhD, Thingg, Sirnaomics, Alboyle, XLinkBot, Twistedswitch, Marsepein, ZTebaykina, Addbot, Mobious179, DOI bot, Silvia3, Microtube, Knight chaik, Tide rolls, ٧٢, Ettrig, Luckas-bot, Yobot, Ptbotgourou, Amirobot, Imtechchd, Materialscientist, Citation bot, LilHelpa, Obersachsebot, Cpicardo, Hammersbach, Aa77zz, Donaldbane, FrescoBot, D'ohBot, Suki77, Citation bot 1, AstaBOTh15, Jonkerz, James atmos, Betterbiologist, Transfectionista, Jesse V., Schekn, Khareldn, RjwilmsiBot, Ripchip Bot, Sheppardj, Dancojocari, Aircorn, EmausBot, WikitanvirBot, John Mackenzie Burke, H3llBot, Brandmeister, Itizen, SsmdZhang, Parageneticist, ClueBot NG, Gilderien, 6ii9, Mesoderm, Meetmadhan, Helpful Pixie Bot, Bibcode Bot, Hurricanefan25, L5tardust, Cadiomals, Harizotoh9, NotWith, Genomernai, Offense9515, Shea150, BattyBot, Tkchafin7153, YFdyh-bot, Squirrelypants, Mogism, Makecat-bot, Szeherezadess, Dmitry Dzhabarov, Jfm5300, Chrislgarry, FallingGravity, Kristenkoz, Olle Terenius (SLU), Noyster, Anrnusna, Meteor sandwich yum, Dicktribe, Monkbot, Orbitaimed, Jigolfer and Anonymous: 176
- **Virus quantification** *Source:* [http://en.wikipedia.org/wiki/Virus\\_quantification?oldid=619104145](http://en.wikipedia.org/wiki/Virus_quantification?oldid=619104145) *Contributors:* Graeme Bartlett, Chowbok, Discospinster, BD2412, Kkmurray, Chris the speller, Acdx, IronGargoyle, Magioladitis, Xenobiologista, France3470, Addbot, Okisa, Citation bot, FrescoBot, SW3 5DL, RjwilmsiBot, Cavitri, Betsy Rogers, TuHan-Bot, Kwhite14, Shannonlrodriguez, Abergabe, Staticd, Callahanjd, MOSNUM Bot, Helpful Pixie Bot, BG19bot, MusikAnimal, Cclehnens, Mogism, Nanoworld111, Monkbot and Anonymous: 6
- **Polymerase chain reaction** *Source:* [http://en.wikipedia.org/wiki/Polymerase\\_chain\\_reaction?oldid=621651935](http://en.wikipedia.org/wiki/Polymerase_chain_reaction?oldid=621651935) *Contributors:* AxelBoldt, Magnus Manske, Ap, Andre Engels, Youscfsan, Rmhermen, Edward, Infrogmation, Lexor, MartinHarper, Ixfd64, MichaelJanich, Ahoersteimeier, Habj, Llull, Mxn, Smack, Hashar, AhmadH, Novum, Ec5618, Charles Matthews, Greatpatton, Ike9898, Dysprosia, Andrewman327, Haukurth, Tpbradbury, E23, Saltine, Thue, BenRG, Donarreiskoffer, Robbot, Senthil, Donreed, Romanm, KellyCoinGuy, Ojigiri, Hadal, Michael Snow, GreatWhiteNortherner, Centrx, DocWatson42, Wolfkeeper, Fastfission, Michael Devore, Jfdwolff, Alvestrand, Jackol, Alan

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