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3rd Edition

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We wish you continued success in your research projects.

Sincerely
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PS: To offer comments and/or suggestions on content, structure and/or layout of this guide, please don't hesitate to contact Bettina Kruchen (e-mail: Bettina.Kruchen@roche.com) or your local representative. Your cooperation in improving Lab FAQs is highly appreciated.

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Working with DNA

Chapter 1

1

1.1. *Precautions for handling DNA*

Handling fresh and stored material before extraction of DNA	➢ For the isolation of genomic DNA from cells and tissues, use either fresh samples or samples that have been quickly frozen in liquid nitrogen and stored at –70°C. This procedure minimizes degradation of the DNA by limiting the activity of endogenous nucleases. ➢ For best results, use fresh blood or blood stored for < 2 days at room temperature. Blood stored for 7 days at 2-8°C or for < 1 month at –15 to –25°C will result in a 10 to 15% reduction of yield of genomic DNA. ➢ Collect blood samples in tubes containing EDTA as anticoagulant, not heparin. Heparin can cause attenuation or inhibition of amplification during PCR. ➢ However, if heparin cannot be avoided, the High Pure PCR Template Preparation Kit* can be used to remove the heparin from the sample.
Pipetting DNA	➢ Avoid vigorous pipetting. ➢ Pipetting genomic DNA through small tip openings causes shearing or nicking. Use tips with wide openings, specially designed for genomic DNA. ➢ Regular pipette tips pose no problem for plasmid DNA and other small DNA molecules.

* Product available from Roche Applied Science: Cat. No. 11 796 828 001

Storage of DNA	➢ Store genomic DNA at 2-8°C, storage at –15 to –25°C can cause shearing of the DNA. ➢ Plasmid DNA and other small DNA molecules can be stored ● at 2-8°C for short term storage ● in aliquots at –15 to –25°C for long term storage. ➢ Keep plasmids for transformation purposes at 2-8°C to avoid nicks. ➢ Store modified DNA at 2-8°C.
Manipulation of DNA	➢ Always keep the DNA sample on ice when preparing an experiment.
Drying DNA	➢ Avoid overdrying of genomic DNA after ethanol precipitation. Let the DNA air dry. ➢ Plasmid DNA and other small DNA molecules can be air or vacuum dried.
Dissolving DNA	➢ Dissolve DNA in Tris buffer (*e.g.*, 10 mM Tris, pH 7.0 – pH 8.0). ➢ To help dissolve the DNA, carefully invert the tube several times after adding buffer and/or tap the tube gently on the side. ➢ Alternatively, let the DNA stand in buffer overnight at 2-8°C. ➢ Do not vortex genomic DNA. ➢ Heat DNA for 10 min. at 65°C to dissolve and inactivate DNases.

1.2. *Commonly used formulas*

Molecular Weight of DNA, Calculation in Dalton*

- Average molecular weight (MW) of a deoxynucleotide: 330 Dalton (Da)*
- Average molecular weight (MW) of a DNA basepair: 660 Dalton (Da)*

MW of dsDNA = [number of basepairs] x [660 Da]

e.g., MW of pBR322 dsDNA (4363 basepairs) $= 4363 \times 660\ Da$
$= 2.9 \times 10^6\ Da$
$= 2.9 \times 10^3\ kDa$

MW of ssDNA = [number of bases] x [330 Da]

e.g., MW of M13mp18 (7249 bases, ssDNA form) $= 7249 \times 330\ Da$
$= 2.4 \times 10^6\ Da$
$= 2.4 \times 10^3\ kDa$

* 1 Da (Dalton) is a unit of mass almost equal to that of a hydrogen atom (precisely equal to 10000 on the atomic mass scale). Named after John Dalton (1766 – 1844) who developed the atomic theory of matter.

Calculation of pmol of 5′ (or 3′) ends

- pmol of ends of a dsDNA molecule

$$= \frac{2 \times 10^6 \times \mu g \text{ (of dsDNA)}}{\text{MW (in Da)}} = \frac{2 \times 10^6 \times \mu g \text{ (of dsDNA)}}{N_{bp} \times 660 \text{ Da}}$$

e.g., pmol of 5′or 3′ends of 1 µg of a 100 basepairs dsDNA fragment $= \frac{2 \times 10^6 \times 1}{100 \times 660} = 30.3$

- pmol of ends of a ssDNA molecule

$$= \frac{1 \times 10^6 \times \mu g \text{ (of ssDNA)}}{\text{MW (in Da)}} = \frac{1 \times 10^6 \times \mu g \text{ (of ssDNA)}}{N_b \times 330 \text{ Da}}$$

e.g., pmol of 5′or 3′ends of 1 µg of a 250 bases ssDNA fragment $= \frac{1 \times 10^6 \times 1}{250 \times 330} = 12.12$

- pmol of ends generated by restriction endonuclease cleavage:
  - circular DNA: 2 x (pmol of DNA) x (number of sites)
  - linear DNA: [2 x (pmol of DNA) x (number of sites)] + [2 x (pmol of DNA)]

N_{bp} = number of basepairs (dsDNA) and N_b = number of bases (ssDNA)

1.2. *Commonly used formulas*

Conversion of µg to pmol

➢ $\text{pmol of dsDNA} = \mu\text{g (of dsDNA)} \times \frac{10^6 \text{ pg}}{1 \mu\text{g}} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{N_{bp}} = \frac{\mu\text{g (of dsDNA)} \times 1515}{N_{bp}}$

e.g., 1 µg of a 100 basepairs dsDNA fragment $= \frac{1 \times 1515}{100} = 15.2 \text{ pmol}$

➢ $\text{pmol of ssDNA} = \mu\text{g (of ssDNA)} \times \frac{10^6 \text{ pg}}{1 \mu\text{g}} \times \frac{1 \text{ pmol}}{330 \text{ pg}} \times \frac{1}{N_b} = \frac{\mu\text{g (of ssDNA)} \times 3030}{N_b}$

e.g., 1 µg of a 1 000 bases ssDNA fragment $= \frac{1 \times 3030}{1\,000} = 3.03 \text{ pmol}$

Conversion of pmol to µg

➢ $\mu g \text{ of dsDNA} = \text{pmol (of dsDNA)} \times \frac{660 \text{ pg}}{1 \text{ pmol}} \times \frac{1 \mu g}{10^6 \text{ pg}} \times N_{bp} =$

$= \text{pmol (of dsDNA)} \times N_{bp} \times 6.6 \times 10^{-4}$

e.g., 1 pmol of a 100 basepairs dsDNA fragment $= 1 \times 100 \times 6.6 \times 10^{-4} = 0.066$ µg

➢ $\mu g \text{ of ssDNA} = \text{pmol (of ssDNA)} \times \frac{330 \text{ pg}}{1 \text{ pmol}} \times \frac{1 \mu g}{10^6 \text{ pg}} \times N_b =$

$= \text{pmol (of ssDNA)} \times N_b \times 3.3 \times 10^{-4}$

e.g., 1 pmol of a 250 bases ssDNA fragment $= 1 \times 250 \times 3.3 \times 10^{-4} = 0.0825$ µg

N_{bp} = number of basepairs (dsDNA) and N_b = number of bases (ssDNA)

1.2. *Commonly used formulas*

Examples

Type	Size	Form	MW (in kDa)	pmol/µg	µg/pmol	pmol of 5´ or 3´ ends/µg
dsDNA fragment	100 bp	Linear	66	15.2	0.066	30.3
dsDNA fragment	500 bp	Linear	330	3.03	0.33	6.06
dsDNA fragment	1000 bp	Linear	660	1.52	0.66	3.03
		RE* Digest, 1 site		1.52	0.66	6.06
		RE* Digest, 2 sites		1.52	0.66	9.12
pUC18/19 dsDNA	2686 bp	Circular	1.8×10^3	0.57	1.77	–
		RE* Digest, 1 site		0.57	1.77	1.14
		RE* Digest, 2 sites		0.57	1.77	2.28
		RE* Digest, 3 sites		0.57	1.77	3.42
pBR322 dsDNA	4363 bp	Circular	2.9×10^3	0.35	2.88	–
		RE* Digest, 1 site		0.35	2.88	0.7
		RE* Digest, 2 sites		0.35	2.88	1.4
		RE* Digest, 3 sites		0.35	2.88	2.1

* RE: restriction enzyme

Notes

1.3. *Isolating and purifying DNA*

Use this chart to select a product according to the type and origin of DNA (see also reference 4).

Type	Origin	Recommendation*
Genomic	tissue, cultured cells, bacteria, yeast	High Pure PCR Template Preparation Kit
	tissue, cultured cells, bacteria, yeast, mouse tail	DNA Isolation Kit for Cells and Tissues
	human blood	High Pure PCR Template Preparation Kit
	mammalian/human blood	DNA Isolation Kit for Mammalian Blood
Plasmid	propagated in *E.coli*	High Pure Plasmid Isolation Kit
		Genopure Plasmid Maxi/Midi Kits
Viral	serum, plasma, blood, other body fluids, cell culture supernatant	High Pure Viral Nucleic Acid Kit
	serum, plasma, cell culture supernatant	High Pure 16 System Viral Nucleic Acid Kit
DNA fragments	PCR mixture, restriction enzyme digests, labeling and modifying reactions, DIG labeled probes.	High Pure PCR Product Purification Kit
		High Pure 96 UF Cleanup Kit
		High Pure PCR Cleanup Micro Kit
	removal of unincorporated nucleotides from labeled DNA molecules	Mini Quick Spin DNA Columns
		Quick Spin Columns
	agarose gel slices	Agarose Gel DNA Extraction Kit
		High Pure PCR Product Purification Kit
		High Pure PCR Cleanup Micro Kit

* The amount of DNA that can be isolated with the kits depends on variables like the amount of sample applied, concentration of DNA within the sample, buffers systems, etc. Please refer to the next pages for a detailed overview.

Use this chart to select a product according to the main application in which the DNA will be used (see reference 4).

Product	PCR	Restriction Enzyme Analysis	Southern Blotting	Labeling/ Modifying Reactions	Clon- ing	Se- quenc- ing	*In Vitro* Tran- scription	Trans fec- tion	Micro- array spotting
DNA Isolation Kit for Cells and Tissues	●	●	●		●				
DNA Isolation Kit for Mammalian Blood	●	●	●		●				
High Pure Plasmid Isolation Kit	●	●	●	●	●	●	●		
High Pure Viral Nucleic Acid Kit	●								
High Pure 16 System Viral Nucleic Acid Kit	●								
High Pure PCR Template Preparation Kit	●	●	●		●				
High Pure 96 UF Cleanup Kit		●		●	●	●			●
High Pure PCR Product Purification Kit	●	●	●	●	●	●			
High Pure PCR Cleanup Micro Kit	●	●	●	●	●	●			
Mini Quick Spin DNA Columns			●			●			
Quick Spin Columns			●						
Agarose Gel DNA Extraction Kit	●	●	●	●	●	●			
Genopure Plasmid Midi Kit Genopure Plasmid Maxi Kit	●	●	●	●	●	●	●	●	

1.3. *Isolating and purifying DNA*

Use this chart to select a product according to its characteristics (see also reference 4)

Product	Quantity of starting material	Typical Yield
Agarose Gel DNA Extraction Kit	➢ Agarose slices: 100 – 200 mg	➢ Recovery: ● 0.1 – 10 kb: ~ 80% ● 10 kb – 100 kb: ~ 60% ● Oligos > 20 bases: ~ 60%
DNA Isolation Kit for Cells and Tissues	➢ Tissue: up to 1 g ➢ Cultured cells: up to 5 x 10^7 cells ➢ Mouse tail: up to 400 mg ➢ Yeast: up to 3 x 10^{10} cells ➢ Gram neg. bacteria: up to 10^{11} cells	➢ depending on tissue type ➢ 700 – 3000 µg ➢ up to 800 µg ➢ up to 300 µg ➢ 1500 – 2700 µg
DNA Isolation Kit for Mammalian Blood	➢ Human whole blood: 10 ml ➢ Rat and mouse whole blood: 10 ml	➢ 350 µg ➢ 570 µg
High Pure 96 UF Cleanup Kit	➢ 100 bp to > 10 kb 20 to 300 µ.	➢ ≥ 25 µl (≥ 150 bp ≥ 40%; 1500 bp ≥ 90%; 4500 bp ≥ 90%; 8000 bp ≥ 80%)
High Pure Plasmid Isolation Kit	➢ E. coli XL1 Blue, pUC19 – 2 ml ➢ E. coli DH5α, pUC19 – 2 ml	➢ 12 µg ➢ 3.5 µg
High Pure Viral Nucleic Acid Kit	➢ 200 – 600 µl of serum, plasma, blood, cell culture supernatant	➢ product detectable by PCR
High Pure 16 System Viral Nucleic Acid Kit	➢ 200 – 600 µl of serum, plasma, blood, cell culture supernatant	➢ product detectable by PCR

Use this chart to select a product according to its characteristics (see also reference 4)

Product	Quantity of starting material	Typical Yield
High Pure PCR Template Preparation Kit	➢ Blood: up to 300 µl ➢ Cultured cells: up to 10^8 cells ➢ Mouse tail: 25 – 50 mg ➢ Yeast: 10^8 cells ➢ Bacteria: 10^9 cells	➢ 3 – 9 µg ➢ 15 – 20 µg ➢ 5 – 10 µg ➢ 10 – 13 µg ➢ 1 – 3 µg
High Pure PCR Product Purification Kit	➢ 100 µl of a modifying, labeling or restriction enzyme digestion reaction	➢ Recovery: > 80% of 5 – 25 µg DNA (fragments > 100 bp)
High Pure PCR Cleanup Micro Kit	➢ up to 100 µl reaction mix up to 100 mg agarose gel slice	➢ Recovery: > 80% of 5 – 25 µg DNA (fragments > 100 bp)
Mini Quick Spin DNA Columns	➢ 20 – 75 µl labeling mixture	➢ Recovery: > 90% ➢ Exclusion limit: ≥ 20 bp
Genopure Plasmid Midi Kit	➢ 10 – 100 ml bacterial culture (low copy plasmid) ➢ 5 – 30 ml bacterial culture (high copy plasmid)	➢ 0.2 – 1 µg/ml culture (low copy plasmid) ➢ 3 – 5 µg/ml culture (high copy plasmid)
Genopure Plasmid Maxi Kit	➢ 100 – 500 ml bacterial culture (low copy plasmid) ➢ 30 – 150 ml bacterial culture (high copy plasmid)	➢ 0.2 – 1 µg/ml culture (low copy plasmid) ➢ 3 – 5 µg/ml culture (high copy plasmid)

Note: For more information on nucleic acid isolation and purification please visit **http://www.roche-applied-science.com/napure**

1.3. *Isolating and purifying DNA*

Perform nucleic acid isolation rapidly and efficiently

The MagNA Pure LC Instrument allows fully automated nucleic acid isolation and PCR setup. True walk-away precision is achieved with the elimination of "hands-on" steps such as pipetting, filtration, and centrifugation. With the MagNA Pure LC Instruments's proven magnetic bead technology, high quality genomic DNA, total RNA, or mRNA is obtained.

The MagNA Pure LC Instrument processes up to 32 samples in less than one hour and isolates pure nucleic acids from a variety of sample types, including:

- Whole blood
- White blood cells
- Cultured cells
- Tissues

Expand the MagNA Pure LC Instrument's flexibility and versatility with the use of various reagent kits, accessories, and software protocols:

- **DNA Isolation Kits** - for blood/cultured cells, tissue, and bacteria/fungi
- **Total Nucleic Acid Isolation Kits** - for blood, plasma, and serum; also available for large volumes
- **RNA Isolation Kits** - for whole blood, blood cells, cultured cells, and paraffin-embedded tissue
- **mRNA Isolation Kits** - for whole blood, blood cells, and tissue

Increase your lab's efficiency by combining the MagNA Pure LC Instrument with the LightCycler Carousel Centrifuge and the LightCycler Instrument.

Note: For more information on MagNA Pure LC please visit **http://www.magnapure.com**

Sizes and weights of DNA from different organism

Organism	Size (bp)	Molecular Weight (in kDa)	Number of Chromosomes
pBR322, *E. coli* plasmid	4363	2.9×10^3	
SV 40, Simian virus	5243	3.5×10^3	
ΦX174, *E.coli* bacteriophage	5386	3.5×10^3	
Adenovirus 2, human virus	35937	23.7×10^3	
Lambda, E.coli bacteriophage	48502	32.0×10^3	
E.coli, bacterium	4.7×10^6	3.1×10^6	
Saccharomyces cerevisiae, yeast	1.5×10^7	9.9×10^6	32 (diploid)
Dictyostelium discoideum, mold	5.4×10^7	3.6×10^7	7 (haploid)
Caenorhabditis elegans, worm	8.0×10^7	5.3×10^7	11/12 (diploid)
Drosophila melanogaster, fruitfly	1.4×10^8	9.2×10^7	8 (diploid)
Mus musculus, mouse	2.7×10^9	1.8×10^9	40 (diploid)
Xenopus leavis, frog	3.1×10^9	2.0×10^9	36 (diploid)
Homo Sapiens, human	3.3×10^9	2.2×10^9	46 (diploid)
Zea mays, maize	3.9×10^9	2.6×10^9	20 (diploid)
Nicotiana tabacum, tabacum plant	4.8×10^9	3.2×10^9	48 (diploid)

1.4. *Analyzing DNA*

Concentration and purity via OD measurement

Concentration of DNA

- 1 A_{260} Unit of dsDNA = 50 µg/ml H_2O
- 1 A_{260} Unit of ssDNA = 33 µg/ml H_2O

Notes

- OD value should range between 0.1 and 1.0 to ensure an optimal measurement.
- The above mentioned values are based on extinction coefficients of nucleic acids in H_2O, please note that these coefficients – and hence the above mentioned values – differ in other buffers and/or solutions.
- Example of calculation:
  - volume of dsDNA sample: 100 µl
  - dilution: 25 µl of this sample + 475 µl H_2O (1+19 dilution)
  - A_{260} of this dilution: 0.44
  - concentration of dsDNA in sample: 0.44 x 50 µg/ml x 20 (=dilution factor) = 440 µg/ml
  - amount of dsDNA in sample: 440 µg/ml x 0.1 ml (= sample volume) = 44 µg

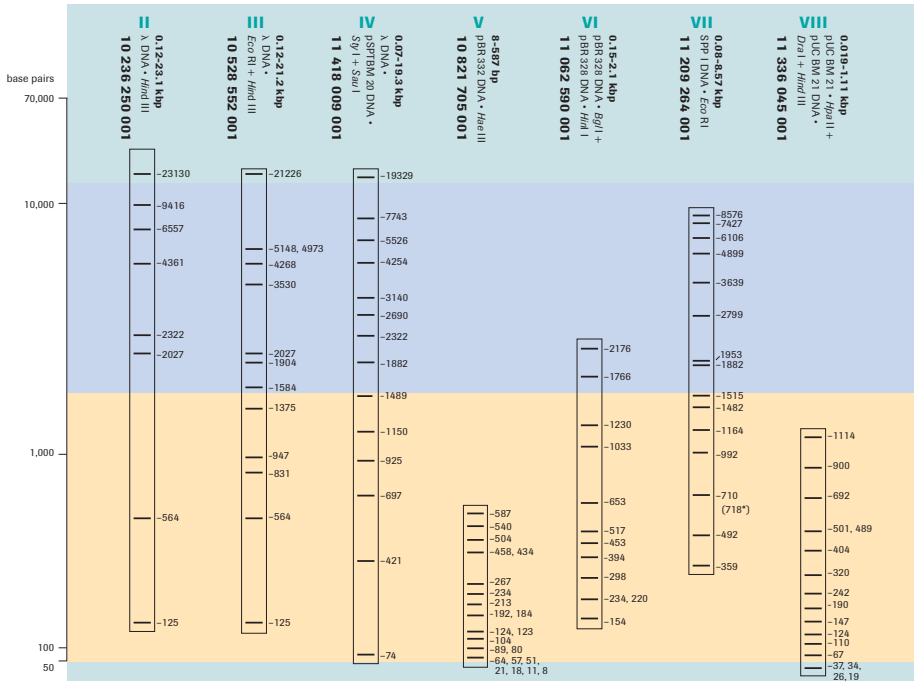
Purity of DNA

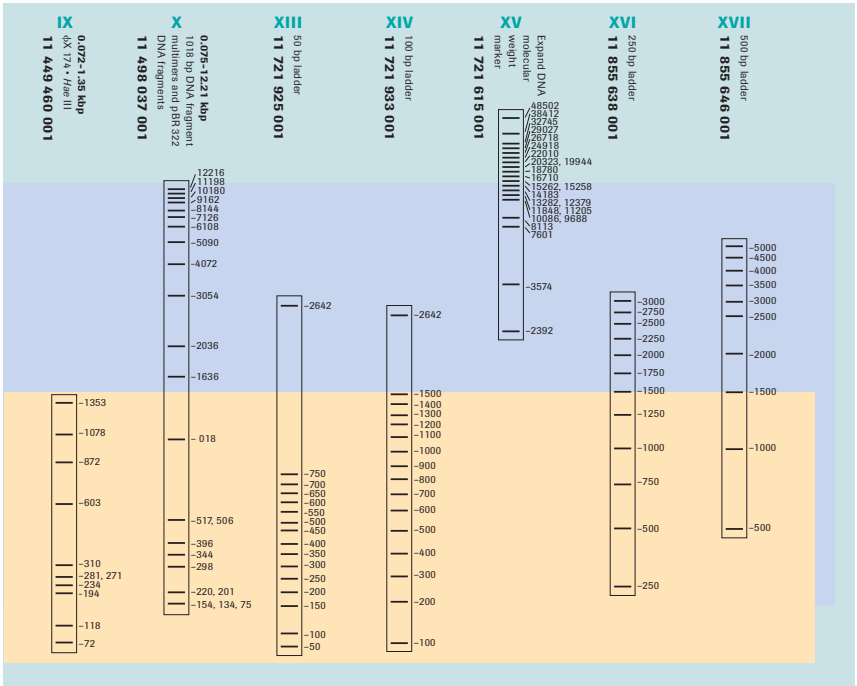
- Pure DNA: $A_{260}/A_{280} \geq 1.8$

Notes

- An $A_{260}/A_{280} < 1.8$ indicates that the preparation is contaminated with proteins and aromatic substances (*e.g.*, phenol).
- An $A_{260}/A_{280} > 2$ indicates a possible contamination with RNA.
- The OD gives no information about the size of the DNA.

Fragment Sizes of DNA Molecular Weight Markers





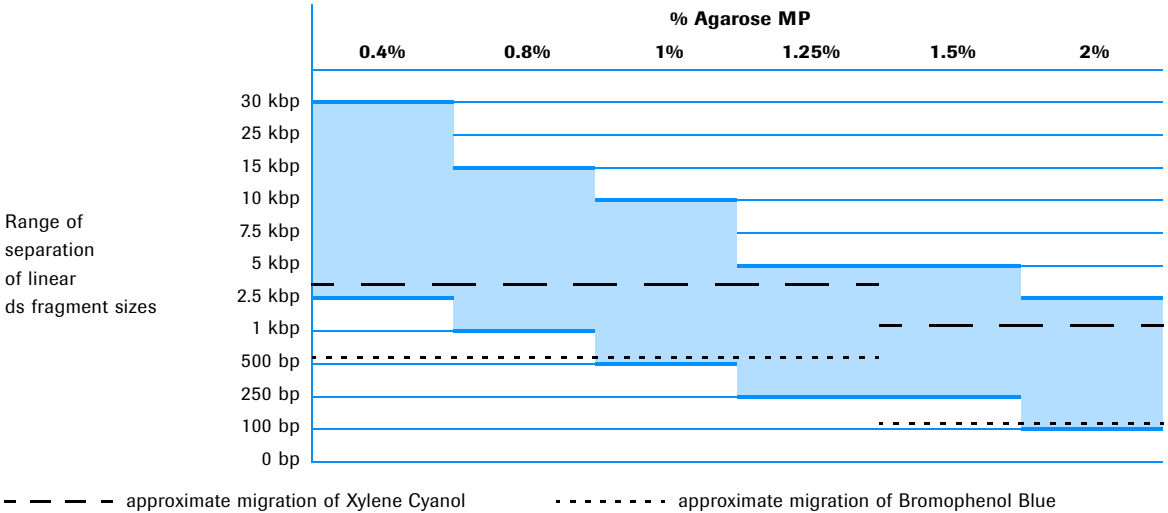
Agarose MS 0.05 Kbp – 1.5 Kbp

Agarose LE 0.2 Kbp – 15 Kbp

Agarose MP 0.1 Kbp – 30 Kbp

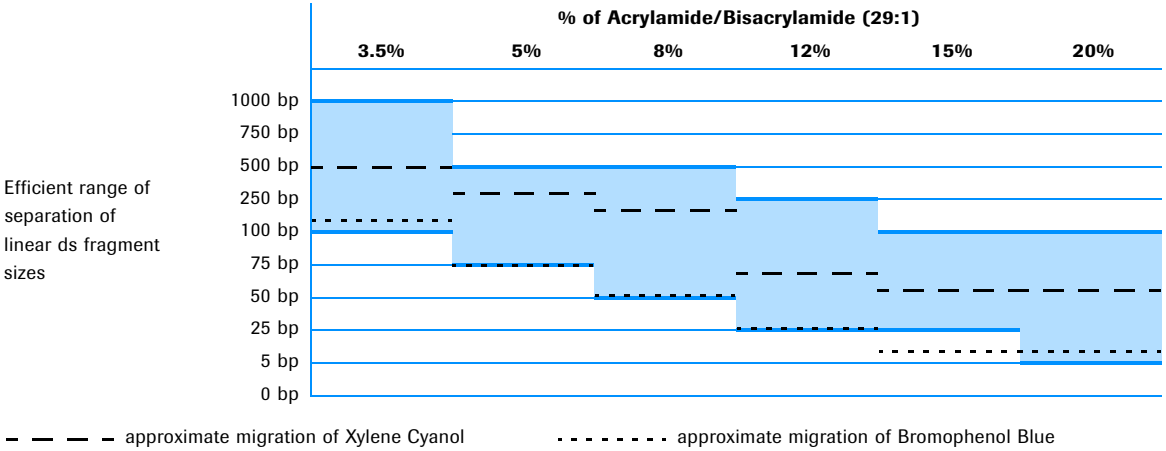
1.4. *Analyzing DNA*

Size estimation of DNA fragments in Agarose MP gels*



* Agarose **M**ulti **P**urpose (Cat. No. 11 388 983 001) – available from Roche Applied Science – is a high gel strength agarose (> 1800 g/cm^2; 1%) permitting the use of very low and very high concentrations, resulting in a broad range of DNA molecules that can be separated in the same type of agarose. Please refer to chapter 5 “Preparing Buffers and Media” for recipes to prepare all necessary buffers.

Size estimation of DNA fragments in Acrylamide gels



Please refer to chapter 5 “Preparing Buffers and Media” for recipes to prepare all necessary buffers.

Restriction Enzymes: General Information

Unit definition	➢ One unit of a restriction endonuclease is the amount of enzyme required to completely digest 1 µg substrate DNA in 60 min. at the appropriate assay conditions stated for each restriction enzyme. ➢ The type of the substrate DNA, the correct reaction temperature and the specific activity of each enzyme are stated in the packinserts that come together with the products. (Compare to page 14 table section "*Sub*")
Stability	➢ All restriction enzymes – supplied by Roche Applied Science – contain expiry dates on the label. 100% activity of the enzyme is guaranteed until that date.
Storage	➢ Restriction enzymes are, like all proteins, susceptible to denaturation and can loose their specific activity when exposed to higher temperatures. ➢ Therefore, the enzymes are supplied as a glycerol solution and should be stored at –15 to –25°C in a non-defrosting freezer. During usage, it is recommended to keep the enzyme on ice or in specially developed bench-top coolers.

Note: For detailed information concerning restriction enzymes please visit: **www.restriction-enzymes.com**

Restriction Enzymes: Buffer System

SuRE/Cut Buffer System* –
buffers supplied as 10x concentrated solutions:

Buffer components	Final Concentration in mM				
	A	B	L	M	H
Tris Acetate	33				
Tris HCl		10	10	10	50
Magnesium Acetate	10				
$MgCl_2$		5	10	10	10
Potassium Acetate	66				
NaCl		100		50	100
1,4-Dithioerythritol (DTE)			1	1	1
1,4-Dithiothreitol (DTT)	0.5				
2-Mercaptoethanol		1			
pH at 37°C	7.9	8.0	7.5	7.5	7.5

Special incubation buffers** –
buffers supplied as 2x concentrated solutions:

Buffer components	Final Concentration in mM			
	Mae I	Mae II	Mae III	Nde II
Tris HCl	20	50	20	50
NaCl	250	220	275	75
$MgCl_2$	6	6	6	5
2-Mercaptoethanol	7	7	7	
Bovine Serum Albumin (BSA)		100 µg/ml		
1,4-Dithiothreitol (DTT)				0,5
pH	8.0 (45°C)	8.8 (50°C)	8.2 (55°C)	7.6 (37°C)

* Buffer system available from Roche Applied Science
**Products available from Roche Applied Science

Restriction Enzymes: Star Activity

Definition	➢ The ability of restriction enzymes to cleave – under non-optimal conditions – DNA sequences that are similar but not identical to the recognition site of the enzyme.
Enzymes affected	➢ The following enzymes can exhibit star activity under certain non-optimized conditions: ● BamH I, BssH II, Dde I, EcoR I, EcoR V, Hind III, Hinf I, Kpn I, Mam I, Pvu II, Sal I, Sau3A I, SgrA I, Taq I ➢ However, reports (see ref. 12) suggest that star activity may be a general property of all restriction enzymes.
Factors causing star activity	➢ High glycerol concentration (> 5% v/v). ➢ Large excess of enzyme. ➢ Non-optimal ionic strength, pH and divalent cations of buffer system. ➢ Presence of organic solvents.
Avoiding star activity	➢ Use the optimal buffer for each enzyme as recommended by its suppliers. ➢ Use the optimal amount of enzyme as recommended by its suppliers. ➢ Make sure that the DNA preparation is free of organic solvents that may have been used during isolation and/or purification of the DNA.

Restriction Enzymes: Reference Materials available

Learn more about our solutions for mapping & cloning by visiting our special interest site at:
www.restriction-enzymes.com

Search for the needed restriction enzyme with the easy-to-use online tool Benchmate RE Finder at:
www.roche-applied-science.com/benchmate

The Restriction Enzymes FAQS and Ordering Guide supplying technical information, including troubleshooting.

The comprehensive **Restriction Enzyme Poster** well known as the information source for commercially available restriction enzymes and their recognition sequences.

The Tools for Mapping and Cloning brochure supplying solutions to support you in your daily research.

available from your local Roche Applied Science representative

1.4. *Analyzing DNA*

Restriction Enzymes: Inactivation and removal

The following procedures can be applied to inactivate restriction enzymes:

Inactivation	➢ By heat treatment: Certain enzymes can be inactivated by heating. Please refer to the product characteristics tables on the next pages for enzyme specific information. ➢ By EDTA treatment: Alternatively, the enzyme can be inactivated by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM.
Removal	➢ By phenol/chloroform extraction: Extract the sample with phenol/chloroform and once with chloroform, precipitate the DNA with ethanol or iso-propanol. ➢ By silica absorption: Alternatively, the tedious and cumbersome phenol/chloroform extraction procedure can be omitted by using the High Pure PCR Product Purification Kit (Cat. No. 11 732 668 001) or High Pure PCR Cleanup Micro Kit (Cat. No. 04 983 955 001) – available from Roche Applied Science.

Abbreviations and icons used in the tables:

A	➢ Percentage activity of enzyme in SuRE/Cut buffer A (100% in bold printed buffer)
B	➢ Percentage activity of enzyme in SuRE/Cut buffer B (100% in bold printed buffer)
L	➢ Percentage activity of enzyme in SuRE/Cut buffer L (100% in bold printed buffer)
M	➢ Percentage activity of enzyme in SuRE/Cut buffer M (100% in bold printed buffer)
H	➢ Percentage activity of enzyme in SuRE/Cut buffer H (100% in bold printed buffer)
🌡	➢ Incubation temperature of enzyme, temperatures in bold differ from the “classical” 37°C
HI	➢ Heat inactivation of enzyme: ● Y: Indicates that the enzyme can be inactivated by heat (15 min at 65°C unless otherwise stated) ● N: Indicates that the enzyme cannot be inactivated by heat (see previous page for alternative procedures)
MS	➢ Indicates sensitivity of enzyme for methylation ● dcm^+ : indicates that the enzyme is blocked by *dcm* methylation ● dam^+ : indicates that the enzyme is blocked by *dam* methylation ● CG^+ : indicates that the enzyme is blocked by eukaryotic methylation ● No indication: the enzyme is not sensitive to any form of methylation
PFGE	➢ Function tested for **P**ulse **F**ield **G**radient **E**lectrophoreses ● 👆: the enzyme is function tested by Roche Applied Science for PFGE experiments ● No indication: the enzyme is not function tested
PCR	➢ Percentage of enzyme activity in a standard PCR Mix (= 10 mM Tris HCl, pH 8.3 at 20°C, 50 mM KCl, 1.5 mM $MgCl_2$, λ-substrate DNA, 200 µM dNTP’s and 2.5 U Taq DNA Polymerase in a final volume of 100 µl)
Sub	➢ Test substrate: λ = phage Lambda; P = pBR332, A = Adeno 2, M = MWM VII / number of cleavage sites

Enzymes available from Roche Applied Science in dual concentrations are indicated in bold.

1.4. *Analyzing DNA*

Restriction Enzymes: Characteristics (for abbreviations, see page 14)

	Sequence	A	B	L	M	H	🌡	HI	MS	PFGE	PCR	Sub
Aat II	GACGT↓C	**100**	0–10	0–10	10–25	0–10	37	Y	CG^+	👍	25%	λ/10
Acc I	GT↓(A,C)(T,G)AC	**100**	0–10	10–25	0–10	0–10	37	N	CG^+		< 5%	P/2
Acs I	(A,G)↓AATT(T,C)	50–75	**100**	0–10	75–100	50–75	50	Y[a]				λ/58
Acy I	G(A,G)↓CG(C,T)C	10–25	**100**	10–25	50–75	25–50	50	N				λ/40
Afl III	A↓C(A,G) (T,C)GT	50–75	75–100	50–75	75–100	**100**	37	N			20%	λ/20
Alu I	AG↓CT	**100**	50–75	25–50	25–50	0–10	37	Y			100%	λ/143
*Alw*44 I	G↓TGCAC	**100**	25–50	75–100	100	10–25	37	N				P/3
***Apa* I**	GGGCC↓C	**100**	10–25	50–75	50–75	0–10	30	Y	dcm^+, CG^+	👍	100%	λ/1
Asp I	GACN↓NNGTC	50–75	**100**	25–50	75–100	75–100	37	N				λ/2
*Asp*700	GAANN↓NNTTC	50–75	**100**	10–25	50–75	0–10	37	N			10%	λ/24
***Asp*718 I**	G↓GTACC	75–100	**100**	0–10	25–50	50–75	37	N	dcm^+		100%	λ/2
*Asp*E I	GACNNN↓NNGTC	10–25	10–25	**100**	25–50	0–10	37	Y				λ/9
Ava I	C↓(T,C)CG(A,G)G	100	**100**	10–25	50–75	10–25	37	Y	CG^+	👍	20%	λ/8
Ava II	G↓G(A,T)CC	**100**	50–75	75–100	100	10–25	37	Y	dcm^+, CG^+		< 5%	λ/35
Avi II	TGC↓GCA	50–75	75–100	10–25	50–75	**100**	37	N		👍	30%	λ/15
***Bam*H I**	G↓GATCC	100	**100**	75–100	100	25–50	37	N			100%	λ/5
Ban II	G(A,G)GC(T,C)↓C	75–100	**100**	50–75	50–75	25–50	37	Y				λ/7
*Bbr*P I	CAC↓GTG	75–100	**100**	75–100	75–100	25–50	37	N		👍	100%	λ/3

	Sequence	A	B	L	M	H	🌡	HI	MS	PFGE	PCR	Sub
***Bcl* I**	T↓GATCA	100	100	25–50	**100**	100	50	N	dam^+			λ/8
Bfr I	C↓TTAAG	25–50	25–50	75–100	**100**	25–50	37	Y			100%	λ/3
***Bgl* I**	$GCC(N)_4$↓NGGC	25–50	50–75	10–25	25–50	**100**	37	Y			30%	λ/29
***Bgl* II**	A↓GATCT	100	100	25–50	100	**100**	37	N				λ/6
Bln I	C↓CTAGG	25–50	50–75	0–10	25–50	**100**	37	N		👍		λ/2
*Bpu*A I	$GAAGAC(N)_{2/6}$	10–25	**100**	25–50	25–50	50–75	37	N				λ/24
*Bse*A I	T↓CCGGA	75–100	**100**	0–10	50–75	25–50	55	N		👍		λ/24
BsN I	C↓GTACG	25–50	100	10–25	75–100	**100**	55	Y[a]	CG^+	👍		M/1
BsiY I	CCNNNNN↓NNGG	100	100	50–75	**100**	25–50	55	N				λ/176
Bsm I	GAATGCN↓N	0–10	50–75	0–10	25–50	**100**	65	N				λ/46
*Bsp*LU11 I	A↓CATGT	100	100	25–50	50–75	**100**	48	N				λ/2
*Bss*H II	G↓CGCGC	**100**	100	75–100	100	75–100	50	N	CG^+	👍	100%	λ/6
*Bst*1107 I	GTA↓TAC	25–50	50–75	0–10	25–50	**100**	37	N	CG^+			λ/3
*Bst*E II	G↓GTNACC	75–100	**100**	25–50	50–75	50–75	60	N			100%	λ/13
*Bst*X I	$CCA(N)_5$↓NTGG	10–25	100	0–10	10–25	**100**	45	N				λ/13
Cel II	GC↓TNAGC	25–50	50–75	25–50	25–50	**100**	37	N		👍		λ/6
Cfo I	GCG↓C	75–100	50–75	**100**	50–75	25–50	37	N			100%	λ/215

[a] Inactivation by heating to 75°C for 1 hour

Restriction Enzymes: Characteristics (continued) (for abbreviations, see page 14)

	Sequence	A	B	L	M	H	🌡	HI	MS	PFGE	PCR	Sub
***Cla* I**	AT↓CGAT	100	100	75–100	100	**100**	37	N	CG^+, dam^+	👍	100%	λ/15
Dde I	C↓TNAG	50–75	75–100	25–50	25–50	**100**	37	N			40%	λ/104
Dpn I	GA↓TC	**100**	75–100	50–75	75–100	75–100	37	N			100%	P/22
***Dra* I**	TTT↓AAA	100	75–100	100	**100**	50–75	37	Y		👍	100%	λ/13
Dra II	(A,G)G↓GNCC(T,C)	100	50–75	**100**	50–75	0–10	37	Y	dcm^+			P/4
Dra III	CACNNN↓GTG	50–75	75–100	50–75	75–100	**100**	37	N			50%	λ/10
Eae I	(T,C)↓GGCC(A,G)	**100**	25–50	75–100	50–75	10–25	37	Y	CG^+, dcm^+			λ/39
***Ecl*X I**	C↓GGCCG	25–50	**100**	25–50	25–50	50–75	37	N		👍		λ/2
*Eco*47 III	AGC↓GCT	25–50	50–75	0–10	25–50	**100**	37	Y	CG^+	👍		λ/2
***Eco*R I**	G↓AATTC	100	100	25–50	50–75	**100**	37	Y		👍	50%	λ/5
*Eco*R II	↓CC(A,T)GG	50–75	75–100	0–25	50–75	**100**	37	Y	dcm^+			A/136
***Eco*R V**	GAT↓ATC	25–50	**100**	0–10	25–50	50–75	37	N			10%	λ/21
Fok I	$GGATG(N)_{9/13}$	100	50–75	75–100	**100**	25–50	37	Y				P/12
Hae II	(A,G)GCGC↓(T,C)	**100**	50–75	25–50	50–75	10–25	37	N	CG^+			λ/48
Hae III	GG↓CC	50–75	50–75	75–100	**100**	25–50	37	N			100%	λ/149
Hind II	GT(T,C)↓(A,G)AC	100	100	25–50	**100**	50–75	37	Y			100%	λ/35
***Hind* III**	A↓AGCTT	50–75	**100**	25–50	100	50–75	37	Y			10%	λ/6
***Hinf* I**	G↓ANTC	100	100	50–75	75–100	**100**	37	N			50%	λ/148

	Sequence	A	B	L	M	H	🌡	HI	MS	PFGE	PCR	Sub
Hpa I	GTT↓AAC	**100**	25–50	25–50	50–75	25–50	37	N	CG^+		100%	λ/14
***Hpa* II**	C↓CGG	50–75	25–50	**100**	50–75	10–25	37	Y	CG^+		40%	λ/328
Ita I	GC↓NGC	0–10	25–50	0–10	0–10	**100**	37	Y				λ/380
Kpn* I	GGTAC↓C	75–100	10–25	**100**	25–50	0–10	37	N			50%	λ/2
Ksp I	CCGC↓GG	0–10	0–10	**100**	0–10	0–10	37	N		☝		λ/34
*Ksp*632 I	$CTCTTC(N)_{1/4}$	**100**	0–10	25–50	25–50	0–10	37	N				λ/4
Mae I**	C↓TAG	25–50	25–50	0–10	0–10	10–25	45	N				λ/14***
Mae II**	A↓CGT	0–10	25–50	0–10	25–50	75–100	50	N	CG^+			λ/143
Mae III**	↓GTNAC	0–10	10–25	0–10	0–10	10–25	55	N				λ/156
Mam I	GATNN↓NNATC	75–100	75–100	75–100	75–100	**100**	37	Y	dam^+		20%	λ/22
***Mlu* I**	A↓CGCGT	10–25	25–50	0–10	10–25	**100**	37	N	CG^+	☝	< 5%	λ/7
*Mlu*N I	TGG↓CCA	**100**	0–10	10–25	10–25	0–10	37	Y		☝		λ/18
Mro I	T↓CCGGA	**100**	0–10	50–75	50–75	0–10	37	N		☝		λ/24
***Msp* I**	C↓CGG	100	100	**100**	100	50–75	37	Y			40%	λ/328
Mun I	C↓AATTG	50–75	0–10	100	**100**	10–25	37	N		☝		λ/8
Mva I	CC↓(A,T)GG	100	50–75	25–50	25–50	**100**	37	N				λ/71
Mvn I	CG↓CG	50–75	0–10	50–75	**100**	10–25	37	N			30%	λ/157

* Requires addition of bovine serum albumin, 100 µg/ml
** Mae I, Mae II, Mae III and Nde II require special incubation buffers which are supplied with each enzyme
*** referred to λcl 857Sam7

Restriction Enzymes: Characteristics (continued) (for abbreviations, see page 14)

	Sequence	A	B	L	M	H	🌡	HI	MS	PFGE	PCR	Sub
Nae I	GCC↓GGC	**100**	0–10	100	0–10	0–10	37	Y	CG^+	👍		P/ 4
Nar I	GG↓CGCC	**100**	75–100	75–100	50–75	0–10	37	Y	CG^+	👍		A/ 20
***Nco* I**	C↓CATGG	50–75	50–75	50–75	50–75	**100**	37	Y			50%	λ/ 4
Nde I	CA↓TATG	25–50	75–100	10–25	50–75	**100**	37	Y				λ/ 7
Nde II**	↓GATC	10–25	10–25	0–10	0–10	10–25	37	N	dam^+			λ/ 116
***Nhe* I**	G↓CTAGC	100	25–50	100	**100**	10–25	37	Y	CG^+		100%	λ/ 1
***Not* I**	GC↓GGCCGC	10–25	50–75	0–10	25–50	**100**	37	Y		👍		A/ 7
Nru I	TCG↓CGA	10–25	**100**	0–10	10–25	75–100	37	Y	dam^+, CG^+	👍	75%	λ/ 5
Nsi I	ATGCA↓T	50–75	100	10–25	50–75	**100**	37	Y			100%	λ/ 14
Nsp I	(A,G)CATG↓(T,C)	25–50	50–75	75–100	**100**	0–10	37	N				λ/ 32
*Pin*A I	A↓CCGGT	100	**100**	10–25	50–75	50–75	37	Y				λ/ 13
***Pst* I**	CTGCA↓G	25–50	25–50	10–25	25–50	**100**	37	N			90%	λ/ 28
Pvu I	CGAT↓CG	50–75	75–100	25–50	50–75	**100**	37	N	CG^+	👍	< 5%	λ/ 3
Pvu II	CAG↓CTG	25–50	25–50	25–50	**100**	25–50	37	N	CG^+		100%	λ/ 15
Rca I	T↓CATGA	75–100	**100**	25–50	50–75	25–50	37	Y				λ/ 8
***Rsa* I**	GT↓AC	100	50–75	**100**	50–75	0–10	37	Y	CG^+		100%	λ/ 113
Rsr II	CG↓G(A,T)CCG	75–100	10–25	**100**	75–100	0–10	37	N	CG^+	👍		λ/ 5
***Sac* I**	GAGCT↓C	**100**	0–10	100	50–75	0–10	37	Y			100%	λ/ 2

	Sequence	A	B	L	M	H	🌡	HI	MS	PFGE	PCR	Sub
***Sal* I**	G↓TCGAC	0–10	25–50	0–10	10–25	**100**	37	Y	CG^+	👍		λ/2
*Sau*3A I	↓GATC	**100**	25–50	25–50	75–100	0–10	37	N	CG^+		100%	λ/116
*Sau*96 I	G↓GNCC	**100**	50–75	25–50	25–50	25–50	37	N	dcm^+, CG^+			λ/74
***Sca* I**	AGT↓ACT	0–10	100	0–10	75–100	**100**	37	N			< 5%	λ/5
*Scr*F I	CC↓NGG	10–25	**100**	10–25	10–25	50–75	37	Y	dcm^+			λ/185
*Sex*A I	A↓CC(A,T)GGT	100	**100**	50–75	50–75	25–50	37	Y	dcm^+	👍		λ/5
***Sfi* I**	$GGCC(N)_4$↓NGGCC	25–50	25–50	75–100	**100**	25–50	**50**	N		👍	10%	A/3
Sfu I	TT↓CGAA	25–50	50–75	10–25	25–50	**100**	37	N		👍		λ/7
*Sgr*A I	C(A,G)↓CCGG(T,C)G	**100**	0–10	100	10–25	0–10	37	N		👍		λ/6
***Sma* I**	CCC↓GGG	**100**	0–10	0–10	0–10	0–10	**25**	Y	CG^+	👍	100%	λ/3
*Sna*B I	TAC↓GTA	75–100	25–50	100	**100**	10–25	37	N	CG^+	👍	50%	λ/1
***Spe* I**	A↓CTAGT	75–100	75–100	75–100	100	**100**	37	Y		👍		A/3
***Sph* I**	GCATG↓C	50–75	75–100	25–50	**100**	75–100	37	Y			< 5%	λ/6
***Ssp* I**	AAT↓ATT	75–100	75–100	10–25	75–100	**100**	37	Y		👍		λ/20
***Stu* I**	AGG↓CCT	100	**100**	100	75–100	50–75	37	Y	dcm^+		30%	λ/6
Sty I	C↓C(A,T)(A,T)GG	50–75	100	10–25	75–100	**100**	37	Y			< 5%	λ/10

[b] Inactivation by heating to 75°C for 15 min
** Mae I, Mae II, Mae III and Nde II require special incubation buffers which are supplied with each enzyme

Restriction Enzymes: Characteristics (continued) (for abbreviations, see page 14)

	Sequence	A	B	L	M	H	🌡	HI	MS	PFGE	PCR	Sub
Swa I	ATTT↓AAAT	0–10	10–25	0–10	0–10	**100**	**25**	N		👍		A / 1
Taq I	T↓CGA	50–75	**100**	25–50	50–75	50–75	**65**	N	dam^+		100%	λ / 121
*Tru*9 I	T↓TAA	100	25–50	100	**100**	25–50	**65**	N				λ / 195
*Van*91 I	$CCA(N)_4$↓NTGG	25–50	**100**	0–10	25–50	0–10	37	Y				λ / 14
***Xba* I**	T↓CTAGA	100	75–100	75–100	75–100	**100**	37	N	dam^+	👍	60%	λ / 1
***Xho* I**	C↓TCGAG	25–50	75–100	10–25	25–50	**100**	37	N	CG^+	👍	< 5%	λ / 1
Xho II	(A,G)↓GATC(T,C)	50–75	25–50	**100**	75–100	0–10	37	N				P / 8
*Xma*C I	C↓CCGGG	50–75	0–10	**100**	75–100	0–10	37	N				λ / 3

Please note: For detailed information concerning restriction enzymes, please consult our Benchmate tool at **http://www.roche-applied-science.com/benchmate**

Double Digestion Table

Enzyme	100% Activity in Sure/Cut Buffers	Asp718 I	BamH I	Bgl II	Bln I	Bst1107 I	BstX I [2]	Cla I	Dpn I	Dra III	Eco47 III	EcoR I	EcoR V	Hind III	Kpn I [1]	Mlu I	Nae I	Nco I	Nde I	Nhe I	Not I	Nsp I	Pst I	Pvu I	Pvu II	Sac I	Sal I	Sfi I [3]	Sma I [4]	Spe I	Sph I	Stu I	Xba I
		B	ABM	ABMH	H	H	BH	ABMH	A	AL	H	ABH	B	BM	L	H	AL	H	H	ALM	H	M	H	H	M	AL	H	M	A	MH	M	ABL	AH
BamH I	A B M	B																															
Bgl II	A B M H	B	M																														
Bln I	H	H	B	H																													
Bst1107 I	H	H	B	H	H																												
BstX I [2]	B H	B	B	H	H	H																											
Cla I	A B M H	B	B	H	H	H	H																										
Dpn I	A	A	A	A	H	H	H	A																									
Dra III	A L	A	A	A	B	B	B	A	A																								
Eco47 III	H	H	B	H	H	H	H	H	SD																								
EcoR I	A B H	B	A	H	H	H	H	H	A	A	H																						
EcoR V	B	B	B	H	H	B	B	B	B	B	H	B																					
Hind III	B M	B	B	B	H	H	B	B	B	A	H	B	B																				
Kpn I [1]	L	A	L	A	SD	SD	SD	A	A	L	SD	A	SD	A																			
Mlu I	H	H	H	H	H	H	H	H	H	SD	H	H	H	H	SD																		
Nae I	A L	A	A	A	A	A	SD	A	A	L	SD	A	SD	A	L	SD																	
Nco I	H	H	B	H	H	H	H	H	H	L	H	H	H	H	L	H	L																
Nde I	H	B	B	H	H	H	H	H	H	B	H	H	H	B	SD	H	A	H															
Nhe I	A L M	A	M	A	A	M	B	M	A	A	SD	A	M	M	L	SD	L	L	M														
Not I	H	H	B	H	H	H	H	H	H	SD	H	H	H	H	SD	H	SD	H	H	SD													
Nsp I	M	B	M	M	B	B	B	M	M	L	B	M	B	M	L	SD	L	M	M	M	B												
Pst I	H	H	H	H	H	H	H	H	A	H	H	H	H	H	SD	H	A	H	H	M	H	M											
Pvu I	H	B	B	H	H	H	H	H	H	A	H	H	B	B	L	H	A	H	H	A	H	M	H										
Pvu II	M	M	M	H	H	H	M	M	M	SD	M	M	M	M	M	H	A	M	M	H	M	M	M	M								
Sac I	A L	A	A	A	A	A	SD	A	A	A	SD	A	A	A	L	SD	L	A	M	L	SD	L	A	A	M							
Sal I	H	H	H	H	H	H	H	H	SD	H	H	H	H	H	SD	H	SD	H	H	SD	H	SD	H	H	H	SD							
Sfi I [3]	M	M	M	M	M	H	B	M	M	L	SD	M	M	M	L	H	L	M	M	M	M	M	M	M	M	L	H						
Sma I [4]	A	A	A	A	A	SD	A	A	A	SD	A	A	A	A	A	SD	A	A	A	A	SD	A	A	A	A	A	SD	A					
Spe I	M H	B	M	H	H	H	H	H	H	L	H	H	H	M	L	H	A	H	H	M	H	M	H	H	M	A	H	M	A				
Sph I	M	B	M	M	H	H	H	M	M	A	H	M	B	M	A	H	A	H	H	M	H	M	H	H	M	M	H	M	A	M			
Stu I	A B L	B	A	A	H	H	B	B	A	L	H	A	B	B	L	H	L	H	B	L	H	M	H	B	M	L	H	M	A	L	M		
Xba I	A H	B	A	A	H	H	H	H	A	A	H	H	B	B	A	H	A	H	H	M	H	M	H	H	H	A	H	M	A	H	M	A	
Xho I	H	B	B	H	H	H	H	H	H	B	H	H	B	M	L	H	A	H	H	M	H	M	H	H	H	A	H	M	A	H	H	B	H

Please remember that star activity may be activated by high glycerol concentration, significant overdigestion, and long incubation times (>16 h).

Legend:

- SD Sequential digest is recommended.
- One enzyme is less than 50% active in specified buffer.

1 Enzyme requires addition of BSA.
2 Enzyme requires incubation at 45°C.

3 Enzyme requires incubation at 50°C.
4 Enzyme requires incubation at 25°C.

Double Digest Information – Use this table to select the most suitable buffer for a given double digest. The table indicates the percentage activity of Roche Applied Science's restriction enzymes in each of the five buffers. The recommended buffer is printed in green (■). Correct usage of the buffer system will prevent the occurrence of side effects (*e.g.*, star activity) that are often observed under suboptimal conditions. Each restriction enzyme is delivered with its own function-tested SuRE/Cut Buffer.

1.4. *Analyzing DNA*

Restriction Enzymes: Cross index of recognition sequences

Palindromic tetra nucleotide sequences:

	AATT	ACGT	AGCT	ATAT	CATG	CCGG	CGCG	CTAG	GATC	GCGC	GGCC	GTAC	TATA	TCGA	TGCA	TTAA
▼□□□□	Tsp509 I								Dpn II Nde II Sau3A I							
□▼□□□		Mae II				Msp I Hpa II		Mae I		HinP1 I		Csp6 I		Taq I		Tru9 I
□□▼□□			Alu I CviJ I				Mvn I FnuD II		Dpn I		Hae III CviJ I	Rsa I			CviR I	
□□□▼□										Cfo I						
□□□□▼		Tai I			Nla III				Cha I							

Sequences are written in 5´ to 3´direction, arrows indicate the point of cleavage
Enzymes in blue recognize only 1 sequence, enzymes in black recognize multiple sequences

Palindromic penta nucleotide sequences:

	AA□TT	AC□GT	AG□CT	AT□AT	CA□TG	CC□GG	CG□CG	CT□AG	GA□TC	GC□GC	GG□CC	GT□AC	TA□TA	TC□GA	TG□CA	TT□AA
▼□□N□□						BssK I						Mae III				
□▼□N□□								Dde I	Hinf I		Sau96 I					
□□▼N□□						ScrF I				Ita I						
□□N▼□□		Tsp4C I														
□□N□▼□											Fmu I					
□□N□□▼																
▼□□A/T□□						EcoR II										
□▼□A/T□□									Tfi I	Tse I	Ava II					
□□▼A/T□□						Mva I										
□□A/T▼□□																
□□A/T□▼□																
□□A/T□□▼																
▼□□G/C□□												Tsp45 I				
□▼□G/C□□																
□□▼G/C□□						Nci I										
□□G/C▼□□																
□□G/C□▼□																
□□G/C□□▼																

Sequences are written in 5´ to 3´direction, arrows indicate the point of cleavage
Enzymes in blue recognize only 1 sequence, enzymes in black recognize multiple sequences

Restriction Enzymes: Cross index of recognition sequences (continued)

Palindromic hexa nucleotide sequences:

	AATT	ACGT	AGCT	ATAT	CATG	CCGG	CGCG	CTAG	GATC	GCGC	GGCC	GTAC	TATA	TCGA	TGCA	TTAA
G▼□□□□C	EcoR I Acs I					NgoM IV Cfr10 I	BssH II	Nhe I	BamH I Xho II	Kas I Ban I	Bsp120 I	Asp718 Ban I		Sal I	Alw44 I	
G□▼□□□C		Acy I								Nar I Acy I			Acc I	Acc I		
G□□▼□□C			Ecl136 II	EcoR V		Nae I				Sfo I			Bst1107 I	Hinc II		Hpa I Hind II
G□□□▼□C																
G□□□□▼C		Aat II	Sac I Ban II AspH I Bsp1286 I		Sph I Nsp I					Bbe I Hae II	Apa I Ban II Bsp1286 I	Kpn I			Bsp1286 I Asp HI	
T▼□□□□A					Rca I	BseA I Mro I BsaW I		Xba I	Bcl I		Eae I	SspB I				
T□▼□□□A														Sfu I		
T□□▼□□A		SnaB I BsaA I					Nru I			Avi II	MluN I					Dra I
T□□□▼□A																
T□□□□▼A																

Sequences are written in 5´ to 3´direction, arrows indicate the point of cleavage
Enzymes in blue recognize only 1 sequence, enzymes in black recognize multiple sequences

Palindromic hexa nucleotide sequences:

	AATT	ACGT	AGCT	ATAT	CATG	CCGG	CGCG	CTAG	GATC	GCGC	GGCC	GTAC	TATA	TCGA	TGCA	TTAA
A▼□□□□T	Acs I		Hind III		BspLU11 I Afl III	PinA I Cfr10 I BsaW I	Mlu I Afl III	Spe I	Bgl II Xho II							
A□▼□□□T		Psp1406 I												Cla I BspD I		Asn I
A□□▼□□T				Ssp I						Eco47 III	Stu I	Sca I				
A□□□▼□T																
A□□□□▼T					Nsp I					Hae II					Nsi I	
C▼□□□□G	Mun I				Nco I Sty I Dsa I	XmaC I Ava I BsoB I	Dsa I	Bln I Sty I			EclX I Eae I	BsiW I	Sfc I	Xho I Ava I BsoB I Sml I	Sfc I	Bfr I Sml I
C□▼□□□G				Nde I												
C□□▼□□G		BbrP I BsaA I	Pvu II MspA1 I			Sma I	MspA1 I									
C□□□▼□G							Ksp I		Pvu I BsiE I		BsiE I					
C□□□□▼G															Pst I	

Sequences are written in 5´ to 3´direction, arrows indicate the point of cleavage
Enzymes in blue recognize only 1 sequence, enzymes in black recognize multiple sequences

1.4. *Analyzing DNA*

Partial Digest

Please keep in mind that this method could be of limited usefulness because the number of possible partial digestion products quickly becomes unmanageable as the number of restriction sites increases.

- For example, in a linear DNA module:

number of restriction sites	1	2	3	4	5	6
number of possible partial-digestion products	0	2	5	9	14	20

- In general, the number of partial-digestion products (F) of a linear DNA molecule that contains N restriction sites is given by the formula

$$F_{N+1} = \frac{N^2 + 3N}{2}$$

Principle

- Ethidium bromide intercalates into dsDNA thereby reducing enzymatic activity of most restriction endonucleases and resulting in a partial digestion of DNA.
- Pipetting scheme for Restriction digest
  Total reaction volume 50.0 µl
  plasmid DNA 2.0-5.0 µg
  Restriction enzyme 5 Units
- Distribute reaction mixture in 5 Eppendorf tubes containing ethidium bromide to final concentrations of 0, 0.02, 0.01, 0.002, 0.005 mg per ml
- Incubate at 37 °C for 20 min
- Load on gel

1.5. *Cloning of DNA*

*Ligation with T4 DNA Ligase**

Properties

- Catalyzes the formation of phosphodiester bonds between adjacent 3´-OH and 5´-P ends in dsDNA.
- Closes single-stranded nicks in dsDNA.
- Needs ATP as co-factor.

Ligation buffer, 10 x

- 660 mM Tris, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP, pH 7.5 at 20°C.
- Buffer is stable at –15 to –25°C, store in aliquots.
- *Note:* ATP is not stable and decreased concentrations of ATP negatively influence the ligation efficiency: aliquot Ligation buffer, store at –20°C and add to ligation mix before use.

Tip

- The new Rapid DNA Dephos & Ligation Kit (Cat. No. 04 898 117 001) available from Roche Applied Science enables fast and efficient dephosphorylation and ligation of sticky- or blunt-end DNA fragments. The convenient kit enables ligation of DNA in 5 minutes and dephosphorylation in 10 minutes.
- *Note:* this kit contains PEG and cannot be used – without further purification of the ligation mix – for transformation of bacteria via electroporation.

Molar ratio of vector and fragment DNA

- Sticky ends:
  - when vector DNA and insert DNA are ~ similar in length: a molar ratio of 1:3 (vector versus insert DNA) is recommended.

- when vector DNA and insert DNA are not similar in length: a molar ratio of 1:1 or 1:2 (vector versus insert DNA) is recommended.

➢ Blunt ends: a molar ratio of vector DNA to insert DNA of 1:5 is recommended.

Application and typical results

➢ Standard Assay: Ligation of DNA fragments:

Components	Sticky ends	Blunt ends
Template DNA	up to 1 µg digested DNA	up to 1 µg digested DNA
10 x Ligation buffer	3 µl	3 µl
T4 DNA Ligase	1 – 5 units	1 – 5 units
H_2O	Add up to 30 µl	Add up to 30 µl
Incubation	4 to 16°C, overnight	16 to 25°C, overnight

➢ Sticky ends: > 95% of the DNA is ligated, depending on type and quality of restriction enzyme.

➢ Blunt ends: > 80% of the DNA is ligated, depending on type and quality of restriction enzyme.

Inactivation of enzyme

➢ T4 DNA Ligase* can be completely inactivated by a 10 min incubation at 65°C. Heat inactivation should only be applied if the ligation reaction mixture is used in experiments other than transformation assays. Otherwise, a drastic decrease of (> factor 20) transformants is possible.

* Product available from Roche Applied Science (Cat. No. 10 481 220 001→1000 U; Cat. No. 10 716 359 001 → 500 U)

1.5. *Cloning of DNA*

Dephosphorylation with rAPid Alkaline Phosphatase

Properties	➢ Catalyzes the dephosphorylation of 5´-protruding, 5´-recessive and 5´ blunt ends from ssDNA, dsDNA, ssRNA and dsRNA. ➢ rAPid Alkaline Phosphatase catalyzes the dephosphorylation of 5' phosphates from DNA and RNA, nucleotides, and proteins. ➢ Is active in restriction enzyme buffers; therefore, restriction enzyme digestion, dephosphorylation, enzyme inactivation, ligation, or 5'-end labeling can be performed without purification steps.
Dephosphorylation buffer, 10 x	➢ 0.5 M Tris, 50 mM $MgCl_2$, pH 8.5 at 20°C. ➢ Buffer is stable at –15 to –25°C.
Inactivation of enzyme	➢ Unlike calf intestinal phosphatase, rAPid Alkaline Phosphatase is rapidly, completely, and irreversibly inactivated by heat treatment for two minutes at +75°C. It is therefore an excellent alternative to Shrimp Alkaline Phosphatase. ➢ The total procedure including restriction enzyme digestion, dephosphorylation, enzyme inactivation and ligation can be performed in one single tube by using Roche Applied Science's Rapid DNA Dephos & Ligation Kit (Cat. No. 04 898 117 001)

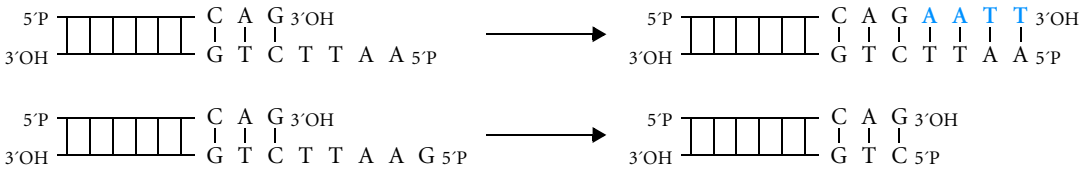
Application and typical results

➢ Standard Assay: Dephosphorylation of 5´ends of dsDNA fragments to prevent self-annealing of vector DNA prior to the insertion of DNA fragments.

Components	recessive or protruding ends	blunt ends
Template DNA	1 pmol	0.2 pmol
10 x rAPid Alkaline Phosphatase buffer, 10x conc.	2 µl	2 µl
rAPid Alkaline Phosphatase	1 unit	1 unit
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	10 min at 37°C	30 min at 37°C

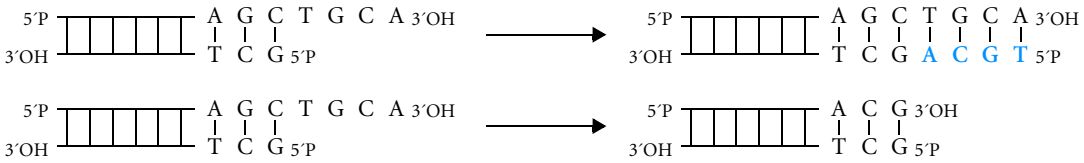
1.5. *Cloning of DNA*

Modifying sticky ends to blunt ends: 5´protruding and 3´recessed ends:



	Klenow	T4 DNA Polymerase	T7 DNA Polymerase	Mung Bean Nuclease
Fill-in 3´recessed ends	👍	👉	👉	👎
Remove 5´protruding ends	👎	👎	👎	👍

Modifying sticky ends to blunt ends: 3´protruding and 5´recessed ends:



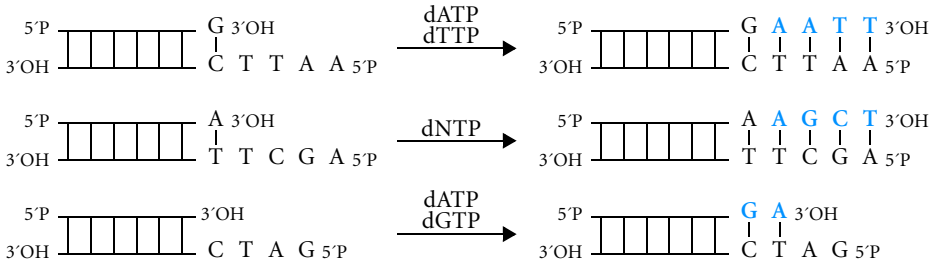
	Klenow	T4 DNA Polymerase	T7 DNA Polymerase	Mung Bean Nuclease
Fill-in 5´recessed ends	👎	👎	👎	👎
Remove 3´protruding ends	👎	👉	👉	👍

1.5. *Cloning of DNA*

Klenow – for partial or complete filling of 3´recessed ends*

Properties

- DNA dependent 5´-3´polymerase with 3´-5´exonuclease activity catalyzing the addition of mononucleotides from dNTPs to the 3´OH terminus of a primer/template DNA.



Filling buffer, 10 x

- 500 mM Tris (pH 7.5), 100 mM $MgCl_2$, 10 mM DTT, 500 µg/ml BSA.
- Buffer is stable at –15 to –25°C, store in aliquots.

Application and typical results

➢ Standard Assay: Partial or complete filling of 3´recessed ends.

Components	Complete filling	Partial filling
Template DNA	1 µg DNA	1 µg DNA
Nucleotides*, final concentration	1 mM of desired[a] dNTPs each	1 mM of desired[a] dNTPs each
10 x Filling buffer	2 µl	2 µl
Klenow*	1 unit	1 unit
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	15 min at 37°C	15 min at 37°C

Inactivation of enzyme

➢ Add 2 µl 0.2 M EDTA and/or heat to 65°C for 10 min.

* Products available from Roche Applied Science (Klenow: Cat. No. 11 008 412 001→ 500 U; Cat. No. 11 008 404 001→ 100 U; Set of dNTPs, PCR-Grade: Cat. No. 11 969 064 001→ 4 x 250 µl (25 µmol); Cat. No. 03 622 614 001→ 4 x 1,250 µl (125 µmol).

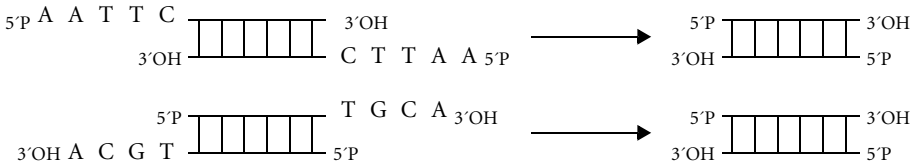
[a] **Note:** Please only add the desired dNTPs as needed according to the sequence.

1.5. *Cloning of DNA*

Mung Bean Nuclease – for removing of 3´ and 5´ protruding ends

Properties

- Degrades ssRNA and ssDNA to produce 5´-phosphoryl oligo- and mononucleotides.
- dsDNA, dsRNA and DNA: RNA hybrids are relatively resistant to the enzyme.



Nuclease buffer, 10x

- 300 mM sodium acetate, pH 4.6, 500 mM NaCl, 10 mM zinc acetate, 0.01% Triton X-100.
- Buffer is stable at –15 to –25°C, store in aliquots

Application and typical results

➢ Standard Assay:
Removing 5´ and 3´protruding ends of DNA creating blunt ends.

Components	Removing ends
Template DNA	1 µg DNA
10 x Nuclease buffer	10 µl
Mung Bean Nuclease	5 units
H_2O	Add up to 100 µl
Incubation	1 hour at 25°C

Inactivation of enzyme

➢ Add EDTA to a final concentration of 1 mM or SDS to a final concentration of 0.01%.

1.5. *Cloning of DNA*

Miscellaneous

Competent cells and transformation

- For an overview of the different procedures, please refer to
  - Hanahan, D. (1983)
    Studies on transformation of *E. coli* with plasmids.
    J. Mol. Biol. **166**, 557–579
  - Hanahan, D. *et al.* (1991)
    Plasmid tranformation of *E. coli* and other bacteria.
    Methods in Enzymology **204**, 63–113
  - Hengen, P. N. (1996)
    Methods and reagents, Preparing ultra-competent *Escherichia coli*.
    Trends in Biochemical Sciences **21**(2), 75–76
- Ready-to-use competent cells are available from different suppliers commercially.

Commercially available cloning kits

- A wide variety of different cloning systems and kits are available from different suppliers.
- Roche Applied Science offers three systems:
  - PCR Cloning Kit (Cat. No. 11 939 645 001): for cloning blunt-end PCR products up to 10 kb
  - Expand Cloning Kit (Cat. No. 11 940 392 001): for cloning blunt-end PCR products from 7 to 36 kb

- Rapid DNA Dephos & Ligation Kit (Cat. No. 04 898 117 001): enabling ligation of sticky and blunt-end fragments within 5 minutes.

Please refer to reference 6 (page 175–191) for a complete overview.

Commonly used bacterial strains

Strain	Genotype
BL21	*E. coli B F⁻ dcm ompT hsdS(r_B- m_B-) gal* (Studier, F.W. et al (1986) J. Mol. Biol., **189**, 113.)
C600[e]	*supE44 hsdR2 thi-1 thr-1 leuB6 lacY1 tonA21*; (Hanahan, D. (1983) J. Mol. Biol. **166**, 557.)
DH5α	*supE44* Δ(*lac*U169 (ϕ80d*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1; (Hanahan, D. (1983) J. Mol. Biol. **166**, 557.)
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*; (Hanahan, D., (1983) J. Mol. Biol. **166**, 557.)
JM108	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-pro*AB); (Yanisch-Perron, C. et al., (1985) *Gene* **33**, 103.)
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-pro*AB) F'[*traD36proAB*$^+$, *lacI*^{q} *lacZ*ΔM15]; (Yanisch-Perron, C. et al., (1985) *Gene* **33**, 103.)
JM110	*rpsL (Str^r) thr leu thi-l lacY galK galT ara tonA tsx dam dcm supE44 Δ (lac-proAB) F'[traD36proAB$^+$, lacI^q lacZΔM15];* (Yanisch-Perron, C. et al., (1985) *Gene* **33**, 103.)

Strain	Genotype
K802	*supE hsdR gal metB*; (Raleigh, E. et al., (1986) Proc.Natl. Acad.Sci USA, 83, 9070.; Wood, W.B. (1966) J. Mol. Biol., **16**,118.)
SURE^{r}	*recB recJ sbc C201 uvrC umuC:Tn5(kan^{r}) lac*, Δ(hsdRMS) endA1 gyrA96 thi relA1 supE44 F'[proAB^{+} lacI^{q} lacZΔM15 Tn10 (tet^{r}); (Greener, A. (1990) Strategies, **3**, 5.)
TG1	*supE hsd Δ5 thi* Δ(*lac-pro*AB) F'[*traD36proAB*$^{+}$, *lacI*^{q} *lacZ*ΔM15]; (Gibson, T.J. (1984) PhD Theses. Cambridge University, U.K.)
XL1-Blue^{r}	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F'[proAB^{+}, *lacI*^{q} *lacZ*ΔM15 Tn10 (*tet*^{r})]; (Bullock et al., (1987) BioTechniques, **5**, 376.)

Commonly used *E. coli* strains

➢ For a detailed overview on the genotypes and references of the most commonly used *E. coli* strains, please refer to "Molecular Biology LABFAX, edited by T.A. Brown, Bios scientific publishers, ISBN 1 872748 00 7".

Notes

1.6. *Labeling of DNA and Oligonucleotides*

General Considerations

Template

- The higher the purity of the DNA template, the better the labeling efficiency.
- For the random primed DNA labeling method, it is critical that the template is linearized and completely heat-denatured prior to the labeling reaction.

Choice of labeling method

- The choice of labeling method depends on:
  - the type of application (*e.g.*, Southern, Dot Blot, …)
  - the available template (*e.g.*, cloned insert, oligonucleotide, …)
  - the requested sensitivity (*e.g.*, single copy gene detection, …)
- The table on page 30 gives an overview of the different methods and their sensitivity for a given application.

Purification of labeled probe

- Unincorporated labeled nucleotides should be removed from the labeling mix:
  - removal from DNA fragments via the High Pure PCR Product Purification Kit* (Cat. No. 11 732 676 001→ up to 250 purifications) or the High Pure PCR Cleanup Micro Kit (Cat. No. 04 983 955 001→ up to 50 purifications) or via ethanol precipitation.
  - removal from oligonucleotides: via ethanol precipitation with glycogen* (20 µg/reaction) or Quick Spin columns*.
- This enables a more accurate quantification of the incorporated label and reduces background in hybridization experiments.

* Products available from Roche Applied Science

Type of label

- Several non-radioactive methods have been developed. The use of these labels (*e.g.*, digoxigenin, biotin, fluorescein, …) offer several advantages:
  - The technology is safe and highly sensitive
  - Results can be achieved much faster (in minutes rather than in hours or days)
  - Probes can be stored for a longer period of time compared to radioactive probes
  - Hybridization solutions can be reused several times
- For an overview of the non-radioactive systems available from Roche Applied Science, please refer to reference 3, 10 and 14.

Note: For detailed information concerning labeling and detection of DNA and RNA please visit:
http://www.roche-applied-science.com/dig

1.6. *Labeling of DNA and Oligonucleotides*

Overview of different techniques

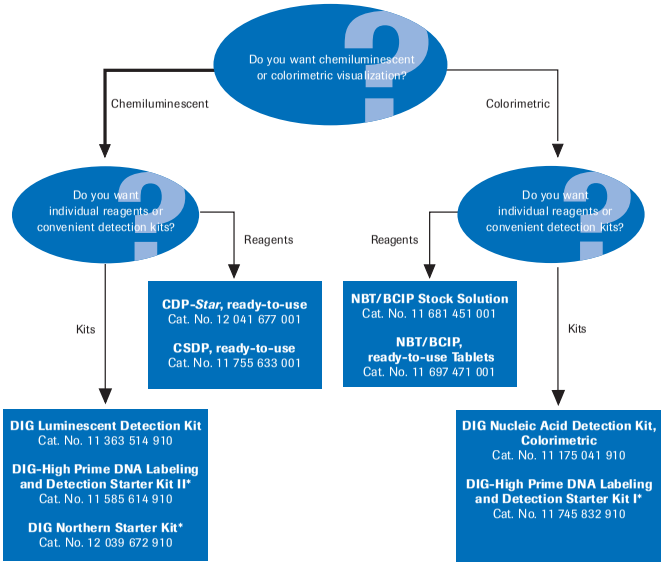
Application	Labeling Methods	Relative sensitivity
Southern Blotting	Random Primed Labeling	+++
Northern Blotting	PCR labeling	+++
	5′ End labeling	++
	3′ End labeling	++
Dot/Slot Blotting	Random Primed Labeling	+++
	PCR labeling	+++
	5′ End labeling	+++
	3′ End labeling	+++

Application	Labeling Methods	Relative sensitivity
Colony/Plaque hybridisation	➢ Random Primed Labeling ➢ PCR labeling ➢ 5´ End labeling ➢ 3´ End labeling	+++ +++ +++ +++
In Situ Hybridisation	➢ PCR labeling ➢ 5´ End labeling ➢ Nick Translation ➢ 3´ End labeling	+++ ++ ++ ++

1.6. *Labeling of DNA and Oligonucleotides*

DIG Labeling of Nucleic Acids

*Note: These kits include labeling reagents and detection substrate.



* Note: These kits include labeling reagents and detection substrate.

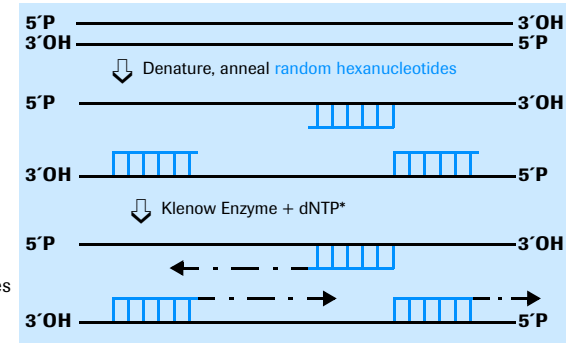
For a complete overview, please refer to references 3, 10 and 14 thus visit: http://www.roche-applied-science.com/dig

1.6. *Labeling of DNA and Oligonucleotides*

Random Primed Labeling with Klenow

Principle

- Based on the random hybridization of a mixture of all possible hexanucleotides to the ssDNA form of the DNA that needs to be labeled.
- The complementary strand is synthesized from the 3´OH termini of the hexanucleotide primers using the 5´-3´polymerase activity of Klenow



* Incorporation of
1. Non-radioactive nucleotides: digoxigenin, biotin, fluorochromes
2. Radioactive nucleotides: [^{32}P], [^{35}S], [^{3}H]

Labeling buffer, 10 x

- 500 mM Tris (pH 7.2), 100 mM $MgCl_2$, 1 mM DTT and 2 mg/ml BSA
- Buffer is stable at –15 to –25°C, store in aliquots.

Application and typical results

- DNA is denatured by heating for 10 min at 95°C and **immediately** put on ice

- Standard assay – prepare reaction mix on ice :

Components	Non-radioactive	Radioactive*
Template DNA	10 ng – 3 µg DNA	10 ng – 2 µg DNA
Nucleotides, final concentration	100 µM of dATP, dCTP, dGTP each, 65 µM dTTP	25 µM of dATP, dGTP, dTTP each
Labeled nucleotide, final concentration	35 µM DIG-, Biotin- or ** Fluorochrome-dUTP	[α^{32}P]dCTP (3,000 Ci/mmol), 50 µCi (1.85 MBq)
10 x Hexanucleotide mix (62.5 A_{260} units/ml)	2 µl	2 µl
Klenow enzyme	2 units	2 units
10 x Labeling buffer	2 µl	2 µl
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	at least 60 min at 37°C	30 min at 37°C

- Size of DNA fragment to be labeled: from 200 up to 50,000 bp.
- Size of labeled DNA fragment ranges from 80 – 200 bp.

Inactivation of enzyme

- Add 2 µl 0.2 M EDTA (pH 8.0) and/or heat to 65°C for 10 minutes.

Tip

- Do not solubilize the DNA to be labeled in buffers containing EDTA since EDTA inhibits the reaction.
- Optimized kits (*e.g.*, Random Primed DNA Labeling Kit Cat. No. 11 004 760 001) are available from Roche Applied Science.

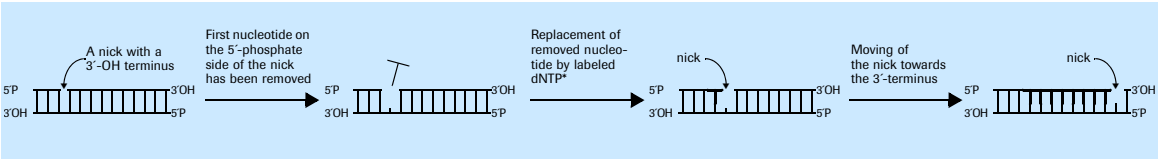
** For a complete overview, please refer to references 10 and 14.

1.6. *Labeling of DNA and Oligonucleotides*

Nick Translation with DNA Polymerase I and DNAse I

Principle

- Based on the ability of DNAse I to introduce random nicks in dsDNA at low enzyme concentrations and in the presence of Mg^{2+}.
- The *E. coli* DNA Polymerase I synthesizes DNA complementary to the intact strand in the 5´-3´direction using the 3´OH termini of the nicks as primers. The 5´-3´exonuclease activity of the enzyme simultaneously removes nucleotides in the direction of synthesis that are replaced by nucleotides supplemented to the reaction.



* Incorporation of
1. Non-radioactive nucleotides: digoxigenin, biotin, fluorochromes
2. Radioactive nucleotides: [^{32}P], [^{35}S], [^{3}H]

NT Buffer, 10 x

- 500 mM Tris (pH 7.4), 100 mM $MgCl_2$, 10 mM DTT, 500 µg/ml BSA.
- Buffer is stable at –15 to –25°C, store in aliquots.

Application and typical results

➢ Standard Assay – prepare reaction mix on ice:

Components	Non-radioactive	Radioactive*
Template DNA	30 ng – 2 µg	30 ng – 2 µg
Nucleotides, final concentration	100 µM of dATP, dCTP, dGTP each, 60 µM dTTP	20 µM of dATP, dGTP, dTTP each
Labeled nucleotide, final concentration	40 µM DIG-, Biotin- or ** fluorochrome-dUTP	[α^{32}P]dCTP (3,000 Ci/mmol), 20 µCi (0.74 MBq)
Mixture of DNA Polymerase I and DNAse I[a]	2 µl	2 µl
10 x NT buffer	2 µl	2 µl
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	90 min at 15°C	30 min at 15°C

* An incorporation rate of > 65% (~3 x 10^8 dpm/µg) is obtained after 30 min.
[a] The ratio of DNA Polymerase I versus DNAse I determines the efficiency of the reaction. A special optimized mixture is available from Roche Applied Science.

➢ Size of DNA fragment to be labeled: from 400 bp to 800 bp.

Inactivation of enzyme

➢ Add 2 µl 0.2 M EDTA (pH 8.0) and/or heat to 65°C for 10 minutes.

Tip

➢ Premixed Nick Translation mixes (*e.g.*, Cat. No. 11 745 808 910) and different kit systems (Cat. No. 10 976 776 001) are available from Roche Applied Science.

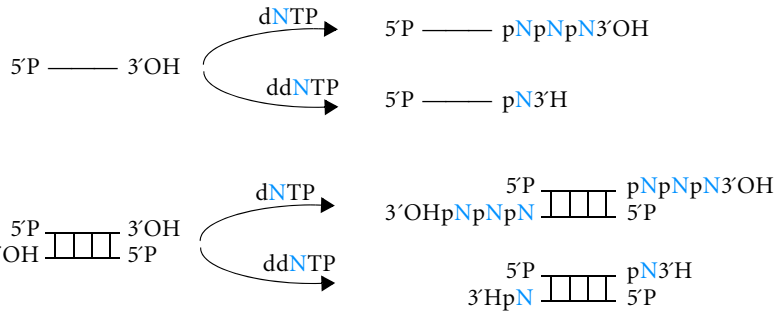
** For a complete overview, please refer to references 10 and 14.

1.6. *Labeling of DNA and Oligonucleotides*

3´End labeling with Terminal Transferase

Principle

- Catalyzes the template independent addition of dNTPs or ddNTPs to the 3´OH ends of ds and ssDNA and oligonucleotides.



incorporation of
- Non-radioactive deoxy- or dideoxynucleotides: digoxigenin, biotin, fluorochromes
- Radioactive deoxy- or dideoxynucleotides: [^{32}P]

Transferase buffer, 10 x

- 2 M potassium cacodylate, 250 mM Tris, 2.5 mg/ml BSA, pH 6.6 at 25°C.
- Buffer is stable at –15 to –25°C, store in aliquots.

Application and typical results

➢ Standard Assay – prepare reaction mix on ice:

Components	Tailing with dNTPs	3´ End labeling with ddNTPs
Template DNA	10 to 100 pmol 3´ends	10 to 100 pmol 3´ends
Radioactive nucleotides, final concentration	[α^{32}P]dNTP (3000 Ci/mmol), 50 µCi (1.85 MBq)	[α^{32}P]ddNTP (3000 Ci/mmol), 50 µCi (1.85 MBq)
Non-radioactive nucleotides, final concentration	– 50 µM Dig, Biotin or fluorochrome dUTP mixed with 500 µM dATP* – 6.25 µM of dATP or dTTP* – 5 µM of dGTP or dCTP*	50 µM Dig, Biotin or fluorochrome dUTP
$CoCl_2$	A or T Tails: 1.5 mM G or C Tails: 0.75 mM	5 mM
Terminal Transferase*	400 units	400 units
10 x transferase buffer	2 µl	2 µl
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	15 min at 37°C	15 min at 37°C

* For more information on length of tail, type of tail (homo- or heteropolymeric), please refer to reference 10.

Inactivation of enzyme

➢ Add 2 µl 0.2 M EDTA (pH 8.0) and/or heat to 75°C for 10 minutes.

Tip

➢ DIG Tailing and DIG 3'End Labeling kits are available from Roche Applied Science.

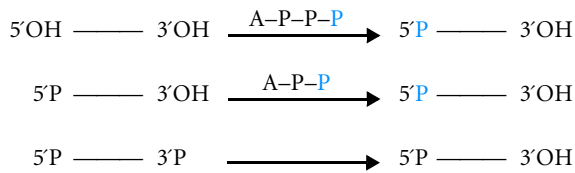
Product available from Roche Applied Science: Terminal Transferase, recombinant (Cat. No. 03 333 574 001→ 24,000 U; Cat. No. 03 333 566 001→ 8,000 U)

1.6. *Labeling of DNA and Oligonucleotides*

5´End labeling with Polynucleotide Kinase

Principle

- Catalyzes the transfer of the terminal phosphate group of ATP to the 5´OH termini of ds and ssDNA and RNA.
- Catalyzes the exchange of terminal 5´P groups of ds and ssDNA and RNA.
- Catalyzes the removal of phosphate groups from the 3´termini of ds and ssDNA and RNA.



Phosphorylation buffer,10 x

- 500 mM Tris, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM dithiothreitol, 1 mM spermidine, pH 8.2 at 25°C.
- Buffer is stable at –15 to –25°C, store in aliquots.

Exchange buffer, 10 x

- 500 mM Imidazole-HCl, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM dithiothreitol, 1 mM spermidine, 3 mM ADP, pH 6.6 at 25°C.
- Buffer is stable at –15 to –25°C, store in aliquots.

Application and typical results

- Standard Assay – prepare reaction mix on ice:

Components	Phosporylation of 5´OH groups	Exchange of 5´P groups
Template DNA	20 pmol 5´OH ends	20 pmol of 5´P termini
nucleotides, final concentration	20 pmol [γ^{32}P]ATP	40 pmol [γ^{32}P]ATP
Polynucleotide Kinase	10 units	10 units
buffer	2 µl, Phosphorylation buffer,10 x	2 µl, Exchange buffer, 10 x
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	30 min at 37°C	30 min at 37°C

- Phosphorylation: More than 30% of [^{32}P] from [γ^{32}P]ATP is incorporated.
- Exchange: More than 10% of [^{32}P] from [γ^{32}P]ATP is incorporated.

Inactivation of enzyme

- Stop phosphorylation and exchange reaction by putting the sample on ice.

Tip

- A special Polynucleotide Kinase, lacking the 3´phosphatase activity is available from Roche Applied Science (Cat. No. 10 709 557 001→ 200 U; Cat. No. 10 838 292 001 → 1,000 U).

1.7. *Amplification of DNA*

Avoiding Contamination

Sources	➢ Laboratory benches, equipment and pipetting devices can be contaminated by previous DNA preparations, plasmid DNA or by purified restriction enzyme fragments. ➢ Cross-contaminations between samples during isolation of nucleic acids. ➢ Products from previous PCR amplifications.
Sample handling	➢ Use sterile techniques and always wear fresh gloves. ➢ Always use new and/or sterilized glassware, plasticware and pipettes to prepare the PCR reagents and template DNA. ➢ Sterilize all reagents and solutions by filtration through a 0.22 µm filter. ➢ Have your own private set of PCR reagents and solutions and use them only for PCR reactions. Store these reagents in small aliquots. ➢ Always include a negative (all reaction components without DNA) and a positive control (*e.g.*, a PCR that has been successfully used in previous experiments).

Laboratory facilities

- Set up physically separated working places for
  - template preparation
  - setting up PCR reactions
  - post-PCR analysis
- Use dedicated (PCR use only) pipettes, micro-centrifuges and disposable gloves.
- Use aerosol resistant pipette tips.
- Set up a PCR reaction under a fume hood (with UV light).

1.7. *Amplification of DNA*

Reaction Components: Template

Purity

- The purity of the template largely influences the outcome of the PCR.
- Large amounts of RNA in the sample can chelate Mg^{2+} and reduce the yield of the PCR.
- Impure templates may contain inhibitors (organic substances: phenol, chloroform, ethanol) that decrease the efficiency of the reaction.
- Always use a purification product and/or procedure specially designed to purify DNA for PCR reactions (see page 7).

Integrity

- Template DNA should be of high molecular weight.
- To check the size and integrity of the DNA, run an aliquot on an agarose gel.

Amount

- The amount of template in a reaction strongly influences the performance of the PCR.
- The recommended amount of template for a standard PCR is
  - maximum 500 ng of human genomic DNA
  - 1 – 10 ng of bacterial DNA
  - 0.1 – 500 ng of plasmid DNA
- The optimal amount of template depends on the application and the allowed error rate within the amplified DNA: the lower the amount of template, the higher the cycle number and the higher the probability for errors within the amplified DNA.

- Lower amounts of template will require specific reaction modifications like changes in cycle numbers, redesign of primers, etc.

Tip

- When testing a new template, include a positive control with primers that amplify a product of about the same size and produce a good yield.
- Avoid to dissolve the template in TE buffer since EDTA chelates Mg^{2+}.
- Use 5–10 mM Tris (pH 7 – 8) or water to dissolve the template.

Reaction Components: Primers

General	➢ Length: 18 to 24 nucleotides. ➢ GC content: 40 – 60%, with a balanced distribution of G/C and A/T rich domains. ➢ Contain no internal secondary structure. ➢ Are not complementary to each other at the 3´ends to avoid primer-dimer forming.
Melting temperature	➢ Estimation of melting temperature T_m: T_m = [2°C x (number of A and T bases)] + [4°C x (number of G and C bases)] see page 164 (Chapter 6.2. Formulas to calculate melting temperature) for more information. ➢ Design primers with similar T_m values.
Annealing temperature	➢ Optimal annealing temperatures are ~5 to 10°C lower than the T_m values of the primers and have to be determined empirically. ➢ Design primers as such that an annealing temperature of 55 – 65°C is allowed, for maximum specificity, use temperatures of 62 – 72°C.

Concentrations of primers	➢ Concentrations between 0.1 and 0.6 µM are generally optimal. ➢ Refer to page 39 to convert µM values into pmol values of primers. ➢ Higher concentrations may promote mispriming and accumulation of non-specific products. ➢ Lower concentrations may be exhausted before the reaction is completed, resulting in lower yields of the desired product.
Storage	➢ Stock solutions: dissolve lyophilized primers in a small volume of 5 mM Tris, pH 7.5 and store at –15 to –25°C. ➢ Working solution: to avoid repeated freezing and thawing, prepare small aliquots of 10 pmol/µl and store at –15 to –25°C.
Tip	➢ When testing new primers, include a positive control reaction with a template that has been tested in PCR.

Concentration

- 1 A_{260} Unit of an Oligonucleotide: 20 – 30 µg/ml H_2O

Molecular Weight (in Dalton)

- $MW = (N_A \times 312.2) + (N_G \times 328.2) + (N_C \times 288.2) + (N_T \times 303.2) + P$

  N_X = number of residues of respective nucleotide in the oligonucleotide

  P = + 17 for phosphorylated oligonucleotide

  P = – 61 for dephosphorylated oligonucleotide

Conversion of µg to pmol

- $\text{pmol of oligo} = \text{µg (of oligo)} \times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{330 \text{ pg}} \times \frac{1}{N} = \frac{\text{µg (of oligo)} \times 3{,}030}{N}$

e.g., 1 µg of a 20 bases oligo = 151.5 pmol

Conversion of pmol to µg

- $\text{µg of oligo} = \text{pmol (of oligo)} \times \frac{330 \text{ pg}}{1 \text{ pmol}} \times \frac{1 \text{ µg}}{10^6 \text{ pg}} \times N =$

$= \text{pmol (of oligo)} \times N \times 3.3 \times 10^{-4}$

e.g., 1 pmol of a 20 bases oligo = 0.0066 µg

N = number of bases

Conversion of µM to pmol

- 1 µl of a X µM primer solution = X pmol primers
- example:
  - Question:
    - given primer solution: 2.5 µM
    - amount of primers needed for experiment: 2 pmol
  - Answer:
    - 1 µl of a 2.5 µM primer solution = 2.5 pmol
    - 2 pmol = 0.8 µl

Conversion of pmol to µM

- 1 µl of a X pmol/µl primer solution = X µM primers
- example:
  - Question:
    - given primer solution: 20 pmol/µl
    - amount of primers needed for experiment: 5 µM
  - Answer:
    - 1 µl of a 20 pmol/µl primer solution = 20 µM primers
    - 5 µM = 0.25 µl

1.7. *Amplification of DNA*

Software

Software*

- Sample of web sites that provides tutorials, lectures and tips on primer design:
  - Primer design software:
    - http://www.premierbiosoft.com
    - http://www.universalprobelibrary.com
    - http://molbiol-tools.ca/PCR.htm
  - Calculating T_m for a given primer:
    - http://www.roche-applied-science.com/benchmate

* Web sites continually appear, disappear and change addresses, quickly invalidating any list of "useful" sites. All of the web sites given here were operating in May, 2007.

Reaction Components: $MgCl_2$ concentration

Function of Mg^{2+} ions	➢ Mg^{2+} ions form soluble complexes with dNTPs and template DNA to produce the actual substrate that the polymerase recognizes.
Concentration of Mg^{2+} ions	➢ The concentration of free Mg^{2+} ions depends on the concentrations of compounds like dNTPs, free pyrophosphates (PPi) and EDTA (*e.g.* from TE buffer). These compounds bind to the ions via their negative charges. ➢ Therefore, the concentration of Mg^{2+} should always be higher than the concentration of these compounds. ➢ The most optimal concentration should be determined empirically and may vary from 1 mM to 5 mM. ➢ The most commonly used $MgCl_2$ concentration is 1.5 mM, with a dNTP concentration of 200 µM each. ➢ Excess Mg^{2+} in the reaction can increase non-specific primer binding and increase the non-specific background of the reaction. ➢ Too little Mg^{2+} in the reaction can result in a lower yield of the desired product.
Tip	➢ A PCR Optimization Kit (Cat. No. 11 636 138 001) – available from Roche Applied Science – allows an easy and straightforward optimization of this reaction component

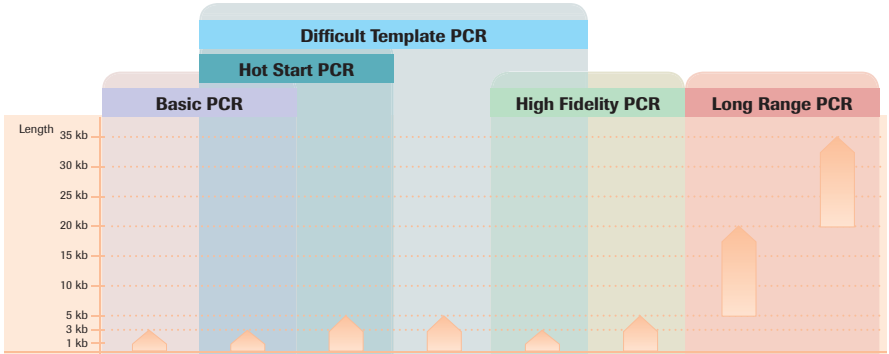
1.7. *Amplification of DNA*

Reaction Components: dNTP concentration

Concentration of dNTP	➢ Always use balanced solutions of all four dNTPs to minimize the error rate. ➢ The final concentration of each dNTP should be between 50 and 500 µM, the most commonly used concentration is 200 µM. ➢ Increase concentration of Mg^{2+} when increasing the concentration of dNTPs. Increases in dNTP concentrations reduces the concentration of free Mg^{2+} thereby interfering with the activity of the polymerase enzyme. ➢ For carry-over prevention, dTTP is substituted by dUTP. A three times higher concentration of dUTP (*e.g.*, 600 µM) compared with the other nucleotides normally results in a good amplification. For more information on this technique, please refer to reference 6.
Storage	➢ Store stock solutions at –15 to –25°C. ➢ Working solutions (*e.g.*, 100 µl mixture of 10 mM of each nucleotide) are also stored at –15 to –25°C.
Tip	➢ Special PCR grade nucleotides* – available from Roche Applied Science – are specially purified and tested to ensure optimal results and maximum sensitivity in all PCR applications.

* see reference 7.

Reaction Components: Choice of Polymerase (see also reference 6)



	Taq DNA Polymerase	FastStart Taq DNA Polymerase	FastStart High Fidelity PCR System	GC-RICH PCR System	Pwo SuperYield DNA Polymerase	Expand High Fidelity^{PLUS} PCR System	Expand Long Template PCR System	Expand 20kb^{PLUS} PCR System
Specificity	■■	■■■■	■■■■	■■■	■■	■■■	■■■	■■
Sensitivity	■■	■■■■	■■■■	■■■	■■	■■■	■■	■■
Robustness	■■	■■■	■■■	■■■■	■■	■■■	■■■	■■■
Accurracy*		1 x	4 x	3 x	18 x	6 x	3 x	2 x
Carryover Prevention	yes	yes	yes	no	no	yes	no	no
Master Mix available	yes	yes	no	no	yes	yes	no	no

* compared to Taq DNA Polymerase

1.7. *Amplification of DNA*

Reaction Components: Hot Start Techniques

Principle

- Taq DNA Polymerase has a low activity at temperatures used to mix the different reaction components (*e.g.*, at room temperature or at 4 °C).
- At these temperatures, the primers may anneal non-specifically and the polymerase may elongate these primers prior to the initial cycle resulting in a series of non-specific amplification products.
- The hot start technique prevents these non-specific products.

Techniques

- Manual: 1 component – such as the polymerase or Mg^{2+} - is only added to the reaction tube after the temperature is > 70 °C.
- Polymerase Antibodies: The polymerase is inactivated by a heat-sensitive antibody. As the temperature rises in the tube, the antibody is inactivated, setting the active polymerase free.
- Chemical Modification of Polymerases: Addition of heat-labile blocking groups to some amino acids renders the Taq DNA Polymerase inactive at room temperature. At higher temperatures, these blocking groups are removed and the enzyme is activated (*e.g.*, FastStart Taq DNA Polymerase (Cat. No. 12 032 937 001) available from Roche Applied Science).

Reaction Components: Other factors

Concentration of Polymerase

- For most assays, the optimal amount of thermostable DNA Polymerase – or blend of polymerases – is between 0.5 and 3.5 units per 50 µl reaction volume.
- Increased enzyme concentrations sometimes lead to decreased specificity.

pH

- In general, the pH of the reaction buffers supplied with the corresponding thermostable DNA Polymerase (pH 8.3 – 9.0) gives optimal results.
- For some systems, raising the pH may stabilize the template and improve the reaction.

Reaction additives

- In some cases, the following compounds can enhance the specificity and/or efficiency of a PCR:
  - Betaine (0.5 – 2 M)
  - Bovine Serum Albumin (100 ng/50 µl reaction mix)
  - Dimethylsulfoxide (2 – 10%, v/v)
  - Glycerol (1 – 5%, v/v)
  - Pyrophosphatase (0.001 – 0.1 units/reaction)
  - Spermidine, Detergents, Gelatine, T4 Gene 32 protein
  - Formamide (2 – 10%)

1.7. *Amplification of DNA*

Cycling Profile for standard PCR

Initial denaturation	➢ It is very important to denature the template DNA completely by initial heating of the PCR mixture. Normally, heating for 2 minutes at 94 – 95°C is enough to denature complex genomic DNA. ➢ If the template DNA is only partially denatured, it will tend to "snap-back" very quickly thereby preventing efficient primer annealing and extension, or leading to "self-priming" which can lead to false positive results.
Denaturation during cycling	➢ Denaturation at 94 – 95°C for 20 to 30 seconds is usually sufficient but must be adapted for the thermal cycler and tubes being used. ➢ If the denaturation temperature is too low, the incompletely melted DNA "snaps back", preventing efficient primer annealing and extension. ➢ Use a longer denaturation time or higher denaturation temperature for GC rich template DNA. ➢ Never use a longer denaturation time than absolutely required, unnecessary long denaturation times decreases the activity of the polymerase.
Primer annealing	➢ The choice of the primer annealing temperature is a very critical factor in designing a high specificity PCR and – for most purposes – has to be optimized empirically (see page 38). ➢ If the temperature is too high, no annealing occurs.

- If the temperature is too low, non-specific annealing will increase dramatically.
- If the primers have complementary bases, primer – dimer effects will occur.

Primer extension

- For fragments up to 3 kb, primer extension is carried out at 72°C. Taq DNA Polymerase adds approximately 60 bases per second at 72°C.
- A 45 second extension is sufficient for fragments up to 1 kb.
- For extension of fragments up to 3 kb, allow about 45 seconds per kb. However, this may need to be adjusted for specific templates.
- To improve yield, use the cycle extension feature of the cycler:
  - first 10 cycles: a constant extension time (*e.g.*, 45 seconds for a 1 kb product)
  - next 20 cycles: increase the extension time by 2 – 5 seconds per cycle (*e.g.*, 50 seconds for cycle 11, 55 seconds for cycle 12, ...)
  - this allows the enzyme more time to do its job because, as PCR progresses, there is more template to amplify and less active enzyme (due to denaturation of the protein during the prolonged high PCR temperatures) to perform the extension.

Cycling Profile for standard PCR **(continued)**

Cycle number	➢ In an optimal reaction less than 10 template molecules can be amplified in less than 40 cycles to a product detectable by gel electrophoresis. ➢ Most PCR's should only include 25 – 35 cycles. ➢ As cycle number increases, non-specific products can accumulate.
Final extension	➢ Usually, after the last cycle, the reaction tubes are held at 72°C for 5 – 15 minutes to promote completion of partial extension products and annealing of single-stranded complementary products. ➢ After the final extension, immediately put the samples on ice or store at 4°C.

1.7. *Amplification of DNA*

Standard Pipetting Scheme

Mix 1 (for 1 reaction):

Reagent		Final concentration
H_20	variable	
10 mM PCR Nucleotide Mix	1 µl	Each dNTP, 200 µM
Upstream and downstream primer	variable	0.1 – 0.6 µM each
Template DNA	variable	0.1 – 0.25 µg
Volume	**25 µl**	

Mix 2 (for 1 reaction):

Reagent	Taq PCR	Final concentration
H_20	19.75 µl	
Enzyme buffer, 10 x conc	5 µl	1 x
Enzyme (5U/µl)	0.25 µl	1.25 units
Volume	**25 µl**	

Combine mix 1 and 2 in a thin-walled PCR tube. Gently vortex the mixture to produce a homogenous reaction, then centrifuge briefly to collect the sample at the bottom of the tube. Standard pipetting scheme applies for enzymes like Taq DNA Polymerase, and Tth DNA Polymerase, and Pwo SuperYield DNA Polymerase.

Standard PCR Temperature Profile

Profile A:

	Temperature	Time	Cycle number
Initial denaturation	94°C	2 min	
Denaturation	94°C	15 – 30 s	25 – 30
Annealing	50 – 65°C[a]	30 – 60 s	
Elongation	72°C	45 s – 3 min	
Final elongation	72°C	7 min	

Profile B*:

	Temperature	Time	Cycle number
Initial denaturation	94°C	2 min	
Denaturation	94°C	15 – 30 s	10
Annealing	50 – 65°C[a]	30 s	
Elongation	72°C	45 s – 3 min	
Denaturation	94°C	15 – 30 s	15 – 20
Annealing	50 – 65°C[a]	30 s*	
Elongation	72°C	45 s – 3 min +5 added seconds/cycle	
Final elongation	72°C	7 min	

[a] The exact annealing temperature depends on the melting temperature of the primers
* This profile ensures a higher yield of amplification products

Notes

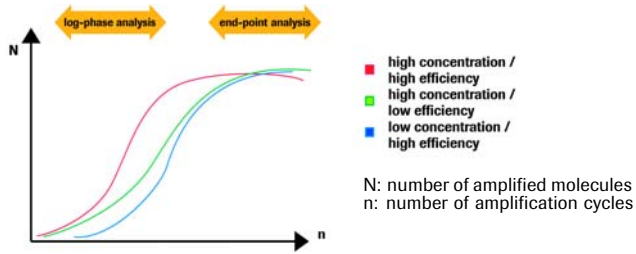
1.8. *Quantitative Real-Time PCR*

Methods

Introduction

- Due to its sensitivity and dynamic range, PCR is the ideal technique to quantify nucleic acids.
- Traditionally, the quantification of a given nucleic acid of interest was determined by methods such as: competitive PCR, PCR ELISA, limiting dilution PCR, radioactive methods.
- These methods are based upon end-point analysis and have several limitations.

End-point analysis



The figure above shows typical amplification curves. Each curve has 3 segments:

- Background phase.
- Exponential - or log-linear - phase
- Plateau phase

End-point analysis is not very suitable for quantitative PCR because it is done in the "plateau phase" of PCR where the reaction no longer follows exponential kinetics. In this phase, the reaction can no longer be described by a mathematical formula. Thus, it is not possible to directly correlate the end-point signal with the initial template amount or target copy number. Further, in the plateau phase, PCR efficiency decreases steadily, because reaction compounds are being consumed and inhibitors are accumulating. These effects vary from sample to sample, resulting in different end-point signals.

Real-Time PCR

- Real-time PCR offers an alternative method for both qualitative and quantitative analysis. This type of analysis allows the amplification and fluorescent detection steps to be performed by a single instrument in a single tube with data recorded online. A real-time PCR instrument measures the accumulation of PCR products during amplification with fluorescent dyes. Because PCR itself and the detection of PCR products occur in the same reaction (vessel), this set-up is also called "homogeneous PCR".

1.8. *Quantitative Real-Time PCR*

Instrument-based Systems

Principles of Real-Time PCR

- Simultaneous amplification and detection of specific nucleic acid sequences via fluorescence-detecting thermocyclers.

LightCycler® System

- The LightCycler® System incorporates several features that make it the ideal tool for qualitative and quantitative PCR as well as mutation analysis in general laboratory applications.
- It includes instrumentation, software, reagents, technical support, and application-specific kits.
- Two real-time PCR systems are available from Roche Applied Science:
  - The Carousel-based LightCycler® Real-Time PCR System (LightCycler® 2.0 Instrument, Cat. No.: 03 531 414 201).
  - The LightCycler® 2.0 Instrument is optimized for two fluorescence detection formats: SYBR Green I and HybProbe probes. In addition, the instrument supports a wide variety of other fluorescence detection formats, such as monocolor SimpleProbe probes, hydrolysis probes, and other formats based on FRET (fluorescence resonance energy transfer).

- The LightCycler® 480 thermal block cycler for either 96- or 384-well plates (LightCycler® 480 Instrument, Cat. No.: 04 640 268 001 96 well, Cat. No.: 04 545 885 001 384 well).
  The LightCycler® 480 System setup enables the use of all current generic DNA dyes (*e.g.*, SYBR Green I, High Resolution Melting dye) and probes (HybProbe probes, SimpleProbe probes, Hydrolysis probes).

➢ For details concerning instrument, reagents and software please refer to **http://www.lightcycler.com** and **www. lightcycler480.com**.

1.8. *Quantitative Real-Time PCR*

Real-Time PCR Assay Formats

All real-time PCR systems detect a fluorescent dye, and then correlate this fluorescence signal to the amount of PCR product in the reaction. There are several methods for detecting and evaluating PCR products fluorimetrically. The most commonly used fluorescent assay methods fall into two classes, sequence-independent detection assays and sequence-specific probe binding assays. Each has its uses and its limitations.

SYBR Green I	➢ Double stranded [ds] - specific intercalating dye.
Hybridization Probes	➢ The presence of a specific amplification product is quantitatively recorded by an increase in fluorescence.
SimpleProbe Probes	➢ Single-label probe with fluorescein.
Hydrolysis Probes	➢ TaqMan® technology using, *e.g.*, FAM, HEX, or VIC.

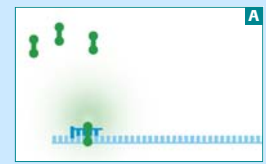
Notes

SYBR Green I Format

Sequence-independent assays rely on fluorophores (typically SYBR Green I) that bind to all double-stranded DNA molecules regardless of sequence.

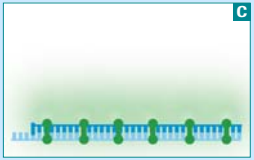
When the SYBR Green I dye intercalates into dsDNA, its fluorescence greatly increases. During the different stages of PCR, the intensity of the fluorescent signal will vary, depending on the amount of dsDNA that is present.

During annealing, PCR primers hybridize to the target and form small regions of dsDNA where SYBR Green I intercalates; the fluorescent signal slightly increases.



In the elongation phase, more dsDNA is formed and more SYBR Green I dye can intercalate; higher fluorescent signal.

At the end of the elongation phase, all DNA has become double-stranded and the maximum amount of SYBR Green I is intercalated. The fluorescence is measured (530 nm) at the end of each elongation phase.



1.8. *Quantitative Real-Time PCR*

Sequence-Specific Probe Binding Assays

FRET Principle

Sequence-specific assays rely on oligonucleotide probes that hybridize to their complementary sequence in the target PCR product and thus only detect this specific product. Commonly used probe formats are hydrolysis probes, hybridization probes (*e.g.*, HybProbe probes) or single-labeled probes (*e.g.*, SimpleProbe probes). The probes are coupled to fluorophores that can be measured by the real-time PCR instrument. Several other detection formats are available as well, but are less frequently used.

The unique LightCycler® HybProbe format is based on the principle of fluorescence resonance energy transfer (FRET).

FRET

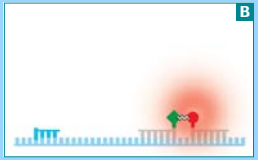
➢ **Fluorescence Resonance Energy Transfer** involves transfer of energy from a donor to an acceptor fluorophore. If the donor and the acceptor are in very close proximity, excitation of the donor by blue light results in energy transfer to the acceptor, which can emit light of a longer wave-length. Excitation spectrum of the acceptor must overlap fluorescence emission spectrum of the donor (see figure below).

HybProbe Format

➢ The HybProbe format is suitable for both quantitative PCR and mutation (SNP) detection assays. It uses two specially designed oligonucleotides that hybridize, side by side, to an internal sequence of an amplified fragment during the annealing phase of PCR.

The donor-dye probe is labeled with fluorescein at the 3´ end and the acceptor-dye probe is labeled with LightCycler® Red at the 5´ end. Hybridization does not take place during the denaturation phase of PCR and, thus, the distance between the dyes is too large to allow energy transfer to occur.

During the annealing phase, the probes hybridize to the amplified DNA fragment in a close head-to-tail arrangement. When fluorescein is excited by the light from the LED, it emits green fluorescent light, transferring the energy to LightCycler® Red, which then emits red fluorescent light. This red fluorescence is measured at the end of each annealing step, when the fluorescence intensity is highest.



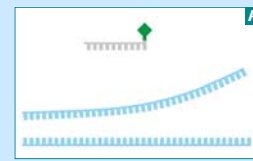
After annealing, the temperature is raised and the HybProbe probe is displaced during elongation. At the end of this step, the PCR product is double-stranded and the displaced HybProbe probes are again too far apart to allow FRET to occur.

One of the advantages of the detection format is that the probes are not altered during PCR. At the end of amplification, both probes are still intact and may be used in a subsequent melting curve experiment (*e.g.*, for mutation detection or SNP analysis).
For the design of primers and hybridization probes for use with the LightCycler® System Roche Applied Science offers the **LightCycler® Probe Design Software**.
For more details please refer to **www.lightcycler.com**

SimpleProbe Format

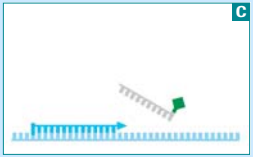
SimpleProbe probes are a special type of hybridization probes. They differ from HybProbe probes in one important way: instead of two probes working together, only a single probe is needed. This single probe hybridizes specifically to a target sequence that contains the SNP of interest. Once hybridized, the SimpleProbe probe emits a greater fluorescent signal than it does when it is not hybridized to its target. As a result, changes in fluorescent signal depend solely on the hybridization status of the probe.

During the denaturation phase no hybridization takes place, thus, only a low fluorescence background is detected at 530 nm.



During the annealing phase, the probe hybridizes to the amplified DNA fragment and is no longer quenched. Fluorescein, when excited by the LightCycler® LED, emits green fluorescent light which is measured only at the end of each annealing step at maximum intensity.

During the subsequent elongation step, the SimpleProbe probe is displaced.

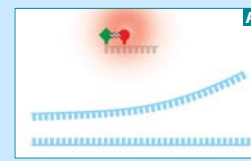


At the end of the elongation step, the PCR product is double stranded and the displaced SimpleProbe probe is again quenched.

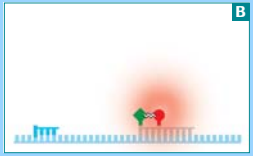
Hydrolysis Probe Format

Hydrolysis probe assays, conventionally called "TaqMan"assays, can technically be described as homogenous 5´nuclease assays, since a single 3´ non-extendable Hydrolysis probe, which is cleaved during PCR amplification, is used to detect the accumulation of a specific target DNA sequence. This single probe contains two labels, a fluorescence reporter and a fluorescence quencher, in close proximity to each other. When the probe is intact, the quencher dye is close enough to the reporter dye to suppress the reporter fluorescent signal (fluorescence quenching takes place via FRET). During PCR, the 5´nuclease activity of the polymerase cleaves the Hydrolysis probe, separating the reporter and quencher. In the cleaved probe, the reporter is no longer quenched and can emit a fluorescence signal when excited.

The probe carries two fluorescent dyes in close proximity, with the quencher dye suppressing the reporter fluorescence signal. The 3' end of the Hydrolysis probe is dephosphorylated, so it cannot be extended during PCR. During denaturation the target double-stranded DNA is separated.



In the annealing phase of PCR, primers and probes specifically anneal to the target sequence.



As the DNA polymerase extends the primer, it encounters the probe. The polymerase then cleaves the probe with its inherent 5´ nuclease activity, displaces the probe fragments from the target, and continues to polymerize the new amplicon.

In the cleaved probe, the reporter dye is no longer quenched and therefore can emit fluorescent light that can be measured by one channel of the LightCycler optical unit, Thus, the increase in fluorescence from the reporter dye directly correlates to the accumulation of PCR products.

Unlike HybProbe probes, hydrolysis probes are digested during PCR. Thus, these probes cannot be used in a subsequent melting curve experiment. This type of assay requires a different experimental approach for detecting a mutation or SNP.

Universal ProbeLibrary

- A sophisticated application of the hydrolysis probe format is the Universal ProbeLibrary from Roche Applied Science. The Universal ProbeLibrary contains 165 prevalidated, double-labeled, real-time PCR probes that can be used to quantify virtually any transcript in the transcriptomes of humans, mice, rats, primates, Drosophila, C. elegans, and Arabidopsis.
- Each of the organism-specific Universal ProbeLibrary Sets can detect 95-99% of all transcripts from that organism. This almost universal coverage is due to the length (only 8-9 nucleotides) of the Universal ProbeLibrary probes. Each probe is much shorter than classic, 25- to 35-nucleotide hydrolysis probes. To maintain the specificity, Tm and assay compatibility that hybridization probes require, the duplex-stabilizing DNA analogue LNA (Locked Nucleic Acid) is included in the sequence of each probe.
- Because it is short, each Universal ProbeLibrary probe can bind to around 7,000 transcripts, while each transcript is detected by around 16 different probes. Yet, only one transcript is detected in a given PCR assay, a specificity ensured by the set of PCR primers chosen.
- Selection of the correct Universal ProbeLibrary probe and specific PCR primers for a given real-time PCR assay is a simple two-step procedure that involves the ProbeFinder Software, which is available online at the Assay Design Center (http://www.universalprobelibrary.com).

- Universal ProbeLibrary assays are compatible with all instruments capable of detecting fluorescein, FITC, FAM and/or SYBR Green I. Universal ProbeLibrary assays have been used successfully on the LightCycler® Carousel-Based System, the LightCycler® 480 System and other real-time PCR instruments from several suppliers.

The powerful combination of the ProbeFinder Software, the Universal ProbeLibrary, and the Transcriptor First Strand cDNA Synthesis Kit, can revolutionize the way you design and perform real-time qPCR assays.

For instruments requiring normalization with Rox reference dye choose in addition the new **FastStart Universal Probe Master (Rox)** from Roche Applied Science. For two-step real-time RT-PCR using the LightCycler® Instruments choose the LightCycler® 480 Probes Master.

For One-Step RT-PCR, the Universal ProbeLibrary probes can be ideally combined on the LightCycler® 480 System Master Hydrolysis probes (Cat.No, 04 991 885 001).

Molecular beacons

- Hairpin-shaped oligonucleotides oligos with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid. The loop portion is complementary to the middle of the expected amplicon and the stem is formed at the annealing temperature of the PCR. For more details refer to http://www.molecularbeacons.com

Scorpions

- Technology based on a stemloop primer with an integral tail which is used to probe an extension product of the primer thus containing a fluorophore and a quencher. The stemloop tail is separated from the PCR primer by a stopper which prevents stemloop sequences to be copied during PCR. During the annealing step the Scorpion tail curls back to hybridize to the target sequence in the PCR product. As the tail of the scorpion and the PCR product are now part of the same strand of DNA, a fluorescence signal is generated.

Notes

1.8. *Quantitative Real-Time PCR*

Principles of Quantification

Quantification analysis in real-time PCR can be subdivided into two basic types, absolute and relative quantification. Each type uses the experimentally determined CP values differently.

Absolute quantification

➢ In absolute quantification assays, the concentration of the target molecule is expressed as an absolute value (*e.g.*, copies, µg/µl, etc.). Absolute quantification methods use a standard curve, calculated from external standard samples of known concentration, to determine the concentration of the target molecule in the unknown. Thus, an absolute quantification assay system produces valid results only if the standards and the unknowns are amplified and detected with the same efficiency.

Application: Absolute Quantification is very suitable for applications in virology and microbiology where you need to determine the copy number of a specific target, or for the determination of absolute gene copy numbers. In a dual-color set-up, absolute quantification can be combined with internal standards to detect any false negative results.

Relative quantification

➢ In relative quantification assays, the target concentration is expressed as a ratio of target-to-reference gene in the same sample, rather than an absolute value. The reference gene is an unregulated nucleic acid that is found at constant copy number in all samples.

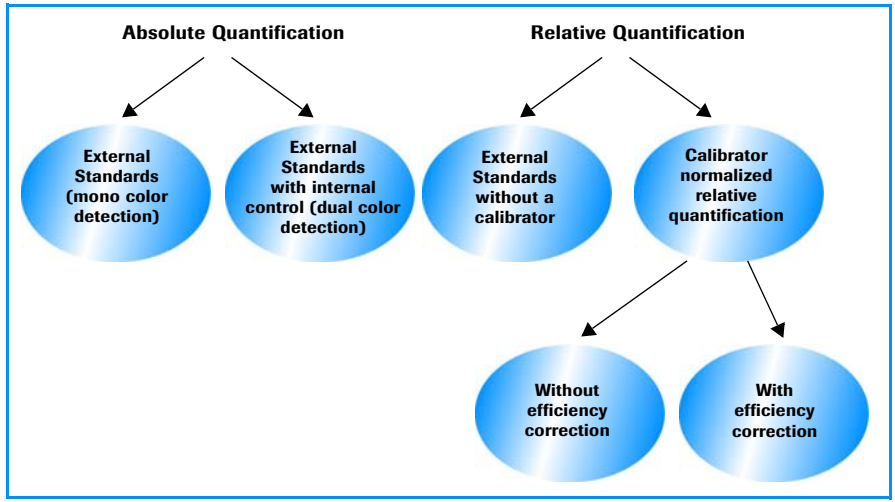
Relative quantification methods correct the sample for differences in quality and quantity like variations in initial sample amount, cDNA synthesis efficiency, or sample loading/pipetting errors. Because the quantity of a target and a reference gene is a function only of the PCR efficiency and the sample crossing point, these assays do not require a standard curve in each analysis run.

Application: Quantification of mRNA expression levels or determination of gene dosis values.

Relative Quantification software, which enables performance of calibrator-normalized relative quantification with PCR efficiency correction, is available for all real-time PCR systems from Roche Applied Science.

For more detailed and up-to-date descriptions of quantitative real-time PCR methods, visit www.lightcycler.com.

Principles of Quantification (continued)



Notes

1.8. *Quantitative Real-Time PCR*

Melting Curve Analysis

Besides monitoring the PCR process online, real-time PCR instruments (including the LightCycler® Carousel-Based System and LightCycler® 480 System) can monitor fluorescence changes during temperature transitions. This allows the annealing and denaturation of nucleic acids to be followed in real-time. This procedure, called melting curve analysis, uses either dsDNA-specific dyes (*e.g.*, SYBR Green I) or sequence-specific oligonucleotide probes (*e.g.*, HybProbe or SimpleProbe probes), and can be added at the end of PCR.

Melting Curve Analysis with SYBR Green I

➢ Melting curve analysis with SYBR Green I is used for product characterization, i.e. to determine whether the desired PCR product is free of nonspecific by-products. PCR products can be characterized by melting curve analysis because each double-stranded DNA molecule has a characteristic melting temperature (Tm), at which 50% of the DNA is double-stranded and 50% is melted, i.e. single-stranded. During a melting curve run, the reaction mixture is slowly heated to 95°C, which causes dsDNA to melt. A sharp decrease in SYBR Green I fluorescence occurs when the temperature reaches the Tm of a PCR product present in the reaction.
For instruments requiring normalization with Rox reference dye choose the new **FastStart Universal SYBR Green Master (Rox)** from Roche Applied Science.

Genotyping with HybProbe and SimpleProbe Probes and SNP Detection

➢ In a genotyping experiment with HybProbe probes, one HybProbe oligonucleotide hybridizes to a part of the target sequence that is not mutated; this probe functions as an anchor. The other HybProbe oligonucleotide (mutation or detection probe) spans the mutation site and has a Tm approx. 5°C lower than the Tm of the anchor probe. As the temperature increases, the shorter mutation probe dissociates first. When this happens, the two dyes are no longer close and the fluorescence signal decreases. Because the Tm of a probe-target hybrid depends not only on the length and the GC content of the probe, but also on the degree of homology in the hybrid, perfectly bound probes separate at a higher Tm than those that are bound to DNA containing a destabilizing mismatch.

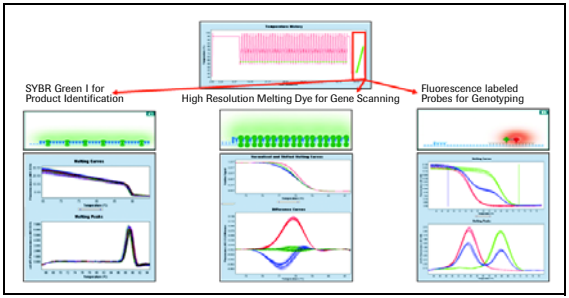
The precise temperature control of the LightCycler® Carousel-Based System and LightCycler® 480 System allow these instruments to monitor specific fluorescent-labeled probes as they melt off a target sequence. When melting curve analysis is used to monitor the melting of short duplexes, such as hybrids between HybProbe or SimpleProbe probes and target, the assay can identify even single base alterations in the amplicon. Thus, this is an ideal tool for detection of Single Nucleotide Polymorphisms (SNPs) or genotyping.

High resolution melting curve analysis

➢ A new class of non-sequence specific DNA dyes (*e.g.*, LightCycler® 480 ResoLight dye)bind to double-stranded DNA Sequence in a saturating manner and therefore give very uniform, sharp signals during melting curve analysis. These binding characteristics can be used to detect subtle differences in sequence between samples (*e.g.*, SNPs, mutations), or within a sample (*e.g.*, to differentiate heteroduplex from homoduplex DNA to identify heterozygotes).

Due to its uniform thermal profile across a plate and its optical system, the LightCycler® 480 System enables the acquisition and analysis of melting curves at high resolution. The LightCycler® 480 High Resolution Melting Master is available to support this method. Sequence variants resulting in different melting curve shapes can be detected using LightCycler® 480 Gene Scanning Software (Cat. No. 05 103 908 001).

An overview of melting curve-based methods supported on the LightCycler® 480 System is shown in the figure below.



For up-to-date information on genotyping methods, visit **www.lightcycler480.com**.

1.9. *References*

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15. LightCycler® System (2004) Roche Applied Science

Working with RNA

Chapter 2

2

2.1. *Precautions for handling RNA*

General Information	➢ Working with RNA is more demanding than working with DNA, because of the chemical instability of the RNA and the ubiquitous presence of RNases. ➢ Unlike DNases, RNases do not need metal ion co-factors and can maintain activity even after prolonged boiling or autoclaving. ➢ Therefore special precautions should be taken when working with RNA.
Gloves and contact	➢ Always wear gloves when working with RNA. ➢ After putting on gloves, don't touch surfaces and equipment to avoid reintroduction of RNases to decontaminated material.
Workspace and working surfaces	➢ Designate a special area for RNA work only. ➢ Treat surfaces of benches and glassware with commercially available RNase inactivating agents. Clean benches with 100% ethanol.
Equipment and disposable items	➢ Use sterile, disposable plasticware. ➢ Electrophoresis tanks for RNA analysis can be cleaned with 1% SDS, rinsed with H_2O, then rinsed with absolute ethanol and finally soaked in 3% H_2O_2 for 10 min. Rinse tanks with DEPC-treated H_2O before use.

Glass and plasticware	➢ Glassware should be baked at 180° – 200°C for at least 4 hours. Autoclaving glassware is not sufficient to eliminate RNases. ➢ Use commercially available RNase free plasticware. ➢ If plasticware should be reused, soak (2 h, 37°C) in 0.1 M NaOH/1 mM EDTA (or absolute ethanol with 1% SDS), rinsed with DEPC treated H_2O and heated to 100°C for 15 min. ➢ Corex tubes should be treated with DEPC treated H_2O overnight at room temperature, then autoclave for 30 min. to destroy unreacted DEPC.
Cleaning of pH electrodes	➢ Incubate for 30 s in 70% ethanol, 5 min in 1 M NaOH, and rinse with DEPC-treated H_2O.

DEPC = diethylpyrocarbonate

2.1. *Precautions for handling RNA*

Reagents	➢ Purchase reagents that are free of RNases. ➢ Reserve separate reagents for RNA work only: Wear gloves, use baked spatulas and untouched weigh boats or weigh paper. ➢ All solutions should be made with DEPC-treated H_2O (see page 155).
Handling fresh and stored material before extraction of RNA	➢ Extract RNA as quickly as possible after obtaining samples. ➢ For best results, use either fresh samples or samples that have been quickly frozen in liquid nitrogen and stored at –70°C. This procedure minimizes degradation of crude RNA by limiting the activity of endogenous RNases. ➢ Blood and bone marrow samples can be stabilized with commercial available stabilization reagents[a]. ➢ All required reagents should be kept on ice.

[a] *e.g.,* RNA/DNA Stabilization Reagent for Blood and Bone Marrow (Cat. No. 11 934 317 001), available from Roche Applied Science

RNase inhibitors	➢ RNase inhibitors* can be used to protect RNA from degradation during isolation and purification and in all downstream applications. ➢ The most commonly used inhibitor is a eukaryotic protein that inactivates RNases, via a non-covalent and reversible binding. ➢ Use an RNase Inhibitor that is active during cDNA synthesis and can protect RNA, like Protector RNase Inhibitor which is active up to 60°C. ➢ To keep the inhibitor active, avoid solutions with strong denaturing agents such as SDS or urea, maintain reducing conditions (1 mM DTT) and hold the temperature below 65°C. ➢ Other inhibitors include macaloid and vanadyl-ribonucleoside complexes.
Storage of RNA	➢ Store RNA, aliquoted in ethanol or isopropanol at – 70°C. Most RNA is relatively stable at this temperature. ➢ Remove ethanol or isopropanol by centrifugation and resuspend in the appropriate RNase-free buffer. ➢ Store cDNA if possible.
Manipulation of RNA	➢ Always keep the RNA sample on ice when preparing an experiment.
Drying RNA	➢ Avoid overdrying of RNA after ethanol precipitation. ➢ Dry in vacuum or for 15 min at room temperature in a clean space.

* Product available from Roche Applied Science: Protector RNase Inhibitor (Cat. No. 03 335 399 001→ 2000 U; Cat. No. 03 335 402 001→ 10000 U)

2.1. *Precautions for handling RNA*

Dissolving RNA	➢ Dissolve RNA by adding RNase-free buffer or H_2O and incubate the tube on ice for 15 minutes. Gently tap the tube or vortex with care.
Pipetting RNA	➢ Use RNase-free tips or autoclave regular tips. ➢ Keep pipettes clean.
Temperature sensitivity	➢ RNA is not stable at elevated temperatures, therefore, avoid high temperatures (> 65°C) since this will affect the integrity of the RNA. ➢ To melt secondary structures, heat RNA to 65°C for 15 min. in the presence of denaturing buffers.

Notes

2.2. *Commonly used formulas*

Calculation of Molecular Weights in Dalton

- Average molecular weight (MW) of a ribonucleotide: 340 Dalton (Da)

MW of ssRNA = [number of bases] x [340 Da]

e.g., MW of tRNA from E. coli (75 bases) = 25.5 x 10^3 Da = 25.5 kDa

Conversion of µg to pmol

- $\text{pmol of ssRNA} = \mu\text{g (of ssRNA)} \times \frac{10^6 \text{ pg}}{1\ \mu\text{g}} \times \frac{1 \text{ pmol}}{340 \text{ pg}} \times \frac{1}{N_b} = \frac{\mu\text{g (of ssRNA)} \times 2{,}941}{N_b}$

e.g., 1 µg of a 100 base ssRNA molecule $= \frac{1 \times 2{,}941}{100} = 29.4$ pmol

- The value calculated with this formula also represents the pmoles of 5′ or 3′ ends.

Calculation of pmol of 5′ (or 3′) ends

- see above calculation of conversion of µg to pmol

N_b = number of bases

Conversion of pmol to µg

➢ $\mu g \text{ of ssRNA} = \text{pmol (of ssRNA)} \times \frac{340 \text{ pg}}{1 \text{ pmol}} \times \frac{1 \mu g}{10^6 \text{ pg}} \times N_b$

$= \text{pmol (of ssRNA)} \times N_b \times 3.4 \times 10^{-4}$

e.g., 1 pmol of a 250 base ssRNA molecule = 0.085 µg

N_b = number of bases

2.2. *Commonly used formulas*

Examples

Organism	Type	Length (in bases)	MW (in kDa)
E. coli, gram negative bacterium	tRNA	75	26
	5S rRNA	120	41
	16S rRNA	1 541	523
	23S rRNA	2 904	987
Drosophila melanogaster, fruitfly	18S rRNA	1 976	672
	28S rRNA	3 898	1.3×10^3
Mus musculus, mouse	18S rRNA	1 869	635
	28S rRNA	4 712	1.6×10^3
Homo sapiens, human	18S rRNA	1 868	635
	28S rRNA	5 025	1.7×10^3
Oryctolagus cuniculus, rabbit	18S rRNA	2 366	804
	28S rRNA	6 333	2.15×10^3

Notes

2.3. *Isolating and purifying RNA*

Use this chart to select a product according to the type and origin of RNA (see also reference 3).

Type	Origin	Recommendation*
mRNA	Cultured cells, tissues, total RNA	mRNA Isolation Kit
		mRNA Capture Kit
	Human whole blood/bone marrow	mRNA Isolation Kit for Blood/Bone marrow
total RNA	Cultured cells, bacteria, yeast, blood	High Pure RNA Isolation Kit
	Tissue	High Pure RNA Tissue Kit
	Cultured cells, tissues, bacteria, yeast, blood	TriPure Isolation Reagent
	Formalin-fixed, paraffin-embedded tissue, fresh-frozen tissue samples	High Pure RNA Paraffin Kit
	Formalin-fixed, paraffin-embedded tissue	High Pure FFPE RNA Micro Kit
Viral RNA	Serum, plasma, other body fluids, cell culture supernatant	High Pure Viral RNA Kit
RNA fragments	Removal of unincorporated nucleotides from labeled RNA molecules	Quick Spin Columns
		Mini Quick Spin Columns

* The amount of RNA that can be isolated with the kits depends on a variety of variables like the amount of sample applied, concentration of RNA within the sample, buffer systems, etc. Please refer to page 66 for a detailed overview.

Use this chart to select a product according to the main application in which the RNA will be used (see reference 3).

Product	RT-PCR	DD*-RT-PCR	cDNA Synthesis	Northern Blotting	RNase protection	*In Vitro* Translation
High Pure RNA Tissue Kit	●	●	●	●	●	●
High Pure RNA Paraffin Kit	●		●			
High Pure FFPE RNA Micro Kit	●	●	●			
High Pure RNA Isolation Kit	●	●	●	●	●	●
High Pure Viral RNA Kit	●					
mRNA Isolation Kit for Blood/Bone Marrow	●		●	●	●	●
mRNA Isolation Kit	●		●	●	●	●
mRNA Capture Kit	●					
TriPure Isolation Reagent	●	●	●	●	●	●
mini Quick Spin RNA Columns				●		
Quick Spin Columns				●		

* DD = differential display

2.3. *Isolating and purifying RNA*

Use this chart to select a product according to its characteristics (see also reference 3).

Product	Quantity of starting material	Typical Yield
High Pure RNA Tissue Kit	➢ Tissue: 1 – 10 mg	➢ 0.5 – 3.0 µg/mg[a]
High Pure RNA Isolation Kit	➢ Blood: 200 – 500 µl ➢ Cultured cells: 10^6 cells ➢ Yeast: 10^8 cells ➢ Bacteria: 10^9 cells	➢ for 10 RT-PCR Reactions ➢ 20 µg[b] ➢ 20 µg ➢ 35 – 50 µg
High Pure Viral RNA Kit	➢ 200 – 600 µl of serum, plasma, urine, cell culture supernatant	➢ Product detectable by RT-PCR
High Pure RNA Paraffin Kit	➢ up to 20 µm FFPE fresh-frozen tissue section	➢ 0.3 to 1.5 µg/5 µm section ➢ 2 – 6 µg/20 mg tissue
High Pure FFPE RNA Micro Kit	➢ 1 – 10 µm FFPE tissue sections	➢ 1.5 – 3.5 µg/5 µm

[a] depends on type of tissue
[b] depends on type of cells

Use this chart to select a product according to its characteristics (see also reference 3).

Product	Quantity of starting material	Yield
mRNA Isolation Kit	➢ Tissue: 50 mg – 1 g ➢ Cells: 2 x 10^5 – 10^8 ➢ total RNA: 250 µg – 2.5 mg	➢ 7 – 14 µg/100 mg tissue[a] ➢ 0.3 – 25 µg/10^7 cells[b] ➢ 1 – 5 µg/100 µg total RNA
mRNA Capture Kit	➢ Tissue: up to 20 mg ➢ Cells: up to 5 x 10^5 ➢ total RNA: up to 40 µg	➢ Product detectable by RT-PCR
TriPure Isolation Reagent	➢ RNA from liver, spleen: 50 mg – 1 g ➢ Cultured epithelial cells, fibroblasts: 10^6 – 10^7	➢ 6 – 10 µg/mg tissue[a] ➢ 8 – 15 µg/10^6 cells[b]
mini Quick Spin RNA Columns	➢ 20 – 75 µl labeling mixture	➢ > 80% ➢ Exclusion limit: ≥ 20 bp
Quick Spin Columns	➢ 50 µl labeling mixture	➢ > 80%

[a] depends on type of tissue
[b] depends on type of cells

Note: For more information on nucleic acid isolation and purification please visit **http://www.roche-applied-science.com/napure**

2.3. *Isolating and purifying RNA*

RNA content in various cells and tissues *(see also reference 3)*

10^7 cells	3T3	Hela	COS
total RNA (µg)	~120	~150	~350
mRNA (µg)	~3	~3	~5

mouse tissue (100 mg)	Brain	Heart	Intestine	Kidney	Liver	Lung	Spleen
total RNA (µg)	~120	~120	~150	~350	~400	~130	~350
mRNA (µg)	~5	~6	~2	~9	~14	~6	~7

Notes

Concentration of RNA

- 1 A_{260} Unit of ssRNA = 40 µg/ml H_2O

Notes

- OD value should range between 0.1 and 1.0 to ensure an optimal measurement.
- The above mentioned value is based on the extinction coefficient of RNA in H_2O.
- Please note that this coefficient – and hence the above mentioned value – may differ in other buffers and/or solutions.
- Cuvettes should be RNase free
  (see chapter 2.1. “Precautions for handling RNA”, page 60)
- Example of calculation:
  - volume of ssRNA sample: 100 µl
  - dilution: 25 µl of this sample + 475 µl H_2O (1/20 dilution)
  - A_{260} of this dilution: 0.56
  - concentration of ssRNA in sample: 0.56 x 40 µg/ml x 20 (= dilution factor) = 448 µg/ml
  - amount of ssRNA in sample: 448 µg/ml x 0.1 ml (= sample volume) = 44.8 µg

Purity of RNA

- Pure RNA: $A_{260}/A_{280} \geq 2.0$

Notes

- Buffered solutions provide more accurate values than water since the A_{260}/A_{280} ratio is influenced by pH. Therefore, it is recommended to measure the ratio in a low salt buffer.
- Pure RNA has a ratio of 1.9 – 2.1 in a 10 mM Tris buffer.
- An A_{260}/A_{280} smaller than 2.0 means that the preparation is contaminated with proteins and aromatic substances (*e.g.*, phenol). In this case, it is recommended to purify the RNA (again) before using it in subsequent applications.
- The OD value gives no indication about the size of the RNA.

2.4. *Analyzing RNA*

Size estimation of RNA fragments

Agarose Electrophoresis

- In most cases, 1% Agarose MP** gels (or variations between 0.8 and 1.2%) are used to separate ssRNA molecules.
- Denaturing conditions*** are crucial for the resolution and visualization of the different fragments in a given gel.

Acrylamide Electrophoresis

- Efficient range of separation of ssRNA molecules:
  - 3.5% of Acrylamide/Bisacrylamide (29/1): 750 – 2,000
  - 5.0% of Acrylamide/Bisacrylamide (29/1): 200 – 1,000
  - 8.0% of Acrylamide/Bisacrylamide (29/1): 50 – 400

Size Markers

- A variety of different molecular weight markers are available from different suppliers. Roche Applied Science offers:
  - **RNA Molecular Weight Marker I, DIG-labeled** (0.3 – 6.9 kb), Cat. No. 11 526 529 910
    9 fragments: 310, 438, 575, 1049, 1517, 1821, 2661, 4742, 6948 bases
  - **RNA Molecular Weight Marker II, DIG-labeled** (1.5 – 6.9 kb), Cat. No. 11 526 537 910
    5 fragments: 1517, 1821, 2661, 4742, 6948 bases

- **RNA Molecular Weight Marker III, DIG-labeled** (0.3 – 1.5 kb), Cat. No. 11 373 099 910
  5 fragments: 310, 438, 575, 1049, 1517 bases

(For more information please refer to reference 10)

➢ Bacterial 23S* (~2,900 b) and/or 16S* (~1,550 b) ribosomal RNA's can also be used as size markers.

* Products available from Roche Applied Sience

** **A**garose **M**ulti **P**urpose – available from Roche Applied Science (Cat. No. 11 388 983 001 – 100 g; Cat. No. 11 388 991 001 – 500 g) – is a high gel strength agarose (> 1,800 g/cm^2: 1%) permitting the use of very low and very high concentrations, resulting in a broad range of linear RNA molecules that can be separated in the same type of agarose. This agarose is also function tested for the absence of DNases and RNases.

*** Please refer to chapter 5 "Preparing Buffers and Media" for recipes to prepare the necessary buffers.

2.5. *RT-PCR*

Reaction Components: Template

Avoiding contamination	➢ Please refer to chapter 1.7. "Amplification of DNA: Avoiding contamination" (page 36) for the different sources of possible contaminations before and during amplification reactions and how to most optimally avoid them.
Type	➢ If possible, use purified mRNA as a template rather than total RNA. ➢ Starting with poly (A^+) mRNA will improve the likelihood of successful amplification of rare mRNA's since the proportion of mRNAs in a total RNA preparation is very low (typically 1 – 5% of total RNA for a mammalian cell).
Integrity	➢ If a mRNA template is used and enough material is available, the integrity of the mRNA can be checked by gel electrophoresis before using it in a RT-PCR. ➢ The mRNA should appear as a smear between 500 bp and 8 kb. Most of the mRNA should be between 1.5 and 2 kb.
Avoiding RNase contamination	➢ To minimize the activity of RNases that are released during cell lysis, include RNase inhibitors* in the lysis mix or use methods (ref. 6 and 7) that simultaneously disrupt cells and inactivate RNases.

- Please refer to chapter 2.1. “Precautions for handling RNA” (pages 60 – 62) for a comprehensive overview how to avoid RNase contamination.
- The most commonly used inhibitor is a eukaryotic protein that inactivates RNases, via a non-covalent and reversible binding.

* Product available from Roche Applied Science: Protector RNase Inhibitor (Cat. No. 03 335 399 001→ 2000 U; Cat. No. 03 335 402 001→ 10000 U)

2.5. *RT-PCR*

Reaction Components: Choice of reverse transcription primers

The primer used for reverse transcription affects both the size and the specificity of the cDNA produced. Four kinds of primers are commonly used in RT-PCR and each has its advantages:

Oligo(dT)$_N$	➢ Generates full-length cDNAs. ➢ If template contains oligo(A) stretches, the primer may bind these and lead to mispriming.
Anchored oligo(dT)$_N$	➢ Prevents priming from internal sites of the poly(A) tail. ➢ Generates full-length cDNA. ➢ Preferred priming method for most two-step RT-PCR applications. ➢ Available as part of the Transcriptor First Strand cDNA Synthesis Kit.*
Random hexamers*	➢ Provides uniform representation of all RNA sequences in mRNA. ➢ Can prime cDNA transcription from RNAs that don't contain a poly(A) tail. ➢ Adjusting the ratio of random primers to RNA in the RT reaction controls the average length of cDNAs formed. Example: A high ratio will generate relatively short cDNAs, which will increase the chances of copying the complete target sequence. ➢ Short cDNA transcripts may be ideal way to overcome difficulties presented by RNA secondary structures.

Sequence-specific

- Selects for a particular RNA, *e.g.*, for diagnostic purposes.
- Greatly increases the specificity of the RT-PCR.
- Only type of priming that can be used for one-step applications.

* Product available from Roche Applied Science: Transcriptor First Strand cDNA Synthesis Kit, Cat. No: 04 379 012 001→ 1 kit (50 rxns, including 10 control rxns); 04 896 866 001→ 1 kit (100 rxns); 04 897 030 001→ 1 kit (200 rxns); Primer "random", Cat. No: 11 034 731 001.

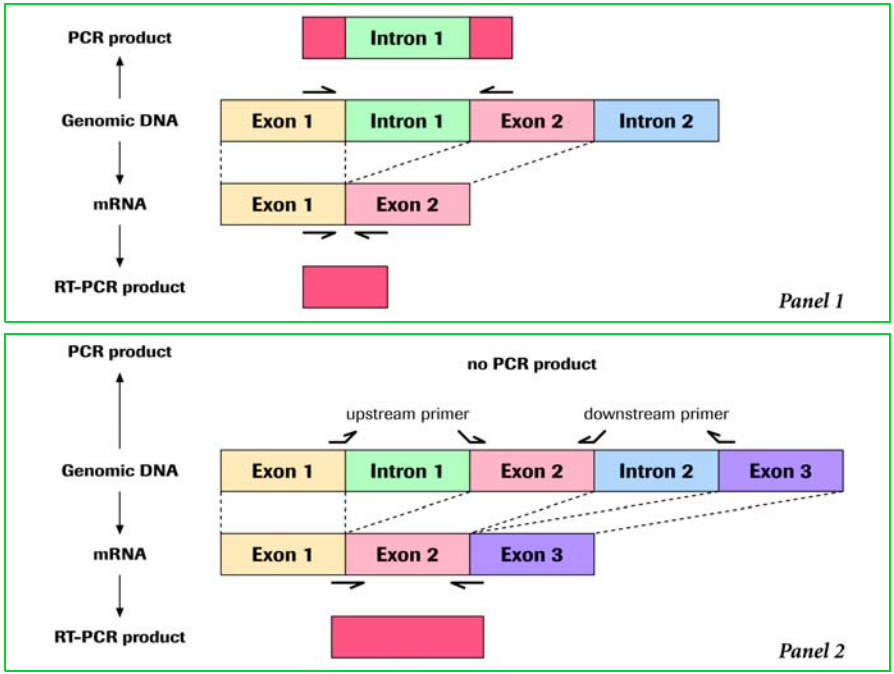
2.5. *RT-PCR*

Reaction Components: Design of reverse transcription primers

RT-PCR amplification of a particular mRNA sequence requires two PCR primers that are specific for that mRNA sequence. The primer design should also allow differentiation between the amplified product of cDNA and an amplified product derived from contaminating genomic DNA. There are two different strategies to design the required primers[a]:

Strategy 1	➢ See panel 1 on next page: ● Design primers that anneal to sequences in exons on both sides of an intron. With these primers, any product amplified from genomic DNA will be much larger than a product amplified from intronless mRNA.
Strategy 2	➢ See panel 2 on next page: ● Design primers that span exon/exon boundaries on the mRNA. Such primers should not amplify genomic DNA.

[a] Please also refer to reference 4.


PCR product
Intron 1
Genomic DNA
Exon 1
Intron 1
Exon 2
Intron 2
mRNA
Exon 1
Exon 2
RT-PCR product
Panel 1
PCR product
no PCR product
upstream primer
downstream primer
Genomic DNA
Exon 1
Intron 1
Exon 2
Intron 2
Exon 3
mRNA
Exon 1
Exon 2
Exon 3
RT-PCR product
Panel 2

Purity of template

Start with the purest RNA template possible

- If your target is mRNA, start with purified mRNA as template, rather than total RNA. (The proportion of mRNA in a total RNA preparation is quite low.) This will greatly increase the likelihood of successfully amplifying rare mRNAs.
- Use a template preparation product that is specifically designed for RNA purification. For details on RNA purification products available from Roche Applied Sciences, see page 65.
- If your target is mRNA, check the integrity of the mRNA by gel electrophoresis. The mRNA should appear as a smear between 500 bp and 8 kb. Most of the mRNA should be between 1.5 kb and 2 kb.
- If your target is eukaryotic total RNA, check the integrity of the total RNA by gel electrophoresis. The total RNA is dominated by ribosomal RNA, so the 18S and 28S rRNA should be appear as clear bands.

Quality of template	**Eliminate RT inhibitors from the template preparation.**
	➢ Before using it as a template, precipitate the RNA using ethanol, then wash it once with 70% ethanol. Be sure to remove all traces of ethanol before using the RNA in the RT reaction. ➢ Use a product specifically designed for RNA purification to prepare the template. Such products can help eliminate potential inhibitors during purification. For details on RNA purification products available from Roche Applied Sciences, see page 65.
Intactness of template	**Take rigorous and repeated precautions to prevent RNase contamination**
during cell lysis	➢ Use isolation methods that simultaneously disrupt cells and inactivate RNases (by adding SDS or guanidine).
during the purification procedure	➢ Even if you don't think RNases could be present at a particular step, act as though they are present. ➢ Include RNase inhibitors (*e.g.*, Protector RNase Inhibitor*) in non-denaturing purification steps. ➢ Use only sterile, RNase- and DNase-free plastic disposables (pipette tips, etc.).

	➢ Sterilize all glassware before use. (RNases can be present on non-sterile glassware.). ➢ Always wear gloves throughout the isolation process. (Skin is a rich source of RNases.). ➢ If necessary, analyze the product of each step in the isolation process by gel electrophoresis to ensure that the RNA is still RNase-free.
after purification	➢ Short-term storage: Store the purified RNA template at +2 to +8°C until use. ➢ Long-term storage: Store the purified RNA template at -70°C. ➢ If possible, store cDNA, it is more stable than RNA.

* Product available from Roche Applied Science: Protector RNase Inhibitor (Cat. No. 03 335 399 001→ 2000 U; Cat. No. 03 335 402 001→ 10000 U)

Notes

2.5. *RT-PCR*

Reaction Components: Choice of enzymes

Temperature optima	➢ Higher incubation temperatures help eliminate problems of template secondary structures. ➢ High temperature improves the specificity of reverse transcription by decreasing false priming. ➢ Thermoactive reverse trancriptases that can be incubated at higher temperatures (50 – 70°C) are more likely to produce accurate copies of mRNA, especially if the template has a high GC content. ➢ Transcribe GC-rich templates with high secondary structures at 55°C - 65°C with the thermostable Transcriptor Reverse Transcriptase* or Transcriptor First Strand cDNA Synthesis Kit*
Divalent ion requirement	➢ Most reverse transcriptases require divalent ions for activity. ➢ Enzymes that use Mg^{2+} are likely to produce more accurate cDNA copies than those that use Mn^{2+} since Mn^{2+} adversely affects the fidelity of the DNA synthesis.
Sensitivity	➢ Reverse transcriptases have different ability to copy smaller amounts of template (see product characteristics table in this chapter, page 78).

* Product available from Roche Applied Science: Transcriptor Reverse Transcriptase (Cat. No: 03 531 317 001→ 250 U, 03 531 295 001→ 500 U, 03 531 287 001→ 2,000 U); Transcriptor First Strand cDNA Synthesis Kit (Cat. No: 04 379 012 001→ 1 kit (50 rxns, including 10 control rxns); 04 896 866 001→ 1 kit (100 rxns); 04 897 030 001→ 1 kit (200 rxns)).

Specificity	➢ Reverse transcriptases differ in their ability to transcribe RNA secondary structures accurately (see product characteristics table in this chapter, page 78).
Reverse Transcriptase – DNA polymerase combinations	➢ Using different reverse transcriptase – DNA polymerase combinations will allow to optimally amplify certain target length.

2.5. *RT-PCR*

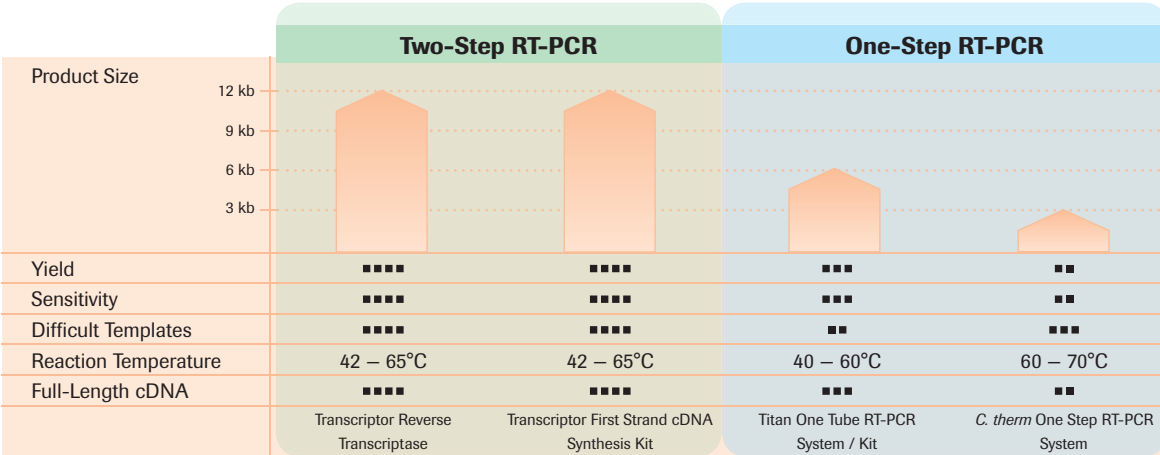
Procedures

Two Step procedure	One tube One Step procedure
➢ **Two tube, two step procedure:** ● In the first tube, the first strand cDNA synthesis is performed under optimal conditions. ● An aliquot of this RT reaction is then transferred to another tube containing all reagents for the subsequent PCR. ➢ **One tube, two step procedure:** ● In the first step, the reverse transcriptase produces first-strand cDNA synthesis in the presence of Mg^{2+} ions, high concentrations of dNTP's and primers. ● Following this reaction, PCR buffer (without Mg^{2+} ions), a thermostable DNA Polymerase and specific primers are added to the tube and the PCR is performed.	➢ Both cDNA synthesis and PCR amplification are performed with the same buffer and site-specific primers, eliminating the need to open the reaction tube between the RT and PCR steps

Advantages of Two Step procedure	Advantages of One tube One Step procedure
➢ **Optimal reaction conditions:** ● The two step format allows both reverse transcription and PCR to be performed under optimal conditions ensuring an efficient and accurate amplification. ➢ **Flexibility:** ● Two step procedures allow the product of a single cDNA synthesis reaction to be used in several PCR reactions for analysis of multiple transcripts. ● Allows choice of primers (oligo (dT), anchored oligo (dT), random hexamer or sequence-specific primers). ● Allows a wider choice of RT and PCR enzymes. ➢ **Amplifies long sequences:** ● With the right combination of reverse transcriptases and thermostable DNA polymerases, two step RT-PCR can amplify RNA sequences up to 14 kb long.	➢ **Minimal time required:** ● The one step reaction has fewer pipetting steps than the two step reaction, thereby significantly reducing the time needed for the experiment and eliminating pipetting errors. ➢ **Reduced risks for contamination:** ● The entire one step reaction takes place in a single tube with no transfers required and no need to open the reaction tube. (steps where contamination can occur). ➢ **Improved sensitivity and specificity:** Two characteristics of the one step reaction provide increased yield and efficiency: ● the cDNA reaction is performed at a high temperature thereby eliminating problems with secondary RNA structures. ● the entire cDNA sample is used as template for the PCR. Use of sequence-specific primers enhance specificity and sensitivity.

2.5. *RT-PCR*

Reaction Components: Choice of enzyme for One-Step and Two-Step PCR



Reaction Components: Choice of enzyme for Two-Step RT-PCR.

	Transcriptor Reverse Transcriptase
Enzyme component	A new recombinant Reverse Transcriptase
Product size	Up to 14 kb
Priming	Oligo d(T), Specific, random hexamers
Reaction temperature	42°C - 65°C
RNase H activity	Yes
Sensitivity	++++
Ability to transcribe difficult templates	++++
Full length cDNA	++++
Incorporation of modified nucleotides	Yes
Product configuration	➢ Enzyme ➢ Reaction buffer
Intended use	➢ Robust reverse transcription of all kinds of templates ➢ Specially usefull for difficult templates ➢ Generation of full length cDNA´s

Reaction Components: Choice of enzyme for One-Step RT-PCR.

	Tth DNA Polymerase	**C. Therm One Step RT-PCR System**	**Titan One Step RT-PCR System**
Enzyme component	Thermostable DNA polymerase with intrinsic reverse transcripase activity	Blend of C. therm and Taq DNA Polymerase	Blend of AMV Reverse Transcriptase, Taq DNA Polymerase and a Proofreading Polymerase
Product size	Up to 1 kb	Up to 3 kb	Up to 6 kb
Priming	Specific	Specific	Specific
Reaction temperature	55°C - 70°C	60°C - 70°C	45°C - 60°C
RNase H activity	No	No	Yes
Sensitivity	+	++	+++
Ability to transcribe difficult templates	+	+++	+
Full length cDNA	–	+	++
Incorporation of modified nucleotides	Yes	Yes	Yes
Carry-over prevention	+	++	–

	Tth DNA Polymerase	C. Therm One Step RT-PCR System	Titan One Step RT-PCR System
Product configuration	➢ Enzyme ➢ PCR reaction buffer ➢ RT-PCR reaction buffer ➢ $Mn(OAc)_2$ solution	➢ Enzyme Blend ➢ Reaction Buffer ➢ DMSO solution ➢ DTT solution	➢ Enzyme Blend ➢ Reaction Buffer ➢ $MgCl_2$ solution ➢ PCR grade Water ➢ DTT solution
Intended use	➢ One-Step RT-PCR of short normal templates	➢ One-Step RT-PCR of difficult (*e.g.* GC-rich) templates	➢ One-Step RT-PCR for fragments up to 6 kb, with high sensivity ➢ Synthesis of full length cDNA´s

- Benefit from the availability of a broad range of reverse transcriptases, to find the best possible product for your application.

- Get the most out of your precious RNA samples and your assays, by using well characterized reverse transcriptases that are function tested over a broad range of different applications.

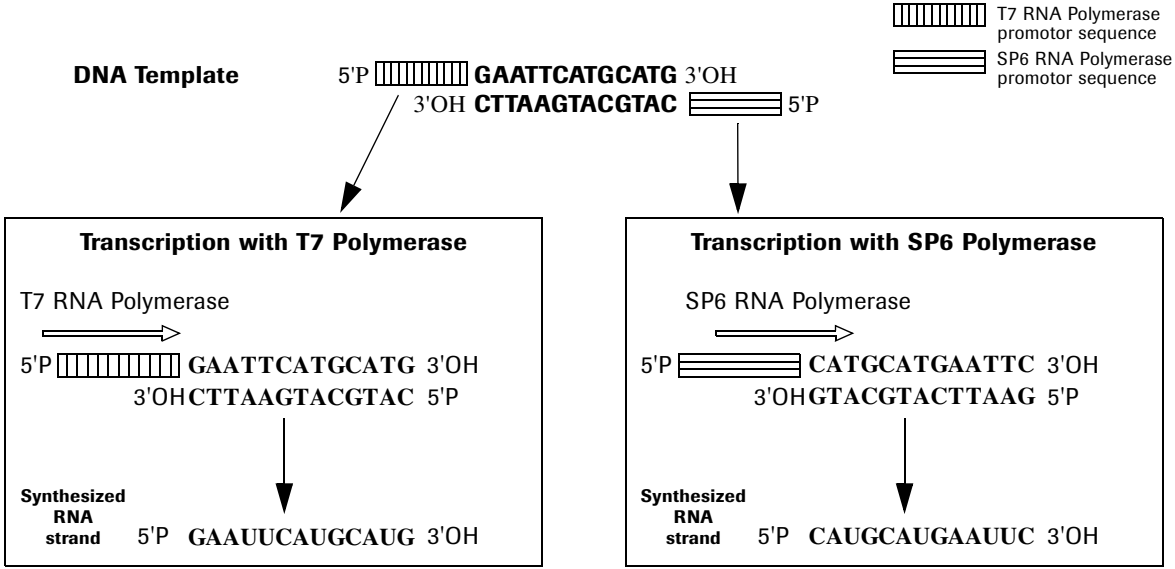
2.6. *Labeling of RNA*

Overview of different techniques

Application	Labeling Method	Relative sensitivity
Northern Blotting	➢ Labeling RNA by *in vitro* Transcription	+++
Southern Blotting	➢ 5'End labeling*	++
	➢ 3'End labeling*	++
Dot/Slot Blotting	➢ Labeling RNA by *in vitro* Transcription	+++
	➢ 5'End labeling*	++
	➢ 3'End labeling *	++
In Situ Hybridisation	➢ Labeling RNA by *in vitro* Transcription	+++
	➢ 5'End labeling*	++
	➢ 3'End labeling*	++

* Note: Please refer references 8, 9, 11 for an overview and a selection help for DIG labeling of nucleic acids.

Principle of In Vitro Transcription with DNA dependent RNA Polymerases



Please refer to pages 88–91 for further information on the conversion of nucleic acid to proteins.

2.6. *Labeling of RNA*

SP6, T7 and T3 Polymerases

Properties	➢ DNA dependent RNA Polymerases, specific for their corresponding promotor.
10 x Transcription buffer	➢ 400 mM Tris (pH 8.0), 60 mM $MgCl_2$, 100 mM DTT, 20 mM spermidine ➢ Buffer without nucleotides is stable at –15 to –25°C, store in aliquots.
Application and typical results	➢ Generate homogeneously labeled single stranded RNA molecules as probes for hybridisation experiments. ➢ Radioactive nucleotides (^{32}P, ^{35}S) and non-radioactive nucleotides (biotin, digoxigenin, fluorochromes) can be incorporated.

➢ Standard Assay:

Components	Radioactive	Non-radioactive*	Cold (without label)
Template DNA	0.5 µg	1 µg	1 µg
Nucleotides, final concentration	ATP, GTP, UTP each 0.5 mM final	ATP, GTP, CTP, each 1 mM final UTP 0.65 mM final	ATP, GTP, CTP, UTP each 1 mM final
Labeled nucleotide, final concentration	[α^{32}P] CTP (400 Ci/mmol), 50 µCi (1.85 MBq/mmol)	DIG, Biotin or fluoro-chrome UTP, 0.35 mM final	
10 x transcription buffer	2 µl	2 µl	2 µl
RNA Polymerase	20 units	40 units	40 units
RNase Inhibitor	20 units	20 units	20 units
H_2O	Add H_2O to 20 µl	Add H_2O to 20 µl	Add H_2O to 20 µl
Incubation	20 min/37°C	2 hours/37°C	2 hours/37°C

➢ This standard assay incorporates > 50% of the input radioactivity

Inactivation of enzyme

➢ Add 2 µl 0.2 M EDTA and/or heat to 65°C for 10 min.

* For a complete overview, please refer to references 8 and 9.

2.7. *References*

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Working with Proteins

Chapter 3

3

3.1. *Precautions for handling Proteins*

Avoiding degradation of proteins	➢ For successful isolation or purification of proteins from biological extracts, include protease inhibitors throughout the procedure. ➢ Proteases are ubiquitous and difficult to separate from proteins. ➢ As a general rule: assume that proteases are present, inhibit early and inhibit often (for more detailed information, please refer to page 83 and/or reference 1).
Avoiding dephosphorylation of proteins	➢ The regulation of many biological processes and pathways is accomplished by the formation and cleavage of phosphate esters. ➢ The phosphorylation of proteins is carefully balanced by an interplay of protein kinases and phosphatases. ➢ Phosphatases are ubiquitous. ➢ A preservation of the phosphorylation pattern is important to get an accurate view about the general or specific phosphorylation status (for more detailed information, please refer to page 84).
Storage of proteins	➢ Aliquot protein solutions and store at different temperatures and in different buffer systems. Test activity of the protein to find out the most optimal storage condition. ➢ Refrigeration – with the appropriate stabilization reagent – is often sufficient to maintain the biological activity of proteins.

- For protein solutions in glycerol (50%, v/v) or suspensions in ammonium sulfate (3.2 M), freezing should be avoided, because of the cold denaturation effect.
- Ice crystals can cause physical shearing and denaturation of proteins.
- Avoid repeated freezing and thawing of enzyme solutions. If the enzyme is to be stored frozen, store it in small portions.
- Shock freezing of proteins in liquid nitrogen and long term storage can be performed after addition of 10 – 20% glycerol.

Manipulation of proteins

- Always keep the protein sample on ice during experiments.
- Use a fresh pipette tip for each sample that has to be removed from the parent vial.
- Never return unused material to the parent vial.
- Always wear gloves to prevent contamination with other proteins and proteases.
- Avoid vigorous vortexing and pipetting of protein solutions to minimize denaturation.
- Avoid dust forming during the experiment – dust contains approximately 60% ceratines (approx. 60 kD).
- Always work with small quantities in small volumes to avoid contamination and work as quickly as possible.

3.1. *Precautions for handling Proteins*

Inhibition of protease activity

Proteases are ubiquitous in all living cells. At the time of cell lysis, proteases are released and can quickly degrade any proteins in the extract. During isolation and purification, proteolytic damage can affect functionality and reduce yields of protein. To get a complete protection against a multitude of proteases different protease inhibitors are needed.

- c●mplete Protease Inhibitor Cocktail Tablets* eliminate the time-consuming search for the right protease inhibitors.
- c●mplete inhibits proteolytic activity in extracts from almost any tissue or cell type, including animals, plants, yeast, bacteria, and fungi (for examples, see www.roche-applied-science.com/proteaseinhibitor).
- The non-toxic c●mplete tablets inhibit a multitude of protease classes, including serine proteases, cysteine proteases, and metalloproteases.
- c●mplete as EDTA-free* version maintain the stability of metal-dependent proteins and effectiveness of purification techniques (*i.e.*, IMAC [immobilized metal affinity chromatography] for isolation of Poly-His-tagged proteins)

* Product available from Roche Applied Science, c●mplete Cat. No. 04 693 116 001, Pack size 20 tablets (each for 50 ml); c●mplete, EDTA-free Cat. No. 04 693 132 001, Pack size 20 tablets (each for 50 ml).

For other pack sizes and more information visit: **www.roche-applied-science.com/proteaseinhibitor**

Notes

3.1. *Precautions for handling Proteins*

Inhibition of phosphatase activity

The phosphorylation states can be the difference between an active and an inactive protein. To avoid that this states change during protein purification or analysis individual phosphatase inhibitors or a cocktail against a broad range of phophatases can be used.

- PhosST●P Phosphatase Inhibitor Cocktail Tablets* eliminate the time-consuming search for the right phosphatase inhibitors.
- PhosST●P protects phosphorylated protein(s) against dephosphorylation. It shows an effective inhibition of a broad spectrum of phosphatases such as acid and alkaline phosphatases, serine/threonine phosphatase classes (*e.g.*, PP1, PP2A and PP2B), tyrosine phosphatase (PTP), and dual-specific phosphatases.
- The tablets inhibit phosphatases in a variety of sample materials including mammalian, insect, or plant cells. PhosST●P is also well-suited for buffers containing formalin for the formalin-fixation of paraffin-embedded (FFPE) tissue sections.
- The non-toxic PhosST●P tablets are easy-to-use and can be combined with c●mplete Protease Inhibitor Cocktail Tablets to simultaneously protect proteins against dephosphorylation and proteolytic degradation.

* Product available from Roche Applied Science, Cat. No. 04906845001 Pack size 10 tablets (each for 10 ml). Cat. No. 04906837001 Pack size 20 tablets (each for 10 ml)

For more information visit: **www.roche-applied-science.com/phoshataseinhibitor**

PHOSSTOP is a trademark of Roche.

3.1. *Precautions for handling Proteins*

Inhibition of protease activity (see reference 1)

General Inhibitors for Classes of Proteases:

Serine proteases[a]	Cysteine proteases[b]	Metalloproteases[c]	Aspartic proteases[d]
PMSF		EDTA	Pepstatin
Pefabloc SC	E-64	Phosphoramidon	
Pefabloc SC PLUS		Bestatin (aminopeptidases	
Aprotinin			
Leupeptin+			
α_2-Macroglobulin			
cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets*			
cOmplete, Protease Inhibitor Cocktail Tablets*			

High-efficiency protease Inhibition

a Contain serine and histidine in the active center

b Contain cysteine (thiol, SH-) in the active center

c Contain metal ions (*e.g.*, Zn^{2+}, Ca^{2+}, Mn^{2+}) in the active center

d Contain aspartic (acidic) group in the active center

\+ Inhibits serine and cysteine proteases with trypsin-like specificity.

* When extractions or single-step isolations are necessary in the acidic pH range, include Pepstatin along with cOmplete Tablets to ensure aspartic (acid) protease inhibition.

Protease-Specific Inhibitors:

Product	for the Inhibition of:
Antipain dihydrochloride	Papain, Trypsin (Plasmin)
Calpain Inhibitor I	Calpain I > Calpain II
Calpain Inhibitor II	Calpain II > Calpain I
Chymostatin	Chymotrypsin
TLCK	Trypsin, other serine and cysteine proteases (*e.g.*, Bromelain, Ficin, Papain)
Trypsin-Inhibitor (chicken egg white, soybean)	Trypsin

3.1. *Precautions for handling Proteins*

Stabilization

General

- Low molecular weight substances such as glycerol or sucrose can help stabilizing proteins.
- However, care has to be taken when choosing these reagents since they can alter the activity of the protein of interest: e. g. sucrose and Poly Ethylene Glycol (PEG) are good stabilizers for invertase, but have denaturing effects on lysozyme.

Addition of salts

- Certain salts (anions and cations) can significantly stabilize proteins in solution.
- Effectiveness of the stabilization effect of ions (according to Hofmeister, see reference 12):

$$(CH_3)_4N^+ > NH_4^+ > K^+ > Na^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$
$$SO_4^{2-} > Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$$

- Note that the widely used stabilization reagent ammonium sulfate contains two of the most effective stabilizing ions: NH_4^+ and SO_4^{2-}.

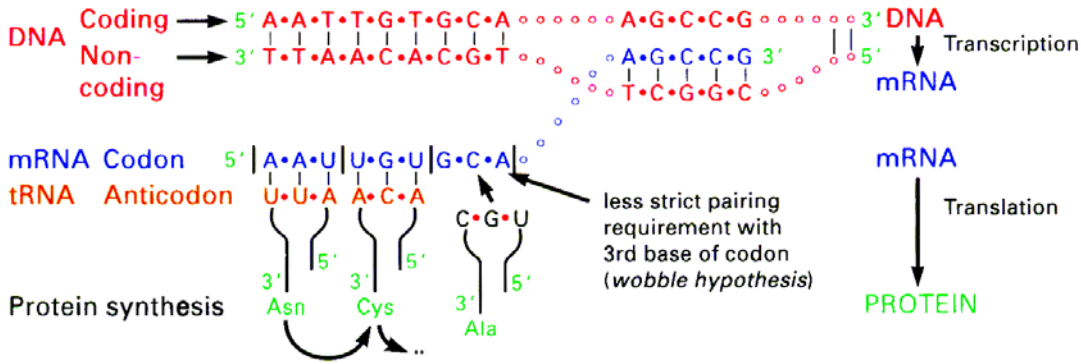
Addition of proteins	➢ Highly diluted protein solutions are generally unstable. ➢ If rapid concentration is not possible, other proteins (*e.g.*, BSA, up to ~1%) may be added to stabilize the sample.
Addition of osmolytes	➢ Poly-alcohols, Mono- and Polysaccharides, neutral polymers and amino-acids are not strongly charged and may not affect enzyme activity. ➢ Typical concentrations for poly-alcohols and sugars are 10 – 40% (w/v). ➢ Use non-reducing sugars or corresponding sugar alcohols (*e.g.*, glycerol, xylitol) to avoid inactivation of proteins by reaction between amino groups and reducing sugars. ➢ Polymers (*e.g.*, **P**oly **E**thylene **G**lycol, PEG) in a concentration range of 1 – 15% increase the viscosity of single phase solvents and help prevent aggregation. ➢ Amino acids without charge (glycine and alanine) can act as stabilizers in a concentration range of 20 to 500 mM. Related compounds such as γ-amino butyric acid (GABA) and trimethylamine N-oxide (TMAO) can also be used in a range of 20 to 500 mM.

Addition of substrates and specific ligands	➢ Addition of specific substrates, cofactors or competitive inhibitors to purified proteins often results in good stabilizing effects. The protein adopts a more tightly folded conformation thereby reducing the tendency to unfold and rendering it less susceptible to proteolytic degradation. ➢ Note that dialysis may be necessary to avoid carryover effects of the substrate or inhibitor when the protein is removed from storage for use in particular situations where maximal activity is desired.
Addition of reducing agents	➢ Metal ions activate molecular oxygen and – as a result – the oxidation of thiol groups of cysteine residues. ➢ Complexation of these metal ions with *e.g.*, EDTA leads to stabilization of proteins. ➢ Destructive oxidative reactions of thiol groups of cysteine can also be prevented by adding reducing agents such as β-mercaptoethanol or dithiotreitol.

- β-Mercaptoethanol (β-ME):
  - Add β-ME to a final concentration of 5–20 mM and keep – if possible – solution under anaerobic conditions (*e.g.*, via an inert gas). β-ME can form disulfide bridges with thiol groups of proteins leading to aggregation and/or inactivation. At pH 6.5 and 20°C, the half-life of β-ME is more than 100 hours. At pH 8.5 and 20°C, the half-life decreases to 4 hours.
- Dithiotreitol (DTT):
  - DTT is effective at lower concentrations ranging from 0.5–1 mM. The concentration should not exceed 1 mM, because it can act as denaturant at higher concentrations and is not soluble in high salt. Oxidation of DTT forms internal disulfide bridges resulting in deactivation, but does not interfere with protein molecules. At pH 6.5 and 20°C, the half-life of DTT is 40 hours. At pH 8.5 and 20°C, the half-life decreases to 1.4 hours.

3.2. *Conversion from Nucleic Acids to Proteins*

Information transfer (see reference 2)

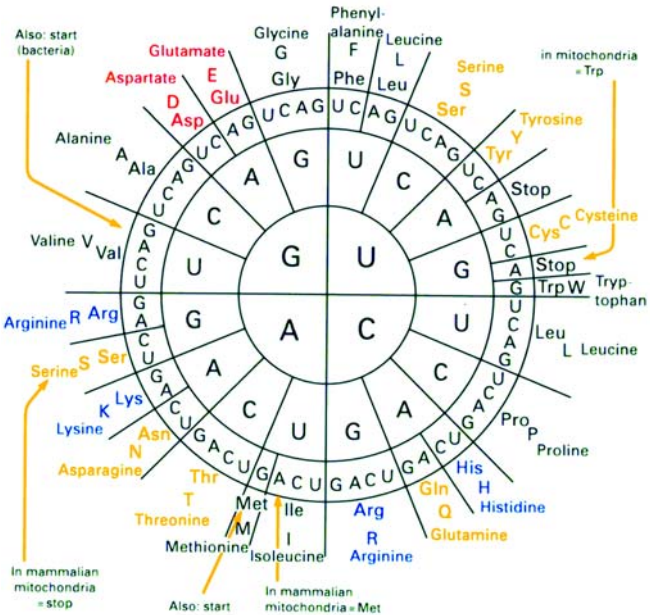


Since there are $4^3 = 64$ possible nucleotide triplets, but only 20 amino acids plus some stop codons, most amino acids are encoded by several nucleotide triplets (synonyms, at maximum 6 in the cases of Arg, Leu and Ser). Most variants occur in the 3rd position of the code ("Wobble hypothesis"). The code is therefore called degenerate. The relationship between triplets and encoded amino acids is shown in the figure on the next page.

Notes

3.2. *Conversion from Nucleic Acids to Proteins*

The Genetic Code (see reference 2)



The triplet sequence is read from the center outwards. The mRNA nucleotide terminology is shown.
For DNA nucleotide sequences, replace U with T. Basic amino acids are shown in blue, acidic amino acids in red, neutral amino acids in black and amino acids with uncharged, polar residues in orange.

Although the code has been assumed to be universal among living species, several exceptions in mitochondria have been found. Furthermore, in mycoplasma, UGA codes for Trp, while in some ciliated protozoa, the normal "stop" codons UAG and UAA specify Gln and UGA specifies Cys instead.

Codon differences in Mitochondria:

	UGA	AUA	CU(A,C,G,U)	AG(A,G)	CGG
General Code	Stop	Ile	Leu	Arg	Arg
Mito-chondria					
Mammals	Trp	Met/ start		Stop	
Drosophila	Trp	Met/ start		Ser (AGA only)	
Protozoa	Trp				
Higher Plants					Trp
S. cerevisiae	Trp	Met/ start	Thr		

Codons with special functions:

	Start Codons	Stop Codons
General code	AUG (codes also for Met)	UAG, UAA, UGA (amber, ochre, opal)
Eukarya	AUG (codes also for Met), CUG (rare), ACG (rare), GUG (rare)	UAG, UAA, UGA
Bacteria	AUG, GUG, UUG (rare)	UAG, UAA, UGA
Mito-chondria	AUA (codes also for Met), AUG	AGA, AGG (mammals)

3.2. *Conversion from Nucleic Acids to Proteins*

Characteristics of Amino Acids

Amino acid	Symbol	MW (in Da)	Side group	Genetic code					
Alanine	A – Ala	89	$-CH_3$	GCU	GCC	GCA	GCG		
Arginine	R – Arg	174	$-(CH_2)_3-NH-CNH-NH_2$	CGU	CGC	CGA	CGG	AGA	AGG
Asparagine	N – Asn	132	$-CH_2-CONH_2$	AAU	AAC				
Aspartic acid	D – Asp	133	$-CH_2-COOH$	GAU	GAC				
Cysteine	C – Cys	121	$-CH_2-SH$	UGU	UGC				
Glutamine	Q – Gln	146	$-CH_2-CH_2-CONH_2$	CAA	CAG				
Glutamic acid	E – Glu	147	$-CH_2-CH_2-COOH$	GAA	GAG				
Glycine	G – Gly	75	-H	GGU	GGC	GGA	GGG		
Histidine	H – His	155	$-C_3N_2H_3$	CAU	CAC				
Isoleucine	I – Ile	131	$-CH(CH_3)-CH_2-CH_3$	AUU	AUC	AUA			
Leucine	L – Leu	131	$-CH_2-CH(CH_3)_2$	CUU	CUC	CUA	CUG	UUA	UUG
Lysine	K – Lys	146	$-(CH_2)_4-NH_2$	AAA	AAG				
Methionine	M – Met	149	$-CH_2-CH_2-S-CH_3$	AUG					
Phenylalanine	F – Phe	165	$-CH_2-C_6H_5$	UUU	UUC				
Proline	P – Pro	115	$-C_3H_6$	CCU	CCC	CCA	CCG		
Serine	S – Ser	105	$-CH_2-OH$	UCU	UCC	UCA	UCG	AGU	AGC
Threonine	T – Thr	119	$-CH(CH_3)-OH$	ACU	ACC	ACA	ACG		
Tryptophan	W – Trp	204	$-C_8NH_6$	UGG					
Tyrosine	Y – Tyr	181	$-CH_2-C_6H_4-OH$	UAU	UAC				
Valine	V – Val	117	$-CH-(CH_3)_2$	GUU	GUC	GUA	GUG		

Basic amino acids are shown in blue, acidic amino acids in red, neutral amino acids in black and amino acids with uncharged, polar residues in yellow. Average molecular weight of amino acid: 110 Daltons.

Notes

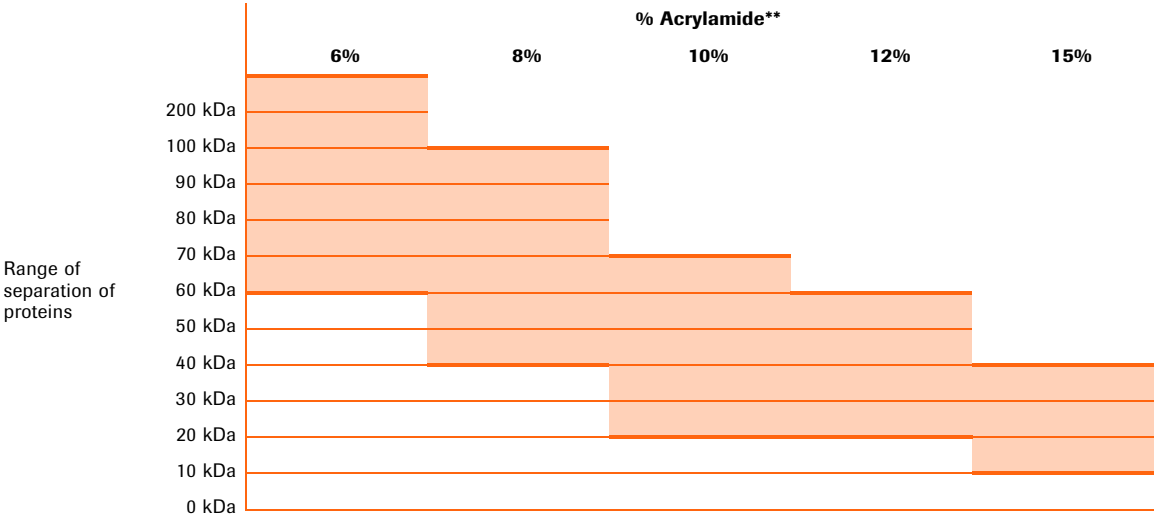
Rarest codons (from *E.coli*) preferred by selected organisms:

Codon frequencies are expressed as codons used per 1000 codons encountered.
A complete compilation of codon usage of the sequences placed in the gene bank database can be found at **http://www.kazusa.or.jp/codon/**
Codon frequencies of more than 15 codons/1000 codons are shown in bold to help identify a codon bias that may cause problems for high level expression in *E. coli*. The arginine codons AGG and AGA are recognized by the same tRNA (product of argU gene) and should therefore be combined. However, regardless of the origin of the coding region of interest, each gene should be addressed individually.

	AGG arginine	AGA arginine	CGA arginine	CUA leucine	AUA isoleucine	CCC proline
Escherichia coli	1.4	2.1	3.1	3.2	4.1	4.3
Homo sapiens	11.0	11.3	6.1	6.5	6.9	**20.3**
Drosophila melanogaster	4.7	5.7	7.6	7.2	8.3	**18.6**
Caenorhabditis elegans	3.8	**15.6**	11.5	7.9	9.8	4.3
Saccharomyces cerevisiae	**21.3**	9.3	3.0	13.4	**17.8**	6.8
Plasmodium falciparium	**26.6**	**20.2**	0.5	**15.2**	**33.2**	8.5
Clostridium pasteurianum	2.4	**32.8**	0.8	6.0	**52.5**	1.0
Thermus aquaticus	13.7	1.4	1.4	3.2	2.0	**43.0**
Arabidopsis thaliana	10.9	**18.4**	6.0	9.8	12.6	5.2

3.3. *Analyzing Proteins*

*Separation ranges of proteins in denaturing SDS-PAGE**



* Glycin buffer
** Molar ratio of acrylamide:bisacrylamide is 29:1.

Please refer to chapter 5 “Preparing Buffers and Media” for recipes to prepare denaturing SDS PAGE gels.

Sizes of commonly used markers in denaturing SDS-PAGE

Marker	Molecular Weight (in kDa)
Myosin heavy chain (rabbit muscle)	205
β-Galactosidase (E.coli)	116
Phosphorylase b (rabbit muscle)	97.4
Fructose 6 phosphate kinase (rabbit muscle)	85.2
Bovine Serum Albumin	66.2
Glutamate dehydrogenase (bovine liver)	55.6
Aldolase (rabbit muscle)	39.2
Triose phosphate isomerase (rabbit muscle)	26.6
Trypsin inhibitor (hen egg white)	28.0
Trypsin inhibitor (soybean)	20.1
Lysozyme (hen egg white)	14.3
Cytochrome c (horse heart)	12.5
Aprotinin	6.5
Insulin chain B	3.4

3.3. *Analyzing Proteins*

Staining of proteins on denaturing SDS-PAGE gels

Method	Detection limit
Coomassie Brilliant Blue R 250 Staining – Standard Method	300 – 1,000 ng per band
Coomassie Brilliant Blue R 250 Staining – Maximal Sensitivity	50 – 100 ng per band
Silver Staining – Neutral Silver Staining	1 – 10 ng per band
Silver Staining – Ammonium Silver Staining	1 – 10 ng per band
Copper Staining	10 – 100 ng per band for 0.5 mm gels 1,000 ng per band for 1 mm gels
Staining with colloidal gold	3 ng per band
Staining with SYPRO fluorescent dyes	1 – 2 ng per band

Please refer to “Harlow and Lane: Antibodies, a Laboratory Manual, page 649 – 654” for the recipes of these staining techniques.

*Staining and detection of proteins on membranes**

Method	Detection limit	Staining →			Destaining →		Immunodetection	
		NC	Nylon	PVDF	NC	PVDF	NC	PVDF
Amido Black	~ 2,000 ng							
Ponceau S	1,000 – 2,000 ng							
Coomassie Blue R 250	500 – 1,000 ng							
								
	300 – 500 ng							
India Ink	10 – 20 ng							
Colloidal Gold	3 ng							

NC = nitrocellulose
PVDF = polyvinylidene difluoride
* Products available from Roche Applied Science

3.3. *Analyzing Proteins*

Buffer exchange via gel filtration

Matrix		Material	pH stability (in aqueous buffer)	Separation range in kDa (for globular proteins)
Sephadex	G – 25	Dextran	2 – 10	1 – 5
	G – 50			1.5 – 30
	G – 75			3 – 80
Sepharose	6B	Agarose	3 – 13	10 – 4,000
	4B			60 – 20,000
	2B			70 – 40,000
Superdex	30	Agarose/Dextran	3 – 12	0 – 10
	75			3 – 70
	200			10 – 600
Sephacryl	S – 100HR	Dextran/Bisacrylamide	3 – 11	1 – 100
	S – 200HR			5 – 250
	S – 300HR			10 – 1,500
Biogel	P – 2	Polyacrylamide	2 – 10	0.1 – 1.8
	P – 4			0.8 – 4
	P – 10			1.5 – 20
	P – 60			3 – 60
	P – 100			5 – 100

Properties of commonly used detergents: Definitions (see reference 3)

Definitions	➢ The MWCO (**M**olecular **W**eight **C**ut **O**ff) is the minimum molecular weight of a molecule that will be retained by the membrane of the dialysis tubes.
Critical micellar concentration (CMC) (in mM)	➢ The minimum concentration at which detergents begin to form micelles. ➢ In practice, the CMC is the maximum concentration of detergent monomers that can exist in H_2O. ➢ The CMC of a detergent may be changed by pH, temperature and the ionic strength of the solution. ➢ The CMC affects the dialysis of a non-ionic detergent. A detergent with a high CMC (and no ionic charge) is readily removed from detergent – protein complexes by dialysis, whereas a detergent with a low CMC dialyzes away very slowly.
Monomeric molecular weight (MMW) (in Da)	➢ Molecular weight of the detergent in its monomeric form.

***Properties of commonly used detergents: Definitions* (continued)**

Micellar molecular weight (MMr) (in Da)	➢ The average size of 1 micelle of a detergent (AuS). AuS = MMW x aggregation number (= average number of monomers in one micelle). ➢ In general, a detergent with a low CMC will have a high micellar molecular weight while a detergent with a high CMC will have a low micellar molecular weight.

Characteristics of detergents

Detergent	CMC	MMW	MMr	Working concentration	Ease of removal	Application
Anionic						
SDS (Sodium dodecylsulfate)	8.3	288.4	18,000	> 10 mg per mg protein		Good denaturing agent for proteins. Ideal for PAGE
DOC (Deoxycholic acid)	1 – 4	416.6	4,200	0.1 – 10 mg membrane lipid		Solubilization of membrane proteins
Zwitterionic						
CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)	4	614.9	6,150	6.5 – 13 mM		Solubilization of membrane proteins.
Nonionic						
Nonidet P-40 [Ethylphenolpoly (ethyleneglycolether)$_n$]	0.25	606.6 (n = 11)	90,000	1–10 mM		Protein solubilization
n-Octylglucoside	14.5	292.4		46 mM		Mild, non-denaturing detergent for the solubilization and reconstitution of membrane proteins. Easily removed via dialysis.
Sucrose monolaurate	0.2	524.6		0.2% – 5% (w/v)		Gentle solubilization and stabilization of membrane proteins
Triton X-100 [Octylphenolpoly (ethyleneglycolether)$_n$]	0.2	647 (n = 10)	90,000	13.5		Solubilization of proteins and PAGE
Tween 20 [Poly(oxyethylene)$_n$ sorbitan-monolaurate]	0.06	1228 (n = 20)		> 10 mg/mg membrane lipid		Used for ELISA and Immunoblots

3.3. *Analyzing Proteins*

Removal of detergents

Removal of detergents from protein solutions depends on three different variables:

- the physical properties of the detergent
- the characteristics (*e.g.*, hydrophobic/hydrophilic) of the protein
- the components of the buffer system

Ionic detergents	➢ Add urea to 8 M, then bind detergent to an ion exchange column. The protein will flow through in 8 M urea, then dialyze to remove urea. ➢ Gel filtration via a G-25 column. For some proteins, the column should be equilibrated in another detergent below its CMC. ➢ For ionic detergents with a relatively low micellar size and low CMC: dilute the sample as much as possible and dialyze. Add a mixed bed resin to the dialysate to increase the exchange rate.
Non-ionic detergents	➢ Gel filtration via a G-200 column. ➢ Dilute the sample and dialyze against deoxycholate, then remove deoxycholate by dialysis. ➢ Velocity sedimentation into sucrose without detergent. ➢ Bind protein to an affinity matrix or ion – exchange column, wash to remove detergent and elute protein. For some proteins, the column should be equilibrated in another detergent below its CMC.

Ammonium sulfate precipitation

Amounts of ammonium sulfate (in g/l solution) to change the concentration of a solution from a initial percentage of saturation to a desired target percentage saturation at 0°C:

Initial percentage saturation	Target percentage saturation																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20		27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25			27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30				28	56	86	117	148	181	214	249	285	323	362	402	445	488
35					28	57	87	118	151	184	218	254	291	329	369	410	453
40						29	58	89	120	153	187	222	258	296	335	376	418
45							29	59	90	123	156	190	226	263	302	342	383
50								30	60	92	125	159	194	230	268	308	348
55									30	61	93	127	161	197	235	273	313
60										31	62	95	129	164	201	239	279
65											31	63	97	132	168	205	244
70												32	65	99	134	171	209
75													32	66	101	137	174
80														33	67	103	139
85															34	68	105
90																34	70
95																	35

Add – step by step – small portions of the required amount of ammonium sulfate allowing to dissolve each portion before the next portion is added. This will prevent accumulation of undesirable high local salt concentrations.

Other precipitation techniques

Trichloroacetic acid (> 5 µg/ml)	➢ Add equal volume of 100% trichloroacetic acid (TCA) to the sample and vortex. ➢ Leave for 20 minutes on ice or for 15 minutes at –20°C. ➢ Centrifuge for 5 min at 6,000 – 10,000 g. ➢ Remove the supernatant. ➢ Resuspend the pellet with 0.1 M NaOH or wash the pellet with an ethanol/ether (1/1) solution and resuspend the pellet in a buffer solution.
Aceton precipitation (< 1 µg/ml)	➢ Add 5 volumes of cold (–20°C) aceton to the sample and vortex. ➢ Leave for 30 min at –20°C. ➢ Centrifuge for 5 min at 6,000 – 10,000 g. ➢ Remove the supernatant and let the pellet air dry. ➢ Resuspend the pellet in buffer.
Chloroform-Methanol precipitation (~ 1 µg/ml)	➢ Add 3 volumes of methanol and 1 volume of chloroform to the sample and vortex. ➢ Add 3 volumes H_2O, vortex for ~ 1 minute. ➢ Centrifuge for 5 min at 6,000 – 10,000 g and discard the upper phase. ➢ Add 3 volumes of methanol and vortex. ➢ Centrifuge for 5 min at 6,000 – 10,000 g. ➢ Remove the supernatant and let pellet air dry. ➢ Resuspend the dry pellet in buffer.

Notes

3.4. *Quantification of Proteins*

OD Measurement

Pure protein solutions (Samples with absorbance > 2.0 should be diluted in the appropriate solvent to obtain absorbances < 2.0).	Read the absorbance of the protein solution versus a suitable control (solvent blank) at 280 nm or 205 nm. A rough approximation for proteins is:		
	1 A_{280} Unit of proteins = 1 mg/ml. (for ranges of protein concentrations from 20 to 3000 µg/ml)		
	Typical A_{280} values for 1 mg/ml protein are :	BSA: IgG: IgM:	0.70 1.35 1.20
	Protein concentration (in mg/ml) = $A_{205}/31$ (for ranges of protein concentrations from 1–100 µg/ml)		
Protein solutions contaminated with nucleic acids (nucleic acid content up to 20% w/v or $A_{280}/A_{260} < 0.6$)	Read the absorbance versus a suitable control (*e.g.,* buffer blank) at 280 nm and 260 nm or 280 nm and 205 nm. Calculate the approximate concentration using one of the following equations:		
	Protein concentration (in mg/ml) = $(1.55 \times A_{280}) - (0.76 \times A_{260})$		
	Protein concentration (in mg/ml) = $A205/(27 + A_{280}/A_{205})$		

Molar conversions for Proteins		Protein/DNA conversions (1 kb of DNA encodes 333 amino acids = 3.7×10^4 Da)	
100 pmol Protein	µg Protein	Protein	DNA
10 kDa	1	10 kDa	270 bp
30 kDa	3	30 kDa	810 bp
100 kDa	10	100 KDa	2.7 kbp

Lowry method

- **Reagent A:**
  - Dissolve 10 g Na_2CO_3 in 500 ml H_2O. Dissolve 0.5 g $CuSO_4 - 5H_2O$ and 1 g Na-tartrate in 500 ml of H_2O. Add the Na_2CO_3 solution slowly to the copper/tartrate solution on a magnetic stirrer.
  - This solution can be stored at 4°C for one year.
- **Before usage: Reagent $A_{activated}$**
  - Combine one volume of the reagent A with 2 volumes of 5% SDS and 1 volume of 0.8 M NaOH.
  - This solution is stable at room temperature for 2 weeks.
- **Reagent B:**
  - Combine 1 volume of 2 N Folin-Ciocalteau Phenol reagent with 5 volumes H_2O.
  - This solution is stable for several months in a dark bottle at room temperature.
- Prepare samples of 100, 50, 25 and 12.5 µg/ml BSA in H_2O. Add 1 ml of reagent $A_{activated}$, mix and incubate for 10 min at room temperature.
- Add 0.5 ml of Reagent B, mix immediately and incubate at room temperature for 30 min.
- Read absorbance at 750 nm, prepare a linear standard curve and calculate concentration.

3.4. *Quantification of Proteins*

Assays **(continued)**

Bicinchoninic acid method	➢ Reagent A and reagent B are commercially available. ➢ Mix 1 volume of reagent A to 50 volumes of reagent B. ➢ Prepare samples of 100, 50, 25 and 12.5 µg/100 µl of BSA. ➢ Add 2 ml of the combined reagent to each sample and incubate for 30 min. at 37°C. ➢ Read the samples versus an appropriate blank at 562 nm. ➢ Prepare a linear standard curve and calculate concentration.
Bradford method	➢ Dissolve 100 mg Coomassie brilliant blue G250 in 50 ml 95% ethanol. Add 100 ml concentrated phosphoric acid. Add H_2O to a final volume of 200 ml. The Bradford dye concentrate is stable for 6 months at 4°C. ➢ Prepare samples of 100, 50, 25, and 12.5 µg/100 µl in the same buffer solution as your protein sample. ➢ Dilute the Bradford dye concentrate 5 x with H_2O, filter if precipitation occurs. ➢ Add 5 ml of the diluted dye to each sample. The red dye will turn blue when binding to the protein, allow color to develop for at least 5 min, not longer than 30 min. ➢ Read the absorbance at 595 nm, prepare a linear standard curve and calculate concentration.

Biuret method

- Biuret Reagent:
  - Dissolve 1.5 g $CuSO_4$ – $5H_2O$ and 6 g sodium potassium tartrate in 500 ml of water.
  - Add 300 ml 10% (w/v) NaOH and make up to 1 liter with H_2O.
  - Store in a plastic container in the dark. This solution will keep indefinitely if 1 g of potassium iodide is added to inhibit the reduction of copper.
- Prepare samples with 0.5, 1.0, 1.5, 2, 2.5 and 3 mg of BSA. Add 2.5 ml of Biuret reagent, allow to react for 30 min.
- Measure absorbance at 540 nm against a blank containing 0.5 ml of sample buffer plus 2.5 ml of Biuret reagent.
- Prepare a linear standard curve and calculate concentration.
  Note that this test has a low sensitivity (range 1 – 6 mg protein/ml).

3.4. *Quantification of Proteins*

Concentration limits of interfering reagents for assays

Use this chart to select the appropriate wavelength depending on the contents of your buffer:

Reagents	A_{205}	A_{280}	Reagents	A_{205}	A_{280}
Ammonium sulfate	9% (w/v)	> 50% (w/v)	NaOH	25 mM	> 1 M
Brij 35	1% (v/v)	1% (v/v)	Phosphate buffer	50 mM	100 mM
DTT	0.1 mM	3 mM	SDS	0.1% (w/v)	0.1% (w/v)
EDTA	0.2 mM	30 mM	Sucrose	0.5 M	2 M
Glycerol	5% (v/v)	40% (v/v)	Tris buffer	40 mM	0.5 M
KCl	50 mM	100 mM	Triton X-100	< 0.01% (v/v)	0.02% (v/v)
β-ME	<10 mM	10 mm	TCA	< 1% (w/v)	10% (w/v)
NaCl	0.6 M	> 1 M	Urea	< 0.1 M	> 1 M

DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
β-ME: β-mercaptoethanol
SDS: sodium dodecylsulfate
TCA: trichloroacetic acid

Compatibility of different buffer systems with protein quantification assays

	BCA	Lowry	Bradford		BCA	Lowry	Bradford
ACES		👎		**HEPPSO**		👎	
ADA	👎	👎		**MES**		👎	
BES		👎		**MOPS**		👎	
Bicine	👎	👎		**MOPSO**		👎	
Bis-Tris	👎			**PIPES**		👎	
CAPS		👎		**POPSO**		👎	
CHES		👎		**TAPS**		👎	
DIPSO		👎		**TAPSO**		👎	
Glycine		👎	👎	**TES**		👎	
HEPES		👎		**Tricin**		👎	
HEPPS		👎		**Tris**	👎	👎	

* Please also note the overview regarding pH buffering ranges in section 5.1, Buffer page 148.

Compatibility of different buffer systems with protein quantification assays (continued)

Abbreviations

- ACES (N-(2-Acetamido)-2-aminoethanesulfonic acid)
- ADA (N-(2-Acetamido)(2-iminodiacetic acid)
- BES (N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid)
- Bicine (N,N-bis-(2-hydroxyethyl)-glycine)
- Bis-Tris (bis-(2-hydroxyethyl)-iminotris-(hydroxymethyl)-methane)
- CAPS (3-Cyclohexylamino)-1-propansulfonic acid)
- CHES (2-(N-Cyclo-hexylamine)-ethanesulfonic acid)
- DIPSO (3-N,N-Bis-(2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid)
- HEPES 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid)
- HEPPS (4-(2-hydroxyethyl)-piperazine-1-propanesulfonic acid)
- HEPPSO (4-(2-hydroxyethyl)-piperazine-1(2-hydroxypropanesulfonic acid)
- MES (2- Morpholinoethanesulfonic acid)
- MOPS (3-Morpholinopropanesulfonic acid)
- MOPSO (3-Morpholino-hydroxypropanesulfonic acid)
- PIPES (Piperazine-1, 4-bis-(2-ethanesulfoic acid)
- POPSO (Piperazine-1, 4-bis-(2-hydroxypropanesulfonic acid))
- TAPS (N-[Tris-(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid)
- TAPSO (N-[Tris-(hydroxymethyl)-methyl]-3-amino-2-hydroxypropanesulfonic acid)
- TES (N-[Tris-(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid)
- Tricin (N-[Tris-(hydroxymethyl)-methyl]-glycine)

Notes

3.5. *Purifying Proteins*

Characteristics of different expression systems

Characteristics	*E. coli*	Yeast	Mammalian cells	Insect cells	RTS*
Expression of toxic proteins					
Expression from PCR templates					
Proteolytic cleavage****					
Glycosylation					
Secretion					
Folding					**
Phosphorylation					 ***
Percentage Yield (dry weight)	1 – 5%	1%	< 1%	30%	up to 6 mg/ml
Labeling					

* Rapid Translation System available from Roche Applied Science (please refer to p. 103)
** with chaperones
*** with suitable kinase and substrate
**** inhibited by *e.g.* c●mplete Protease Inhibitor Cocktail Tablets

Sources of antibodies

Source	Type	Quantity of total antibody	Quantity of specific antibody	Contaminating antibodies	Purity of specific antibody	Comments
Serum	Poly-clonal	10 mg/ml	< 1 mg /ml	Other serum antibodies	10% (except for antigen affinity column)	Antigen-affinity purification procedure
Culture supernatant with 10% FCS	Mono-clonal	1 mg/ml	0.01 – 0.05 mg/ml	Background from calf serum	> 95%, no cross reaction high quality	Purification with protein A or G columns*
Culture supernatant with serum-free medium	Mono-clonal	0.05 mg/ml	0.05 mg/ml	None	> 95%, no cross reaction high quality	Purification with protein A or G columns*
Ascites	Mono-clonal	1 – 10 mg/ml	0.9 – 9 mg/ml	Background from mouse antibodies	Max. 90%, cross reactions possible	Antigen affinity purification procedure

* not recommended for IgM.

3.5. *Purifying Proteins*

Expressing Proteins: Cell-free – Rapid Translation System

The Rapid Translation System (RTS) from Roche Applied Science is a scalable *in vitro* protein expression platform that produces large amounts of protein for characterization studies, functional assays, or structural analysis. It is available based on bacterial (*E. coli*) as well as eukaryotic (wheat germ) cell lysates.

RTS enables both transcription and translation utilizing the unique HY (High-Yield) and the patented CECF (continuous-exchange, cell-free) technologies. RTS offers much higher levels of protein expression compared to other in vitro transcription/translation systems, with yields of up to 6 mg/ml of expressed protein.

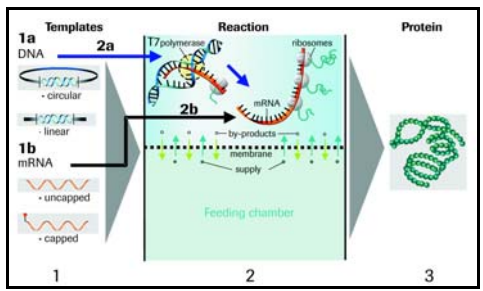


Fig. 1: Schematic illustration of the continuous-exchange cell-free (CECF) reaction principle

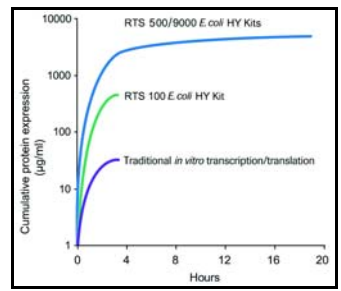


Fig. 2: GFP (Green Fluorescent Protein) expression compared to a conventional *in vitro* expression system

- Transcription and translation take place simultaneously in the reaction compartment.
- Substrates and energy components needed for a sustained reaction are continuously supplied through a semi-permeable membrane.
- Potential inhibitory reaction by-products are diluted via diffusion through the same membrane into the feeding compartment.
- High levels of protein expression by allowing protein synthesis to continue for up to 24 hours.

Conquer complex problems with ease

- Expression of several DNA templates in a single reaction to express proteins in parallel
- Overcome the limitations of cell-based systems by expressing toxic proteins in a cell-free RTS lysate.
- Elimination of laborious up- and downstream steps (*e.g.*, host-cell transformation, culturing, or lysis)
- Expression of proteins from PCR-generated linear templates or suitable expression vectors

Prokaryotic cell-free expression kits	mg/ml	mg/µl reaction	hours
RTS 100 *E. coli* HY Kit	up to 0.4 mg/ml	up to 0.02 mg/50 µl	4
RTS 100 *E. coli* Disulfide Kit	up to 1.5 mg/ml	up to 0.08 mg/50 µl	24
RTS 500 *E. coli* Disulfide Kit	up to 2.5 mg/ml	up to 2.5 mg/1 ml	24
RTS 500 ProteoMaster *E. coli* HY Kit	up to 6 mg/ml	up to 6 mg/1 ml	24
RTS 9000 *E. coli* HY Kit	up to 5 mg/ml	up to 50 mg/10 ml	24

Eukaryotic cell-free expression kits	mg/ml	mg/µl reaction	hours
RTS 100 Wheat Germ CECF Kit	up to 1 mg/ml	up to 0.05 mg/50 µl	24
RTS 500 Wheat Germ CECF Kit	up to 1 mg/ml	up to 1 mg/1 ml	24

The **RTS *E. coli* Disulfide** and **RTS Wheat Germ Kits** allow the expression of soluble disulfide-bonded or eukaryotic proteins with high success rates.

If your protein is eukaryotic and ...	you should use ...	or alternatively use ...
didn't express in *E. coli in vitro*	RTS Wheat Germ	RTS *E. coli*
you want a high initial success rate	RTS Wheat Germ	RTS *E. coli* after sequence optimization with ProteoExpert
didn't express in RTS *E. coli*	RTS Wheat Germ	
isn't soluble when expressed in *E. coli* (RTS or *in vivo*)	RTS Wheat Germ	
needs disulfide bonds	RTS *E. coli* Disulfide	
doesn't depend on correct folding for downstream applications	RTS *E. coli*	
needs to be glycosylated	a cell-based system	

If your protein is prokaryotic and ...	you should use ...	or alternatively use ...
doesn't depend on correct folding for downstream applications	RTS *E. coli*	
you want a high initial success rate	RTS *E. coli* after sequence optimization with ProteoExpert	RTS Wheat Germ
needs disulfide bonds	RTS *E. coli* Disulfide	

Many prokaryotic, eukaryotic, and viral proteins have already been expressed with RTS.

For the most current list of successfully expressed proteins and for further information please visit: **www.proteinexpression.com**

3.5. *Purifying Proteins*

Choices of animals for immunization

Animal	Maximal amount of serum*	Possibility to generate monoclonal antibodies	Inbred	Comments
Rabbits	~ 500 ml	👎	👎	Best choice for polyclonal antibodies
Mice	~ 2 ml	👍	👍	Best choice for monoclonal antibodies
Rats	~ 20 ml	👍	👍	Good choice for monoclonal antibodies
Hamsters	~ 20 ml	👎	👎	Good choice for polyclonal antibodies
Guinea Pigs	~ 30 ml	👎	👎	Hard to bleed
Chicken	~ 20 ml/egg	👎	👎	Antibodies are available during entire life of chicken

* during the course of one immunization regime

Notes

3.5. *Purifying Proteins*

Doses of immunogens for Rabbits

Type of antigen	Examples	Routes of injections	Dose
Soluble Proteins	➢ Enzymes ➢ Carrier proteins conjugated with peptides ➢ Immune complexes	➢ Subcutaneous ➢ Intramuscular ➢ Intradermal ➢ Intravenous	50 – 1000 µg
Particulate Proteins	➢ Viruses (killed) ➢ Yeast (killed) ➢ Bacteria (killed) ➢ Structural proteins	➢ Subcutaneous ➢ Intramuscular ➢ Intradermal	50 – 1000 µg
Insoluble Proteins	➢ Bacterially produced from inclusion bodies ➢ Immunopurified proteins bound to beads	➢ Subcutaneous ➢ Intramuscular ➢ Intradermal	50 – 1000 µg
Carbohydrates	➢ Polysaccharides ➢ Glycoproteins	➢ Subcutaneous ➢ Intramuscular ➢ Intradermal ➢ Intravenous	50 – 1000 µg
Nucleic acids	➢ Carrier proteins conjugated to nucleic acids	➢ Subcutaneous ➢ Intramuscular ➢ Intradermal ➢ Intravenous	50 – 1000 µg

Routes of injections for Rabbits

Routes	Maximum volume	Adjuvant	Immunogen	Comments
Subcutaneous	800 µl per site, 10 sites per animal	Possible	Soluble or insoluble	Easy injections
Intramuscular	500 µl	Possible	Soluble or insoluble	Slow release
Intradermal	100 µl per site, 40 sites per animal	Possible	Soluble or insoluble	Injections more difficult, slow release
Intravenous	1000 µl	No	Soluble, ionic detergent < 0.2% Non ionic detergent < 0.5% Salt < 0.3 M Urea < 1 M	Not effective for primary immunizations

3.5. *Purifying Proteins*

Purifying antibodies using Protein A, Protein G* and Protein L*

Use this chart to select the appropriate product to purify antibodies from solutions:

Organism	Antibody	Protein A	Protein G	Protein L
Human	IgG1	++	++	++
	IgG2	++	++	++
	IgG3	–	++	++
	IgG4	++	++	++
	IgM	+	–	++
	IgA	+	–	++
	IgE	–	–	++
	IgD	–	–	++
	Fab	+	+	++
	F(ab)2	+	+	++
	k light chain	–	–	++
	scFv	+	–	++
Mouse	IgG1	+	++	++
	IgG2a	++	++	++
	IgG2b	++	++	++
	IgG3	–	+	++
	IgM	+	–	++

Organism	Antibody	Protein A	Protein G	Protein L
Rat	IgG1	–	+	++
	IgG2a	–	++	++
	IgG2b	–	+	++
	IgG2c	+	++	++
Rabbit	IgG	++	++	+
Horse	IgG	+	++	–
Pig	IgG	++	++	++
Sheep	IgG1	–	++	–
	IgG2	+/-	++	–
Goat	IgG	++	++	–
Chicken	IgY	–	–	++
Cow	IgG1	–	++	–
	IgG2	++	++	–

++ = strong binding
+ = moderate binding
– = no binding

* Protein A Agarose (Cat. No. 11 134 515 001) and G Agarose (Cat. No. 11 243 233 001) are available from Roche Applied Science

Cell Preparation

- Wash cells/tissue at least twice with ice-cold PBS to remove remaining serum proteins from the culture medium.
- A sample volume of 1 to 3 ml per immunoprecipitation reaction is recommended.

Cell Lysis

- Composition of 1 x lysis buffer:
  - 50 mM Tris, pH 7.5; 150 mM NaCl
  - 1% Nonidet P40; 0.5% sodium deoxycholate
  - 1 Complete Protease Inhibitor Cocktail Tablet[a] per 25 to 50 ml buffer
- Stability of lysis buffer:
  - stable for 24 hours at 4°C
  - stable – in aliquots – for at least 4 weeks at –20°C.

[a] Product available from Roche Applied Science: Complete *e.g.,* Cat. No. 04 693 116 001 (more details in reference 14)

Note

- In rare cases, aspartic acid proteases can interfere during isolations in animal tissues. These proteases however only exhibit pronounced activities in the acid pH range. If extractions have to be performed at these pH values, it is recommended to add pepstatin* to the above lysis buffer at a concentration of 0.7 µg/ml.
- Detergents are essential for breaking up the cells and keeping proteins in a soluble state. In most cases, the detergents included in the above mentioned lysis buffer are suitable. However, for some antigens, more sophisticated solubilization protocols may be applied when the protein has to be obtained in a functionally active state.

Wash Buffers

- Low salt wash buffer:
  - same buffer as lysis buffer
- High salt wash buffer (1x concentrated):
  - 50 mM Tris, pH 7.5
  - 500 mM NaCl
  - 0.1% Nonidet P40
  - 0.05% sodium deoxycholate

- Stability of wash buffers:
  - stable for 24 hours at 4°C
  - stable – in aliquots – for at least 4 weeks at – 20°C.
- Add 20% glycerol to these wash buffers if solutions need to be stored at – 20°C.
- *Note:* The tighter the binding between antibody and antigen, the more stringent the washing conditions should be. The washing buffers described are used if low stringency conditions are appropriate. If higher stringency is required, increase salt concentrations and ionic strength by using 0.5 M NaCl or 0.5 M LiCl in the first wash. Additionally, 0.1% SDS may be applied during cell lysis and the first two washes.

Tips

- Keep temperature during the whole procedure between 0 and 4°C to help to reduce enzymatic degradation.

* Available from Roche Applied Science Cat. No. 10 253 286 001 → 2 mg; Cat. No. 11 359 053 001 → 10 mg; Cat. No. 11 524 488 001 → 50 mg

3.5. *Purifying Proteins*

Ion Exchange Chromatography

Principle

- Ion exchange chromatography depends on the reversible absorption of charged solute biomolecules to immobilized ion exchange groups.
- Every soluble biomolecule will have a specific interaction with the resin, depending on its charge under the choosen pH and counterion-conditions.
- These conditions have to be established experimentally to find the optimal proportions to elute the molecule of interest from the resin. Neutralizing the ionic interactions with counterions like salts or by changing the pH desorbs the molecule from the resin.

Types

- There exist two types of ion exchange groups:
  - Anion exchangers with the charged groups Di**e**thyl**a**mino**e**thyl (**DEAE**, weak anion exchanger) and **Q**uaterny ammonium (**Q**, strong anion exchanger)
  - Cation exchangers with the charged groups **C**arboxy**m**ethyl (**CM**, weak kation exchanger) and **S**ulpho**p**ropyl (**SP**, strong kation exchanger).
- Strong ion exchangers are more stable in their behavior under different pH conditions.

- These groups are coupled to a broad variety of immobile phases that define the ligand density and the physical and chemical properties of the resin.
- For a more comprehensive overview of the different types of commercially available products, please refer to references 4, 7, 9, 10 and the product specifications of the different manufacturers of columns and media.

3.5. *Purifying Proteins*

Ion Echange Chromatography: Experimental setup

An easy setup experiment for establishing chromatographic conditions for proteins:

1. Adjust the pH of the sample in 0.5 pH steps between pH 6 and pH 9
2. Incubate 20 µl of the sample with 10 µl chromatographic resin, equilibrated at the same pH and buffer conditions, for 10 minutes at an appropriate temperature with careful mixing.
3. Spin down the chromatographic resin in a desk-top centrifuge.
4. Test the supernatant for the protein in comparison to the load, measure the total content of the supernatant.
5. Choose the pH at which the protein of interest binds to the resin (nothing in the supernatant) and desirably most of the total protein is in the supernatant and test under which counterion conditions the protein will no longer bind to the resin:
6. Adjust the counterion (*e.g.,* KCl or NaCl) concentration of the sample in 50 mM steps between 100 mM and 1 M.

7. Incubate 20 µl of the sample with 10 µl chromatographic resin, equilibrated to the same counterion concentration of the sample, for 10 minutes at an appropriate temperature with careful mixing.

8. Spin down the chromatographic resin in a desk-top centrifuge.

9. Test the supernatant for your protein in comparison to the load, measure the total content of the supernatant.

10. These test should be performed with different ion-exchangers to find the most optimal resin. You can also use the obtained information to find conditions where most contaminants bind but the protein of interest remains unbound, although the binding and elution from a resin is more desirable because it yields concentrated protein.

3.5. *Purifying Proteins*

Tagging

Tag	Size	Remarks/Sequence	Host	Cleavage	Detection via Western Blotting	Immunoprecipitation	Immuno affinity purification	Immunofluorescence
AviTag	15 aa	Mono-biotinylation of proteins via the AviTag sequence/GLNDIFEAQ**K**IEWHE	RTS* (Cell-free) Mammalian cells Bacterial cells Insect cells Yeast	None	Streptavidin-POD*	Streptavidin Mutein Matrix*	Streptavidin Mutein-Matrix*. Lowered biotin dissociation constant ($1.3x10^{-7}$M) allows elution of biotinylated proteins	Avidin-Fluorescein* or Avidin-Rhodamine*
Flag	8 aa	Synthetic peptide Sequence: DYKDDDDK	Mammalian cells Bacterial cells Yeast	Enterokinase	Anti-Flag	Anti-Flag	Anti-Flag agrose affinity gels	Anti-Flag with secondary antibody
β-gal	120 kDa	β-galactosidase	Mammalian cells Bacterial cells	Factor Xa	Anti-β-gal	Anti-β-gal	APTG-Agarose	Anti-β-gal with secondary antibody
GFP	27 kDa	Green fluorescent protein	Mammalian cells	None	Anti-GFP*	Anti-GFP*	Anti-GFP*	Direct green fluorescence of fused proteins or Anti-GFP-Flourescein* antibody
GST	26 kDa	Glutathione-S-transferase	Bacterial cells Insect cells	Thrombin Factor Xa	Anti-GST	GST-Agarose Beads	Glutathion-agarose-beads	Anti-GST with secondary antibody
HA	9 aa	Peptide of human influenza virus Sequence: YPYDVPDYA	Mammalian cells Bacterial cells	None	Anti-HA-POD*	Anti-HA*, Anti-HA Affinity Matrix*	Anti-HA*, Anti-HA Affinity Matrix*	Anti-HA-Biotin*, Flourescein* or Rhodamine*
His_6 or His_{10}	6 aa or 10 aa	Polyhistidine binds metal ligand (affinity chromatography)	Mammalian Cells Bacterial cells Yeast Insect cells	Thrombin enterokinase	Anti-His_6-POD*	Anti-His_6*	Poly-His-Protein purification-Kit	Anti-His with secondary antibody

Tag	Size	Remarks/Sequence	Host	Cleavage	Detection via Western Blotting	Immunoprecipitation	Immuno affinity purification	Immunofluorescence
Intein	55 kDa	Protein splicing element from the Yeast VMA 1 gene	Bacterial cells	Mediated self-cleavage	Anti-Intein	Anti-Intein	Chitin-beads	Anti-Intein with secondary antibody
MBP	44 kDa	Maltose binding protein	Bacterial cells	Factor Xa	Anti-MBP		Amylose Resin	
c-myc	10 aa	Human c-myc protein Sequence: EQKLISEEDL	Mammalian cells Bacterial cells	None	Anti-c-myc-POD*	Anti-c-myc*	Anti-c-myc*	Anti-c-myc with secondary antibody
Protein C	12 aa	Ca^{2+} depending binding of Anti-Protein C antibody Sequence: EDQVDPRLIDGK	Mammalian cells Bacterial cells	None	Anti-Protein C-POD	Anti-Protein C, Anti-Protein C Affinity Matrix*	Anti-Protein C, Anti-Protein C Affinity Matrix*	Anti-Protein C with secondary antibody
VSV-G	11 aa	Vesicular Stomatitis Virus Sequence: YTDIEMNRLGK	Mammalian cells Bacterial cells	None	Anti-VSV-G-POD	Anti-VSV-G*	Anti-VSV-G*	Anti-VSV-G with secondary antibody

* availabel from Roche Applied Science

3.6. *References*

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Working with Cells

Chapter 4

4

4.1. *Precautions for handling Cells*

Tissue culture reagents

General	➢ Optimize growth conditions of your cells: use only fresh media and additives and minimize variations.
Basic medium	➢ Various commercial media are available (*e.g.,* RPMI 1640, DMEM). Medium constituents are nutrients (amino acids, glucose), vitamins, inorganic salts and buffer substances. Some constituents are quite unstable and therefore may cause problems when not freshly added. Many cells need additional factors for proper growth.
Fetal calf serum	➢ Serum is an extremely complex mixture of albumins, globulins, growth promotors and growth inhibitors. The quantity and quality of these components are affected by age, nutrition and health of the animals from which the serum is obtained. It is subject to significant biological variation.
Additives	➢ Some cells are dependent on additional substances/components which are neccessary for viability or dividing activity, *e.g.,* growth factors, trace elements, essential metabolites, proteins.

CO_2 Incubator

- Cells are grown at 37°C in a CO_2-incubator at 100% relative humidity.
- CO_2 is needed to control pH.
- Cell physiology is highly sensitive to pH variations.
- Some media need a concentration of 5% CO_2, other media have to be run at 10% CO_2.
- Inconsistent conditions within an incubator may cause variances between culture plates.
- Pollution, chemicals, fungal or bacterial contamination's from the incubator may affect cell physiology.

4.1. *Precautions for handling Cells*

Tissue culture reagents

Commonly used antibiotics in cell culture

Antibiotic	Concentration [a]	Gram-pos.Bact.	Gram-neg. Bact.	Mycoplasm.	Yeast	Fungi	Stability at 37°C
Ampicillin*	100 U/ml	+	+	-	-	-	3 days
Amphotericine B*	0,25 to 25 µg/ml	-	-	-	+	+	3 day
Cabenicillin	100 U/ml	+	+	-	-	-	3 days
Ciprofloxacin	10 µg/ml			+			5 days
BM-Cyclin* (Pleuronutilin and Tetracyclin derivates)	10 µg/ml 5 µg/ml	+	+	+	-	-	3 days
Erythromycin	100 µg/ml	+	+	+	-	-	3 days
Gentamycin*	5 to 50 µg/ml	+	+	+	+	-	3 days
Kanamycin*	100 µg/ml	+	+	+/-	-	-	5 days
Lincomycin	50 µg/ml	+	-	-	-	-	4 days
Neomycin	50 µg/ml	+	+	-	-	-	5 days
Nystatin	100 U/ml	-	-	-	+	+	3 days
Penicillin G*	50 to 100 U/ml	+	-	-	-	-	3 days
Polymixin B	100 U/ml	-	+	-	-	-	5 days
Streptomycin*	50 to 100 µg/ml	+	+	-	-	-	5 days

[a] two or more antibiotics at the suggested concentration may cause cytopathic effects.
* Product available from Roche Applied Science

Decontamination of cell culture:

- 1) Dilute and seed cells into 96 well plate with antibiotica free medium.
- 2) Ad antibiotica at different concentrations.
- 3) Check surviving rate at highest concentration of antibiotic.
- 4) Reduce the concentration 1– to 2–times in future treatments.
- 5) Cultivate the cells in medium without antibiotic.
- 6) Repeat step 1) to 5) until the culture is free of contamination.

4.1. *Precautions for handling Cells*

Growth areas and yields of cells[a] in a culture vessels

Cell Culture Vessel		Growth area[b] (cm^2)	Numbers of cells[c]
Multiwell plates	96 well	0.32 – 0.6	~ 4×10^4
	48 well	1	~ 1×10^5
	24 well	2	~ 2.5×10^5
	12 well	4	~ 5×10^5
	6 well	9.5	~ 1×10^6
Dishes	Ø 35 mm	8	~ 1×10^6
	Ø 60 mm	21	~ 2.5×10^6
	Ø 100 mm	56	~ 7×10^6
	Ø 145 – 150 mm	145	~ 2×10^7
Flasks	40 – 50 ml	25	~ 3×10^6
	250 – 300 ml	75	~ 1×10^7
	650 – 750 ml	162 – 175	~ 2×10^7
	900 ml	225	~ 3×10^7

[a] Such as 3T3, Hela or CHO cells.
[b] Per well, if multiwell plates are used; varies slightly depending on supplier.
[c] assuming confluent growth.

Introduction

Mycoplasma is a common and serious contamination of cell cultures, and one of the major problems in biological research using cultured cells. It has been shown that up to 30% (variation from 5% to 85%) cell cultures are contaminated with mycoplasma, the main contaminants being the species M.orale, M.laidlawaii, M.arginii and M.hyorhinis.

It is important to keep in mind that mycoplasma do not always reveal their presence with macroscopic alterations of the cells or medium. Many mycoplasma contaminants, particularly in continuous cell lines, grow slowly and do not destroy host cells. They are able, however, to affect various parameters (*e.g.*, changes in metabolism, growth, viability, morphology, etc.) and thus can interfere with experiments in cell culture. Therefore, it is an absolute requirement for routine, periodic assays to detect possible contamination of cell cultures. This is particularly important with continuous or established cell lines.

The major source of Mycoplasma contamination are:

- Cells purchased from outside
- People handling cells
- Serum, medium and additives
- Liquid nitrogen

4.1. *Precautions for handling Cells*

Mycoplasma Contamination

Indication for Mycoplasma contamination

Cells not adhering to culture vessel	Mycoplasma contamination	Segregate culture and test for Mycoplasma infection. Clean fume hood and incubator.
	Overlay trypsinized cells	
	No attachemend factors in medium	
Decreased growth of culture	Mycoplasma contamination	Segregate culture and test for Mycoplasma infection. Clean fume hood and incubator.
	Low level bacterial or fungi contamination	
	Depletion, absence or breakdown of essential growth-promoting components.	
	Inproper storage of reagents	
	Very low initial cell inoculum	

Suspension cells clumping together	Mycoplasma contamination	Segregate culture and test for Mycoplasma infection. Clean fume hood and incubator.
	Presence of calcium and magnesium ions	
	Cell lysis and release of DNA resulting from overdigestion with proteolytic enzymes	

Detection of Mycoplasma contamination

Overview of different assays

Assay	Sensitivity	Specifity
DNA staining with DAPI*	+/-	+/-
DNA-RNA hybridisation	+	+
ELISA[a]	+/-	+
PCR[a]	+	+
Culture	+/-	+/-

4.1. *Precautions for handling Cells*

Detection of Mycoplasma contamination

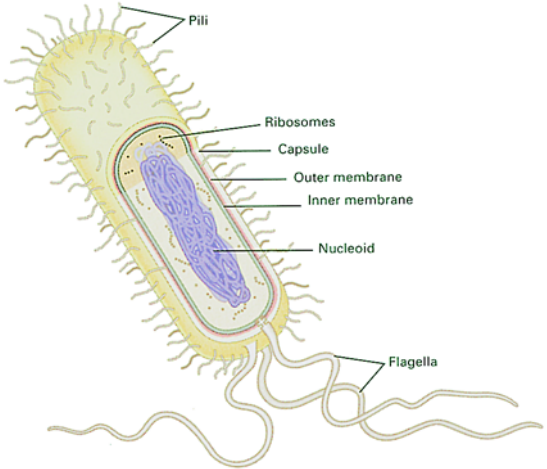
Assay	Principle
DAPI* (4',6-Diamide-2'-phenylindole dihydro chloride)	DAPI is a fluorescent dye that specifically stains double-stranded DNA
Culture	Combination of direct mycoplasma cultivation in aerobic and anaerobic conditions. A species specific assay should follow the isolation.
ELISA**	Determination of each species is use done seperatly.
PCR*	Usely multiplex PCR for the most "popular" mycoplasma strains.

[a] Mycoplasma Detection Kit (Cat. No. 11 296 744 001) and **Mycoplasma PCR ELISA (Cat. No. 11 663 925 910) are available from Roche Applied Science.
* products available from Roche Applied Science.

Elimination of Mycoplasma in cell culture

- BM-Cyclin*: 5–10 µg/ml medium
- Gentamycin*: 50 µg/ml medium
- Ciprofloxacin: 10 µg/ml medium

Typical Properties of Bacterial Cells

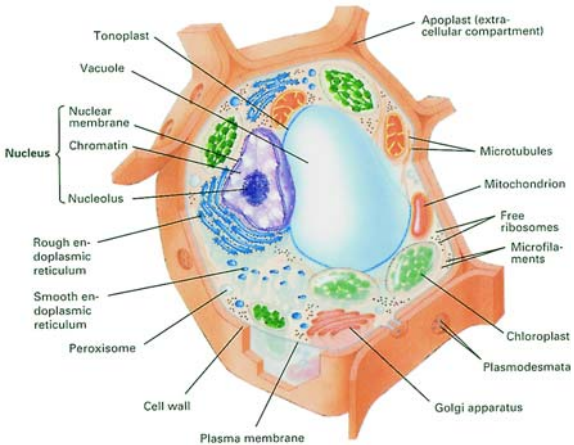


After Campbell, N.A.: Biology 4/e. Benjamin/Cummings 1996.

Domain	Bacteria
Kingdom	Bacteria
Nucleus	no (common term prokarya)
Genome	circular, ca. 10^6 to 5 x10^7 kb, extra plasmids
RNA Polymerase	one type
Starting amino acid for translation	formylmethionine
Reproduction	binary scission
Cellular organization	unicellular (some are aggregated)
Nutrition	chemoorganotrophic, photoautotrophic or photoheterotrophic
Size of cells	average 1 – 5 µm, wide variation
Cell membranes	rigid, contain peptidoglycans
Internal membranes	no

4.2. *Basic Information*

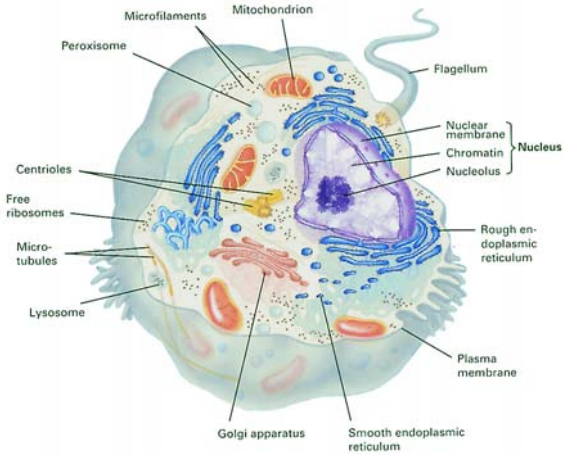
Typical Properties of Plant Cells



After Campbell, N.A.: Biology 4/e. Benjamin/Cummings 1996.

Domain	Eukarya
Kingdom	Plant
Nucleus	yes
Genome	linear, 10^7 to $> 10^{11}$ kb, organized in several chromosomes
RNA Polymerase	several types
Starting amino acid for translation	methionine
Reproduction	asexual/sexual
Cellular organization	multicellular
Nutrition	photoautotrophic
Size of cells	average 10 – 100 µm, wide variation
Cell membranes	rigid, contain cellulose and lignin
Internal membranes	yes, they enclose organelles/vesicles

Typical Properties of Animal Cells



After Campbell, N.A.: Biology 4/e. Benjamin/Cummings 1996.

Domain	Eukarya
Kingdom	Animals
Nucleus	yes
Genome	linear, 10^7 to $> 10^{11}$ kb, organized in several chromosomes
RNA Polymerase	several types
Starting amino acid for translation	methionine
Reproduction	asexual/sexual
Cellular organization	multicellular
Nutrition	chemoheterotrophic
Size of cells	average 10 – 100 µm, wide variation
Cell membranes	soft, lipid bilayer only
Internal membranes	yes, they enclose organelles/vesicles

4.2. *Basic Information*

Nucleic Acid and Protein content of a bacterial cell[a]

Cell data	Per cell	Per liter culture (10^9 cells/ml)
Wet weight	950 fg	950 mg
Dry weight	280 fg	280 mg
Total Protein	155 fg	155 mg
Total genomic DNA	17 fg	17 mg
Total RNA	100 fg	100 mg
Volume	1.15 μm^3 = 1 picoliter	
Intracellular protein concentration	135 µg/ml	

Theoretical maximum yield for a 1 liter culture (10^9 cells/ml) of protein of interest is

- 0.1% of total protein: 155 µg per liter
- 2.0% of total protein: 3 mg per liter
- 50.0% of total protein: 77 mg per liter

[a] values for *Escherichia coli* or *Salmonella typhimurium*

Nucleic Acid content in mammalian cells

Nucleic Acid content in a typical human cell

Cell data	Per cell
Total DNA	~ 6 pg
Total RNA	~ 10 – 30 pg
Proportion of total RNA in nucleus	~ 14%
DNA:RNA in nucleus	~ 2 : 1
Human genome size (haploid)	3.3×10^9
Coding sequences/genomic DNA	3%
Number of genes	$0.5 - 1 \times 10^5$
Active genes	1.5×10^4
mRNA molecules	$2 \times 10^5 - 1 \times 10^6$
Average size of mRNA molecule	1900 b
Different mRNA species	$1 - 3 \times 10^4$

RNA distribution in a typical mammalian cell

RNA Species	Relative amount
rRNA (28S, 18S, 5S)	80 – 85%
tRNAs, snRNAs	15 – 20%
mRNAs	1 – 5%

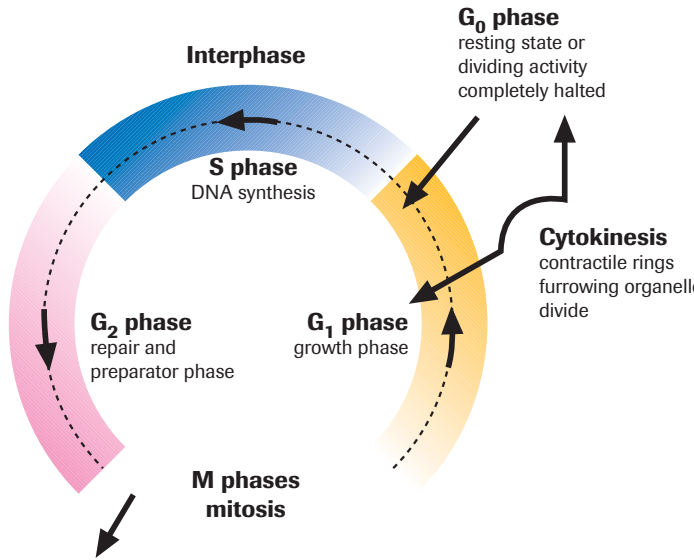
4.2. *Basic Information*

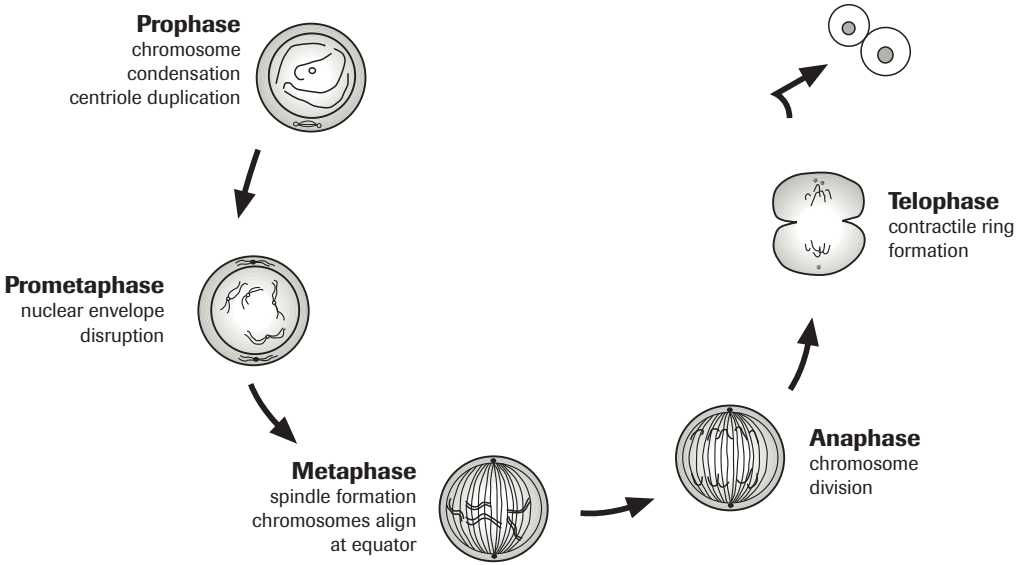
*Nucleic Acid and Protein content in human blood**

Data	Erythrocytes	Leukocytes	Thrombocytes
Function	O_2/CO_2 transport	Immune response	Wound healing
Cells per ml	5×10^9	$4 - 7 \times 10^6$	$3 - 4 \times 10^8$
DNA content	–	30 – 60 µg/ml (6 pg/cell)	–
RNA content	–	1 – 5 µg/ml	–
Hemoglobin content	~ 150 mg/ml (30 pg/cell)	–	–
Plasma protein content	–	–	60 – 80 mg/ml

* From a healthy individual.

Notes


Interphase
G_0 phase
resting state or
dividing activity
completely halted
S phase
DNA synthesis
Cytokinesis
contractile rings
furrowing organelle
divide
G_2 phase
repair and
preparator phase
G_1 phase
growth phase
M phases
mitosis


Prophase
chromosome
condensation
centriole duplication
Prometaphase
nuclear envelope
disruption
Metaphase
spindle formation
chromosomes align
at equator
Anaphase
chromosome
division
Telophase
contractile ring
formation

4.2. *Basic Information*

Arresting Cells

Synchronization of mammalian somatic cells

Production of synchronized cells in cell cycle phase	method	inhibition of	reversibility of block	suitable for
G_0	depriviation of serum from medium	protein synthesis	+	fibroblasts
late G_1	L-mimosine	formation of hyposunine from lysine	+/-	CHO cells, HL-60
early S	hydroxyurea thymidine	synthesis of dNTPs	+/-	transformed human cells
G/S	aphidicolin	DNA polymerase	+	human fibroblasts transformed human cells
G_2	topoisomerase II	toposisomerase II inhibitors	+	transformed human cells
M	nocodazole (followed by shake-off)	depolymerization of microtubuli	+	human fibroblasts transformed human cells

Notes

4.3. *Manipulating Cells*

Transfection of mammalian cells

	Ca^{2+}	lipos. Transfection	Transduction	Electroporation	Microinjection
Principle	Ca phosphate / DNA aggregates are transferred through the cell membrane	DNA / lipophilic agent complexes are transferred through the membrane	Reagent to be coupled to a "translocation peptid"	A high voltage pulse is used to open the membrane of the cells	Injection directly into the nucleus or cytoplasm using micro glass capillaries
use for DNA	👍	👍	👍	👍	👍
use for RNA	👎	👍	👍	👍	👍
use for peptides	👎	👍	👍	👍	👍
use for antibodies	👎	👍	👉	👍	👍
Cells	eukaryotic cells	eukaryotic cells	eukaryotic cells	eukaryotic cells bacteria yeasts	eukaryotic cells
Costs for equipment/ reagents	low / low	low / medium	medium	high / low	high / medium
Efficiency	low	medium - high	low - high	medium - high	very high
Remarks	inefficient but simple, high volumes possible	purity of DNA is important	Trends in Cell Biology 10(2000) 290–295. Nature Biotechnology 19(2001), 360–364	standard method for many bacteria, survival rate is critical for eukaryotic cells	non-random method, comparably few cells can be used, method of choice *e.g.,* for antibodies

General	➢ There exist several well etablished methods for delivery of molecules, especially nucleic acids, into eukaryotic cells. However no single technique alone is suitable for the multitude of different cellular systems used for transfection experiments. Depending on the cell type and the specific experimental requirements like transfection of difficult cell lines, primary cells, easy cell lines, different molecules (DNA, RNA, oligonucleotides, proteins) or even high throughput purposes, each transfer method may possess advantages or disadvantages. ➢ Keep an eye on your cells: take care that they are in good condition and set a suitable plating protocol for optimal cell density from start to end of transfection.
Dividing vs. non-dividing cells	➢ Dividing cells tend to be more accessible for uptake and expression of foreign DNA compared to quiescent cells. Mitogenic stimuli (*e.g.*, virus transformation, growth factors, conditioned media, feeder cells) are often used to activate primary cells.
Adherent vs. suspension cells	➢ Transfection efficiencies differ by orders of magnitude between adherent and suspension cells. It is speculated that the limiting step is the uptake by endocytosis, however, a plausible mechanistic explanation on molecular level does not exist so far. Therefore the search for more efficient transfection reagents is mainly empirical.

Splitting protocol	➢ Before splitting, adherent cells have to be trypsinated in order to remove them from the substrate. This routine step means a severe obstruction of normal cellular functions. Differences in the splitting protocol (*e.g.*, extend of trypsination, inactivation of trypsin, time untill transfection starts) could have an impact on the efficiency of transfection experiments.
Passage number	➢ Cell lines tend to be unstable and therefore features may change with time in culture. The passage number indicates, how often a cell line has been splitted (normally within one lab). The exact passage number untill the line has been established is unknown in most cases. ➢ Different culture conditions could lead to clonal selection. Cell lines with the same name could therefore differ significantly with respect to physiology and morphology (and transfectability).
Cell number (grade of confluency)	➢ Cell lines divide exponentially when there is space on the substrate (tissue culture dish). Growth rate is negatively affected by cell density (contact inhibition), depletion of nutrients or metabolic endproducts (*e.g.*, pH). The extent of reporter gene expression is directly correlated with cell number at transfection start and growth rate until cell lysis.

DNA influences on transfection

General	➢ Control your vector: check the quality of your purified DNA and consider suitability of functional sequences for your special cellular system. ➢ Always use a control vector.
Vector integrity	➢ Functionality of a vector depends on the structural integrity of the plasmid preparation. Supercoiled form versus relaxed form, double strand breaks, degradation by nucleases and physical stress during storage and handling influences transfection efficiency.
Vector preparation	➢ Vectors are produced in bacterial systems and purified according to various protocols. Contaminants in the vector preparation (*e.g.*, CsCl) may influence transfection efficiency.
Vector architecture (Enhancer / Promoter / cDNA / poly A signal)	➢ Often transfection systems are optimized and compared by the use of control vectors with strong viral regulatory elements (*e.g.*, RSV[a], CMV[b], SV40[c]). However, the relative efficiacy of viral promotor/enhancer systems can differ from cell line to cell line in a range of two orders of magnitude. In some cell lines expressing the large T antigen (*e.g.*, COS) the SV40 system is highly efficient due to autonomous plasmid amplification. In many other cell lines the CMV promotor is most efficient.

[a] Respiratory Syncitial Virus [b] Cytomegalo Virus [c] Simian Virus 40

4.3. *Manipulating Cells*

Transfection protocol

General	➢ Establish a suitable transfection protocol: start with the standard protocol and do some tests with variations in ratio and dose for optimization.
Preparation of transfection complex	➢ It is a common feature of all transfection reagents that DNA is packed into compact, highly condensed particles. In this form the DNA tends to be highly resistant to degradation by nucleases and inaccessible to intercalating dyes. Variables like transfection reagent/DNA-ratio, ionic strength, buffer/pH, DNA/lipid- concentrations, temperature affect the composition and the functionality of the transfection complexes.
Transfection reagent / DNA ratio (charge ratio)	➢ Transfection complexes are predominantly formed by charge interaction. Generally, in *in vitro* transfection experiments (cell lines, primary cells), complexes with a positive net-charge seem to give highest expression rates. It has often been argued that positive complexes are more efficiently adsorbed to the negatively charged cell surface. On the other hand, in *in vivo* approaches, complexes with an excess of negative charges proofed to be most efficient.
Dose dependence	➢ Many transfection reagents show an optimum characteristic when the amount of transfection complex is increased. The ascending part of the curve reflects the increasing amount of DNA transfected into the cells.

- The declining part of the curve reflects the cytostatic (some times cytotoxic) effect of larger amounts of transfection complex on cells. Interestingly FuGENE® Transfection Reagents* does not show this decrease at the high-dose end.

Application of transfection complex

- There are two alternative ways to apply the transfection complex to the cells. Either concentrated, dropwise to the medium or in combination with a medium exchange prediluted with medium. The first option is more convenient. The latter option allows a more constant application which avoids local toxic doses.

Transfection medium

- The medium present during the transfection affects transfection efficiency in a positive or negative manner. This is true for different basic medium compositions and especially when calf serum has to be included. With a number of transfection reagents the efficiency is reduced in the presence of FCS (Fetal Calf Serum). Some companies provide optimized serum-free transfection media** to be used in combination with the transfection reagents. However FuGENE® Transfection Reagents* are effective in the presence or absence of serum.

* Available from Roche Applied Science
** Nutridoma-CS; -HU; -NS; -SP are also offered by Roche Applied Science

4.3. *Manipulating Cells*

Time course of transfection

General	➢ Set up a suitable time course for your transfection experiment for optimal expression of your protein of interest.
Start of transfection	➢ 12 hours before transfection split the cells into culture plates. At the beginning of transfection the culture should be about 50% confluent, in order to nearly reach confluency at the end of the experiment. If serum is taken away during transfection cells may arrest for some time. ➢ The uptake of transfection complex by cells takes place within a period of hours (0.5h – 6h). The kinetic has a saturation characteristic which means, that after a certain point no further increase in efficiency can be detected.
Medium exchange	➢ After the application of the transfection complex, the medium has to be replaced by normal growth medium. This step is absolutely essential if transfection has to be performed in the absence of FCS. With non-toxic transfection reagents* which work in the presence of serum (*e.g.,* FuGENE® Transfection Reagents*) this step could be eliminated, if there is enough medium for the following expression period.

Time of analysis	➢ Reporter gene expression is analyzed 24h to 48h after the start of transfection. Within this period, there is a constant increase in concentration of reporter gene product. It mainly depends on the sensitivity of the reporter gene assay (strength of promotor) and the ”lab routine“ when is the best time for harvesting the cells.

* Products available from Roche Applied Science, please visit and bookmark **www.powerful-transfection.com**

Calcium phosphate-DNA coprecipitation

- The day before transfection plate cells from exponential growth phase (1 – 4 x 10^5 cells/ml) into 12-well or 60 mm plates.
- One hour before the precipitate is added, the medium should be changed with fresh medium.
- A solution of 100 µl 2.5 M $CaCl_2$ and up to 25 µg plasmid DNA is diluted with low TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.6) to a final volume of 1 ml.
- One volume of this 2 x Ca^{2+}/DNA solution is added quickly to an equal volume of 2x HEPES solution (140 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.05 at 23°C).
- The cells are precipitated for 2 – 6 h at 37°C (pH of 7.3 – 7.6.).
- After incubation, for some cell lines, *e.g.,* CHO cells, a "glycerol shock" is advised. Cells are exposed to 20% glycerol in PBS. After 1 min the glycerol is removed by adding fresh medium, aspiration of the mixture and replacement with fresh medium. If no "glycerol shock" is used, just replace with fresh medium.

For detailed information see: Jordan M., Schallhorn A., and Wurm F.M., Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation., Nucleic Acids Research, 24 (4) p.p. 596–601, 1996.

Liposomal and non-liposomal Transfection Reagents

Transfection of siRNA oligo´s for gene knockdown applications	**X-tremeGENE siRNA Transfection Reagent:** Proprietary blend of lipids and other components, sterile filtered and free of components derived from animals. It enables the efficient transfection of a wide range of cell lines with low cytoxic side effects and, siRNA- and cotransfection-based gene knockdown experiments. It also functions exceptionally well in the presence and absence of serum.
Transfection of plasmids and linear DNA fragments for cellular analysis, protein expression and gene knockdown applications (using shRNA expressing plasmids)	**FuGENE® HD Transfection Reagent:** Next-generation proprietary non-liposomal reagent that is free of animal-derived components. As proven by many scientists, it combines minimal cytotoxic side effects with excellent transfection efficiency in many cell lines not transfected well by other reagents (*e.g.* cell lines commonly used in oncology research, stem cells and many other difficult-to-transfect cell lines). The reagent functions in up to 100% serum and, requires a limited amount of handling steps (dilute plasmid DNA, mix with FuGENE® HD Transfection Reagent, incubate and pipet the complex directly to the cells).

For more information and a database of successfully transfected cells, please consult **www.powerful-transfection.com**

- Electroporation uses of a transmembrane electric field pulse to induce microscopic pathways (pores) in a bio-membrane. All major manufacturers of electroporation devices offer detailed protocols for many cell lines. Electroporation is not restricted to any substance. Almost any substance that can be solved or dispersed in water can be transferred into living cells. Two very different methods are used, long Millisecond Pulses and short Microsecond Pulses. The latter are much more physiologically compatible.
- The cells should be in the exponential growing phase before harvesting. Adherent cells should not be more confluent than 70%.
- Keep treatment of adherent cells with trypsin as short as possible, otherwise the membrane gets damaged.
- Solve the DNA in water, or in low TE (1mM Tris-HCl, 0.1 mM EDTA, pH 7.6), EDTA inside the cell has very toxic effects.
- After electroporation the cell membrane is open. Handle cells with extreme care. Do no shake, aspirate very slowly with pipette.

- Times and temperature are critical. Resealing of the holes in the membrane occurs during approx. 1 – 3 minutes at room temperature.

Please note: Don't keep cells on ice because after 30 minutes the cells leak out and the ion gradient is destroyed.

Calculating the Required Field Strength for Electroporation

A standard value for the required field strength can be calculated using the following formula:

$$E_c = \frac{V_c}{1.5 \times a}$$

Example:
Cell radius a = 10 µm = $10 \cdot 10^6$ cm
Critical breakdown voltage at room temperature V_c = 1 V [V_c (4°C) = 2 V]

$$\text{Critical field strength } E_c = \frac{1\ \text{V}}{1.5 \times 10 \times 10^{-4}\ \text{cm}} = 666\ \frac{\text{V}}{\text{cm}}$$

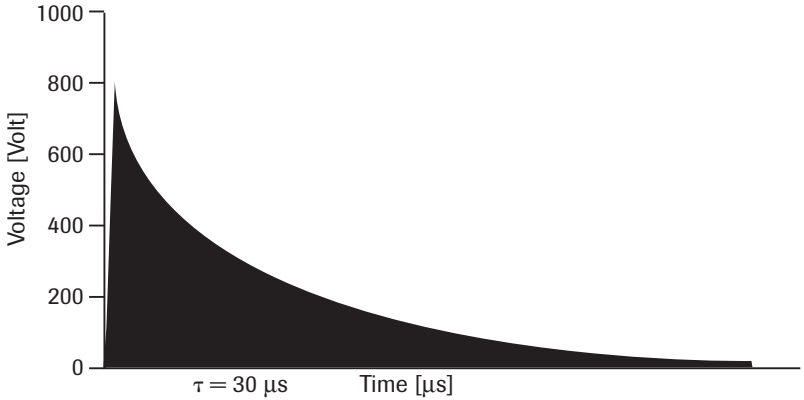
The voltage required for the Multiporator can be calculated using the field strength which has been calculated and the distance between the electrodes in the cuvette (d: Distance between electrodes [cm]):

$$V = E_c \cdot d$$

$$\text{Example for 2 mm cuvettes: } 666\ \frac{\text{V}}{\text{cm}} \times 0.2\ \text{cm} = 133\ \frac{\text{V}}{\text{cm}}$$

Suggestion for a test series:

1. Voltage applied: 130 V
2. Voltage applied: 160 V
3. Voltage applied: 200 V
4. Additional voltage 240 V and in appropriate increments



Voltage Profile of Eppendorf Multiporator in Mammalian Transfection Mode

4.3. *Manipulating Cells*

Microinjection

This physical method offers the best possible transfection efficiency as the DNA is brought directly into the cell nucleus.
Cells are injected using an extremely fine glass capillary (*e.g.*, Eppendorf Femtotips) which is filled with DNA, RNA or any other liquid reagent. With the aid of a micromanipulator (*e.g.*, Eppendorf InjectMan 5179) this glass capillary is moved into the cells and with the aid of a microinjector (*e.g.*, Eppendorf FemtoJet 5247) pressure is applied to force the liquid into the cells.

Microinjection is the only non-random transfection method available. It has some unique advantages and limitations as well.

Advantages

1. It is the only method where the user can actively decide which cells should be transfected.
2. It is possible to inject directly into the nucleus or the cytoplasm of a cell.
3. Cells remain in their cellular context; thus, intra- and extracellular signals or transport processes can be analyzed.
4. Co-transfection of different reagents is possible.
5. The cells can be monitored in real time during the transfection process.
6. In principle any kind of cells or substances can be used for microinjection.

Limitations

1. The number of cells that can be analyzed is limited. Using automatic microinjectors, a maximum of about 1000 adherent cells per hour can be transfected. If suspension cells are used this number is even much lower.
2. The equipment is rather expensive.
3. It takes time to be an expert with this technique.

- Seed cells at 25% confluence, let the cells grow overnight.
- Substitute the cells with medium containing varying concentration of selection reagent
- Replace the selection medium every 3 to 4 days
- Determine the appropiate concentration of selection reagent

Selection Reagent	Concentration	Cell death after
Blasticidin	1 to 20 µg/ml	7 to 10 days
G-418 (Neomycin)	50 to 1000 µg/ml	7 to 14 days
Hygromycin*	10 to 500 µg/ml	7 to 14 days
Zeocin	50 to 1000 µg/ml	7 to 14 days

* Product available from Roche Applied Science

4.4. *Analyzing Cells*

Apoptosis/Necrosis

Terminology of cell death

- Cell death can occur by either of two distinct[6,7] mechanisms: Necrosis or Apoptosis. In addition, certain chemical compounds and cells are said to be cytotoxic to the cell, that is, to cause its death.
- Someone new to the field might ask, what's the difference between these terms? To clear up any possible confusion, we start with some basic definitions.

Necrosis and apoptosis

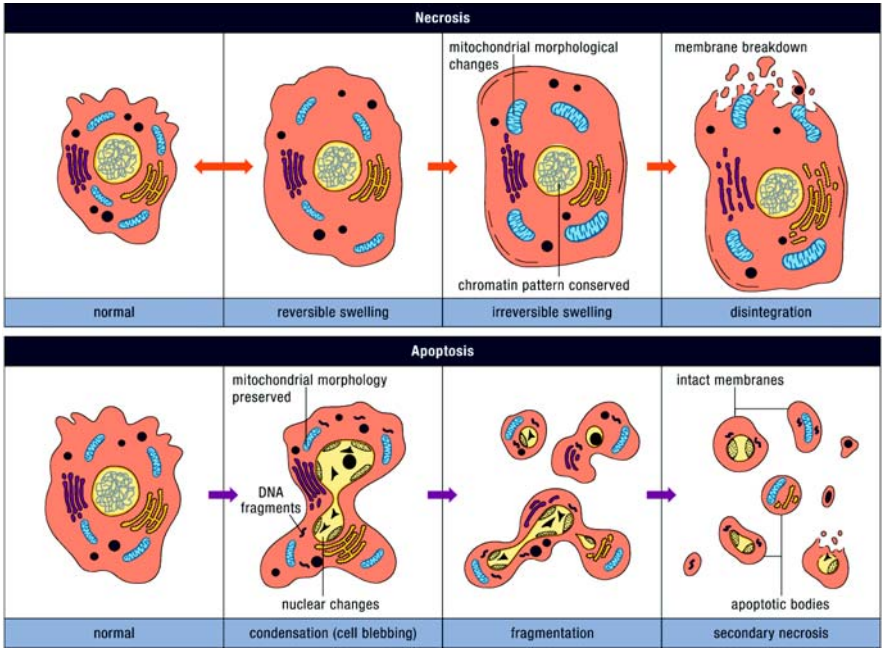
The two mechanisms of cell death may briefly be defined:

- Necrosis ("accidental" cell death) is the pathological process which occurs when cells are exposed to a serious physical or chemical insult.
- Apoptosis ("normal" or "programmed" cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes.

Cytotoxicity

- Cytotoxicity is the cell-killing property of a chemical compound (such as a food, cosmetic, or pharmaceutical) or a mediator cell (cytotoxic T cell). In contrast to necrosis and apoptosis, the term cytotoxicity does not indicate a specific cellular death mechanism.
- For example, cell-mediated cytotoxicity (that is, cell death mediated by either cytotoxic T lymphocytes [CTL] or natural killer [NK] cells) combines some aspects of both necrosis and apoptosis.

Illustration of the morphological features of necrosis and apoptosis



Differences between necrosis and apoptosis

- There are many observable morphological and biochemical differences between necrosis and apoptosis.

- Necrosis occurs when cells are exposed to extreme variance from physiological conditions (*e.g.*, hypothermia, hypoxia) which may result in damage to the plasma membrane. Under physiological conditions direct damage to the plasma membrane is evoked by agents like complement and lytic viruses.

- Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive damage resulting in an intense inflammatory response.

- Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and the cell is an active participant in its own demise ("cellular suicide").

- It is most often found during normal cell turnover and tissue homeo stasis, embryogenesis, induction and maintenance of immune tolerance, developement of the nervous system and endocryne-dependent tissue atrophy.

- Cells undergoing apoptosis show characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material. *In vivo*, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells. Due to this efficient mechanism for the removal of apoptotic cells *in vivo* no inflammatory response is elicited. *In vitro*, the apoptotic bodies as well as the remaining cell fragments ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed “secondary necrosis”.

4.4. *Analyzing Cells*

Apoptosis/Necrosis

	Necrosis	Apoptosis
Morphological features	➢ Loss of membrane integrity ➢ Begins with swelling of cytoplasm and mitochondria ➢ Ends with total cell lysis ➢ No vesicle formation, complete lysis ➢ Disintegration (swelling) of organelles	➢ Membrane blebbing, but no loss of integrity ➢ Aggregation of chromatin at the nuclear membrane ➢ Begins with shrinking of cytoplasm and condensation of nucleus ➢ Ends with fragmentation of cell into smaller bodies ➢ Formation of membrane bound vesicles (apoptotic bodies) ➢ Mitochondria become leaky due to pore formation involving proteins of the bcl-2 family.
Physiological significance	➢ Affects groups of contiguous cells ➢ Evoked by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemic, metabolic poisons) ➢ Phagocytosis by macrophages ➢ Significant inflammatory response	➢ Affects individual cells ➢ Inducted by physiological stimuli (lack of growth factors, changes in hormonal environment) ➢ Phagocytosis by adjacent cells or macrophages ➢ No inflammatory response

Biochemical features	➢ Loss of regulation of ion homeostasis ➢ No energy requirement (passive process, also occurs at 4°C) ➢ Random digestion of DNA (smear of DNA after agarose gel electrophoresis ➢ Postlytic DNA fragmentation (= late event of death)	➢ Tightly regulated process involving activation and enzymatic steps ➢ Energy (ATP)-dependent (active process, does not occur at 4°C) ➢ Non-random mono- and oligo nucleosomal length fragmentation of DNA (Ladder pattern after agarose gel eletrophoresis) ➢ Prelytic DNA fragmentation ➢ Release of various factors (cytochrome C, AIF) into cytoplasm by mitochondria ➢ Activation of caspase cascade ➢ Alternations in membrane asymmetry (i.e., translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)

Originally, to study both forms of cell death, necrosis and apoptosis, cytotoxicity assays were used. These assays were principally of two types:

- Radioactive and non-radioactive assays that measure increases in plasma membrane permeability, since dying cells become leaky.
- Colorimetric assays that measure reduction in the metabolic activity of mitochondria, mitochondria in dead cells cannot metabolize dyes, while mitochondria in living cells can.

However, as more information on apoptosis became available, that both types of cytotoxicity assays vastly underestimated the extent and timing of apoptosis. For instance, early phases of apoptosis do not affect membrane permeability, nor do they alter mitochondrial activity. Although the cytotoxicity assays might be suitable for detecting the later stages of apoptosis, other assays were needed to detect the early events of apoptosis.

In relation with increased understanding of the physiological events that occour during apoptosis, a number of new assay methods have been developed. For instance, these assays can measure one of the following apoptotic parameters:

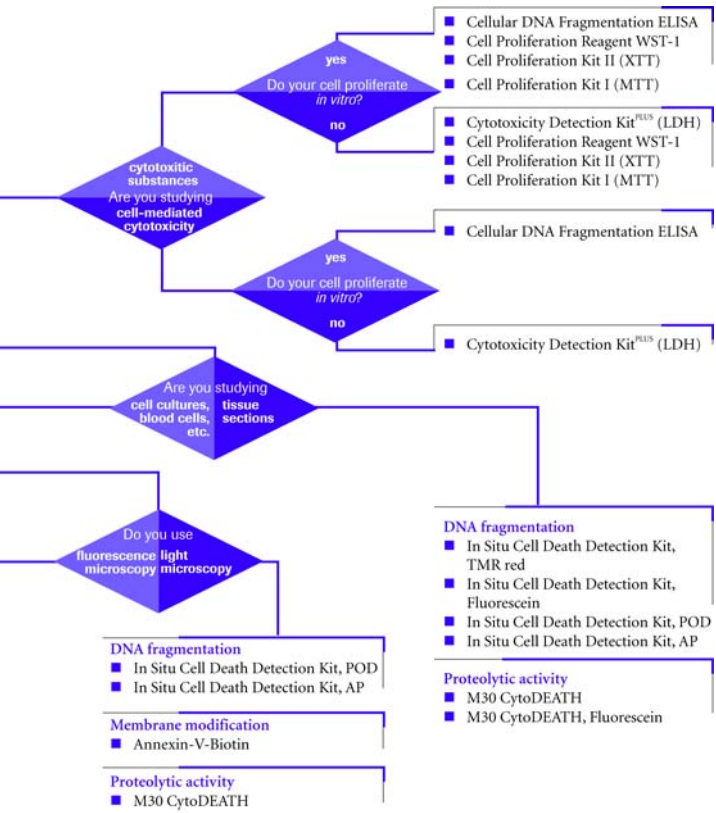
- Fragmentation of DNA in populations of cells or in individual cells, in which apoptotic DNA breaks into different fragments.

- Alternations in membrane asymmetry. Phosphatidylserine translocates from the cytoplasmic to the extracellular side of the cell membrane.
- Activation of apoptotic caspases. This family of protease sets off a cascade of events that disable a multitude of cellular functions.
- Release of cytochrome C and AIF into cytoplasma by mitochondria.

For more details please check out **http://www.roche-applied-science.com/apoptosis**

4.4. *Analyzing Cells*

Apoptosis Assay Methods: Selection Guide



4.4. *Analyzing Cells*

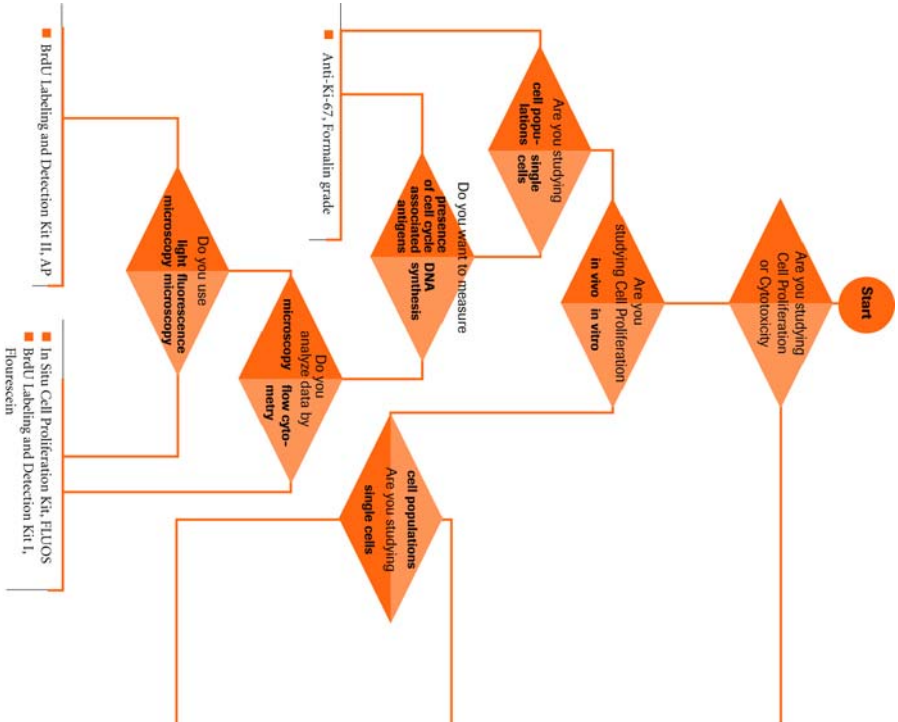
Cell Proliferation and Viability

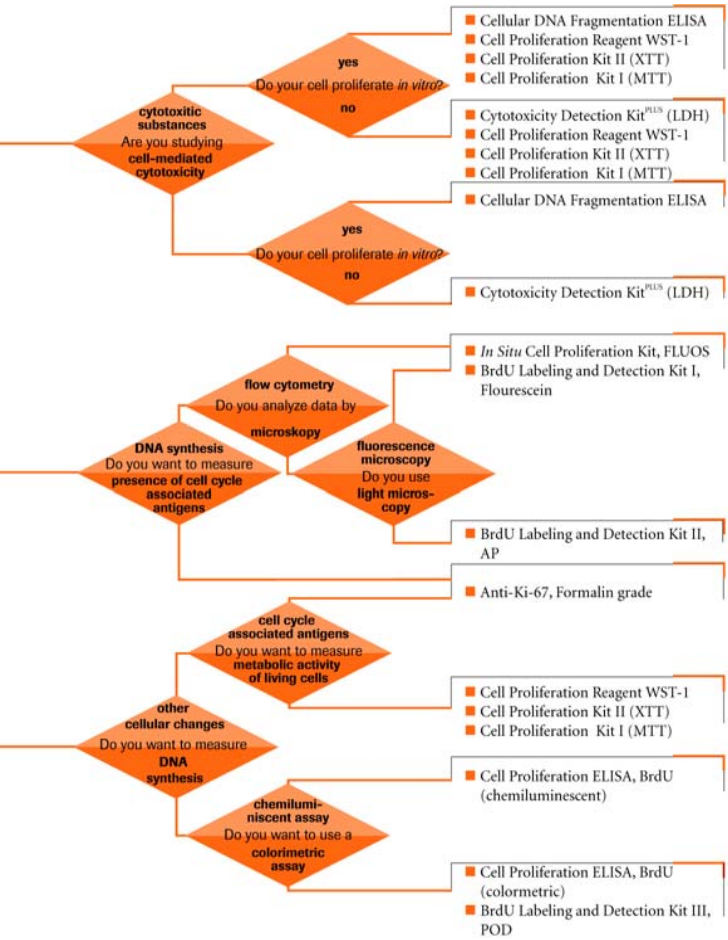
General	➢ Rapid and accurate assessment of viable cell number and cell proliferation is an important requirement in many experimental situations involving *in vitro* and *in vivo* studies. ➢ Usually, one of two parameters are used to measure the health of cells
Cell Viability	➢ Can be defined as the number of healthy cells in a sample. Whether the cells are actively dividing or are quiescent is not distinguished. Cell viability assays are often useful when non-dividing cells (such as primary cells) are isolated and maintained in culture to determine optimal culture conditions for cell populations. ➢ Methods for determining viable cell number is a direct counting of the cells in a hemocytometer, cell morphology (staining), metabolic activity
Cell Proliferation	➢ Is the measurement of the number of cells that are dividing in a culture. Methods for determination: clonogenic assays (plate cells on matrix), establish growth curves (time-consuming), measurement of DNA synthesis (^{3}H-thymidine or bromodeoxyuridine), indirect parameters (measure cell cycle regulating molecules)

Notes

4.4. *Analyzing Cells*

Proliferation Assay Methods: Selection Guide





All Products are available from Roche Applied Science,
http://www.roche-applied-science.com/apoptosis

4.4. *Analyzing Cells*

FACS (Fluorescence activated cell sorting)

General

- powerful technique used in flow cytometry to physically separate and identify specific types of cells from heterogeneous populations.

Procedure: Staining of surface proteins on intact cells

<table>
<tr><td>Sensitivity: +++ Duration: +</td><td>Sensitivity: ++ Duration: ++</td><td>Sensitivity: + Duration: +++</td></tr>
<tr><td>Biotin-Streptavidin enhancement</td><td>Directly labeled 2nd Ab</td><td>Directly labeled primary antibody</td></tr>
<tr><td colspan="3">Harvest Cells (5x10^5) per stain, (For suspending adherent cells, try EDTA (2mM in PBS, incubate at 37°C) first.
Trypsin solutions might damage surface antigens and preclude their detection)</td></tr>
<tr><td colspan="3">Wash cells once with FACS buffer</td></tr>
<tr><td colspan="2">Incubate with first antibody 30 min (1–10 µg/ml)</td><td rowspan="3">Incubate with directly labeled antibody.</td></tr>
<tr><td colspan="2">Wash cells with FACS buffer</td></tr>
<tr><td>Add Biotinylated 2nd Ab (1–10 µg/ml)</td><td rowspan="3">Add labeled 2nd Antibody, incubate 30 min.</td></tr>
<tr><td>Wash cells with FACS buffer</td><td></td></tr>
<tr><td>Add streptavidin linked fluorochrome
(1–5 µg/ml) incubate 15–30 min.</td><td></td></tr>
<tr><td colspan="3">Wash, resuspend in FACS buffer, measure</td></tr>
<tr><td colspan="3">All incubations should be done on ice. Keep solutions in the dark after addition of the fluorochrome.</td></tr>
</table>

- For discrimination of dead cells, Propidium Iodide (PI) or 7-Aminoactinomycin-D (7-AAD) can be added in the final step.
  Propidium Iodide (PI) and 7-Aminoactinomycin-D (7-AAD) selectively enter dead cells and lead to high fluorescence in FL3 allowing to exclude them from analysis (dead cells may bind antibodies unspecifically).

Controls

Control type	Parameter controlled	Influenced by
Cells only in FACS-Buffer	Autofluorescence, used for setting up Instrument	Cell Type, Cell treatment (some chemicals exhibit strong autofluorescence!). Dead cells might exhibit stronger autofluorescence.
Omit first antibody (not for directly labelled)	Controls non-specific binding of secondary reagents.	Immunoglobulin receptors on the cell surface (might bind secondary antibody). Secondary reagents might stick to dead (permeabilized) cells.
Isotype control antibody instead of primary antibody.	Controls specificity of staining for primary antibody	

4.4. *Analyzing Cells*
FACS

Measurements

Name	Measured parameter
FSC (Forward Scatter)	Size of cell
SSC (Sideward scatter)	Granularity of cell
FL 1–4 (Fluorescence channel 1–4)	Detection of antibodies, fluorescent proteins (eg. GFP), and DNA binding molecules

1: Switch on FACS, check levels of sheath, waste etc. (ask operator)

2: With the unstained cells as sample, set up the following

- Set up dot plot view of FSC vs. SSC adjust amplification levels so that bulk of cells is in window.
- Set gate on population to be measured (Fig. 1)
- Set up histogram view of fluorescence channel(s) to be measured. Adjust fluorescence level of channel(s) to be used, so that the bulk of the cells is in the first decade (Fig. 2)

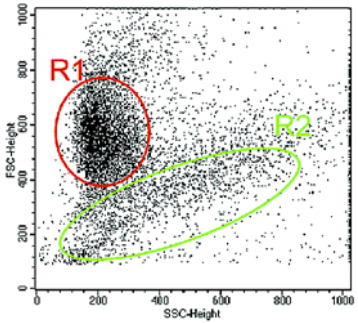


Fig 1

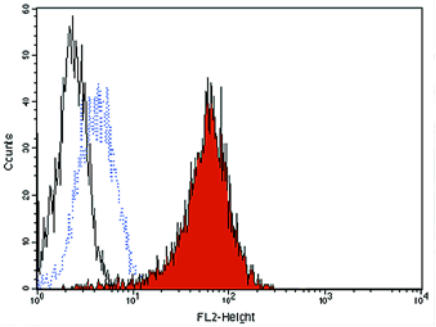


Fig 2

- Fig 1: FSC vs. SSC plot. Intact cells are located in the gate R1 (red). Dead cells and debris are in the gate R2 (green).

- Fig 2: Overlay of measurements of population R1 from Fig.1. Black: Autofluorescence. Blue: Isotype control antibody. Red: Specific antibody.

Troubleshooting

- No signal/no cells visible in FSC/SSC plot:
  Cells lost during staining? Check with a microscope.
  FSC/SSC amplification too high or low (cells are not in the window)?
  Try lowering/raising the amplification.
  Problem with the FACS system. Restart the system.
- No clear population, cells are spread in FSC SSC plot
  Air bubbles are aspirated ? Refill flow path with buffer (´Prime´)
  Cells lost? Check with microscope.

Tips

- Stainings can be conveniently done in 96 well round-bottom plates, for easy handling with a multichannel pipettor.
- Small samples (50 – 200 µl) can be measured in (H3) tubes. Just put one of the small tubes in a larger 'Facs' tube.
- For measurement of small samples the outer needle jacket should be removed to prevent complete sample aspiration.

Notes

4.4. *Analyzing Cells*

Overview: Reporter Gene Assays

REPORTER	👍	👎	CHEMILUMINESCENCE			ELISA			Histology		WESTERN BLOT
			Sensitivity	Material	Time (h)	Sensitivity	Material	Time (h)	Chemical	Immunological	
CAT	non endogeneous activity	narrow linear range	–	–	–	10 pg	E*	4	👍	👍	1 ng/band
β-Gal	bio- and chemical assays	endogeneous activity (mammalian cells)	20 fg	E*	1.5 – 2.5	10 pg	E*	4	👍	👍	1 ng/band
Luc	high specific activity	requires substrate + O_2 + ATP	5 fg – 1 pg	E*	0.5	–	–	–	–	👍	–

* E = Extract

hGH*	secreted – protein	cell lines with defects in the secretary pathway	–	–	–	1 pg	S*	4	–	–	1 ng/ band
SEAP	secreted protein	endogeneous activity in some cells & cell lines with defects in the secretary pathway	10 fg	S*	1	–	–	–	–	–	1 ng/ band
GFP	auto-fluores-cence	requires post-translational modifications	–	–	–	–	–	–	–	–	5 ng/ band

* no cross-reactivity with rat GH

** S = Supernatant

4.4. *Analyzing Cells*

Overview: Reporter Gene Assays

- Reporter systems contribute to the study of eukaryotic gene expression and regulation by joining a promoter sequence to an early detectable "reporter" gene.

- Reporter systems are also used to check transfection efficiency.

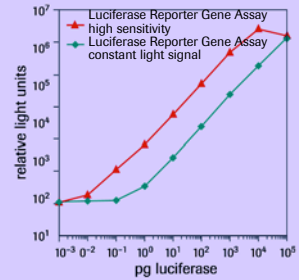
CAT*	Chloramphenicol Acetyltransferase
β-Gal*	β-Galactosidase
Luc*	Luciferase
hGH*	Human Growth Hormon
SEAP*	Secreted Human Placental Alkaline Phosphatase
GFP	Green Fluorescent Protein

* Nonradioactive Reporter Gene Assays available from Roche Applied Science; please visit: **http://www.roche-applied-science.com**

Notes

4.4. *Analyzing Cells*

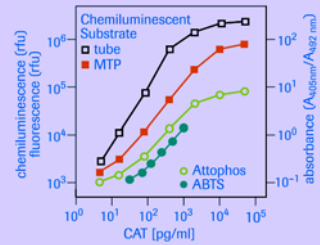
Overview: Reporter Gene Assays

	Method/detection	Advantages	Dynamic range/Sensitivity
Luciferase Reporter Gene Assay, high sensitivity	➢ Quantify enzymatically active luciferase ➢ D-Luciferin/ chemiluminescence	➢ Produces a high intensity light emission ($t_{1/2} \simeq 5$ min) ➢ Optimized lysis buffer for dual reporter genes assays	 Sensitivity comparison of Luciferase Reporter Gene Assays
Luciferase Reporter Gene Assay, constant light signal	➢ Quantify enzymatically active luciferase ➢ Luciferin/chemiluminescence	➢ Produces an extended light emission ($t_{1/2} \simeq 3$h) ➢ Ideal for high throughput screening (HTS) ➢ Optimized lysis buffer for dual reporter genes	
hGH ELISA (secreted human growth hormone)	➢ ELISA for hGH secreted in culture medium ➢ Measure total (active and inactive) hGH ➢ Colorimetric, fluorescent, or chemiluminescent detection	➢ 20 times more sensitive than isotopic hGH assays ➢ No cell lysis required ➢ Study protein expression kinetics over extended periods ➢ Master-lot standardization*	5 ng–400 ng/ml of cell extract

	Method/detection	Advantages	Dynamic range/Sensitivity
β-Gal Reporter Gene Assay, chemiluminescent	➢ Quantify enzymatically active β-Gal ➢ Galacton PlusTM Chemiluminescent Substrate	➢ Highly sensitive ➢ Measures only active enzyme ➢ Extended light emission ($t_{1/2} \simeq 10$ min) ➢ Optimized lysis buffer for dual reporter genes	400 pg–400 pg/ml of cell extract
β-Gal ELISA	➢ ELISA determines total expressed (active and inactive) β-Gal enzyme ➢ Colorimetric, fluorescent, or chemiluminescent detection	➢ Nonradioactive assay ➢ Master-lot standardization* ➢ Specific for bacterial β-glactosidase ➢ Optimized lysis buffer for dual reporter genes	50 pg–1000 pg/ml of cell extract
β-Gal Staining Set	➢ Measures enzymatic activity ➢ Histochemical stain for β-Gal expressing cells ortissue sections	➢ Easy to use. Mix reagents and apply to cells or tissue section	
SEAP Reporter Gene Assay, chemiluminescent (secreted alkaline phosphatase)	➢ Quantify enzymatically active alkaline phosphatase in culture medium ➢ CSPD Chemiluminescent Substrate	➢ No cell lysis required Extended light emission ($t_{1/2} \simeq 1$h) ➢ Assay culture medium for reporter protein ➢ Study protein expression kinetics	500 fg–50 ng/ ml of of cell extract

4.4. *Analyzing Cells*

Overview: Reporter Gene Assays

	Method/detection	Advantages	Dynamic range/Sensitivity
CAT	➢ ELISA determines total expressed (active and inactive) CAT enzyme ➢ Colorimetric, fluorescent, or chemiluminescent detection	➢ Highly sensitive ➢ Master-lot standardization* ➢ Anti-CAT-coated tubes and micro-titer plates available for detection ➢ Optimized lysis buffer for dual reporter genes	200 pg–1000 pg/ml of cell extract  CAT ELISA performed with various detection systems
CAT Staining Set	➢ Measures enzymatic activity ➢ Histochemical stain for CAT expressing cells or tissue sections	➢ Easy to use. Mix reagents and apply to cells or tissue section	

* Master-lot standardized controls and the provided lot specific information enables direct comparison of data from different sets of experiments, even when kits from different production lots are used. As a result, you can compare results from assays run at different times.

All reporter gene assays listed are available from Roche Applied Science.

4.5. *References*

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9. Brochure: FuGENE® HD Transfection Reagent: Cover new ground - one reagent for superior results" (2007), Roche Applied Science

Preparing Buffers and Media

Chapter 5

5

5.1. *Buffers*

Definitions

Term	Symbol	Meaning	Example
Mole	mol	An amount containing Avogadro's* number of whatever units are being considered. Avogadro's* number = 6.023×10^{23}	1 mol H_2O molecules = 6.023×10^{23} H_2O molecules.
Molar or Molarity	mol/liter or M	Concentration of a substance in a liquid. $\text{Molarity} = \frac{\text{moles of substance}}{\text{liter of solution}}$	An 1 M (mol/liter) solution of Tris in H_2O is prepared by dissolving 1 mol Tris molecules in H_2O to a final volume of 1 liter.
Molar weight	g/mol	Weight of 1 mol (= 6.023×10^{23} parts) of a molecule, as defined by the molecular weight of this molecule.	Molecular weight of Tris: 121.1 g/mol. An 1 M (mol/liter) solution of Tris in H_2O is prepared by dissolving 121.1 g Tris in H_2O to a final volume of 1 liter.

Example: How many grams of NaOH are needed to make up a 100 ml solution of 5 M NaOH (= 5 mol/l)?
Formula: Needed grams = Concentration x Molecular weight x Final volume
= 5 mol/liter x 40 g/mol x 0.1 liter
= 20 g

* Named after Amedeo Avogadro (1776–1856), a famous Italian physical chemist.

1gr/100ml = 1%

Precautions

Reagents

- Use the highest possible purity grade of reagents.

H_2O

- Prepare all solutions with fresh double-distilled or deionized H_2O.
- Only use H_2O from a destillation or deionization unit with a resistance value of 18 MOhm/cm.
- Regularly clean the units following instructions from the manufacturers.

Preparing Buffers

- Remove reagents from parent vials by shaking, do not use spatulas.
- Wear gloves.
- Take the water of crystallization into account when calculating the amount of reagent needed to make solutions with a given molarity.
  - *e.g.*, Molecular weight of $MgSO_4 - 7H_2O$
    = 120.37 (MW of $MgSO_4$) + 126.11 (MW of 7 molecules H_2O)
    = 246.48

Sterilizing

- By autoclaving:
  - 0.5 liter solution: ~15 minutes at 121°C at 1 bar
  - 1 liter solution: ~20 minutes at 121°C at 1 bar
- *Caution:* certain buffers (*e.g.*, containing glucose, SDS, β-mercaptoethanol) should **not** be autoclaved.
- Use filtration through an 0.22 µm filter as an alternative.

Precautions (continued)

pH measurement

- Always fix the pH electrode in a vertical position.
- Gently stir the solution when adjusting the pH.
- Never put the electrode in solutions containing SDS.
- Periodically calibrate the electrode with at least two calibration solutions.
- Store the pH electrode in solution, keep the diaphragm wet and make sure that there is always electrolyte solution inside the electrode.
- Clean the pH electrode with distilled water after usage.
- *Caution:* the pH of most solutions is temperature-dependent:
  e.g.,: 5°C: pH of a 0.05 M Tris solution = 8.07
  25°C: pH of a 0.05 M Tris solution = 7.50
  37°C: pH of a 0.05 M Tris solution = 7.22

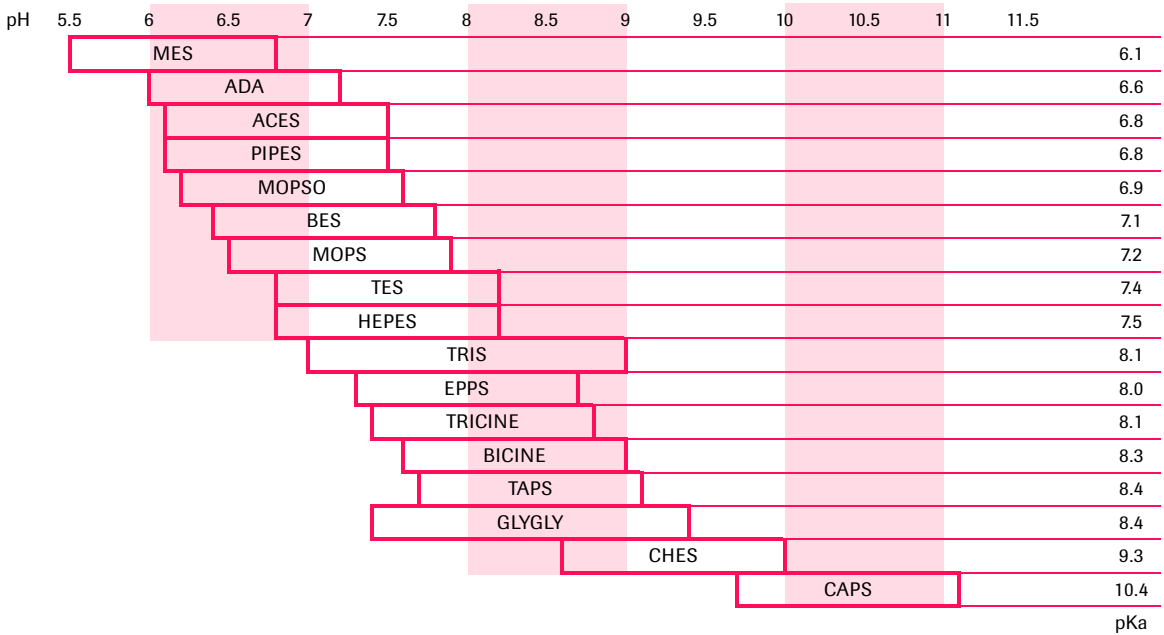
Safety

- Carefully consult the product safety information and material safety data sheets of chemicals.
- Take local safety and laboratory regulations into account when working with chemicals.

Notes

5.1. *Buffers*

Buffering ranges of commonly used buffers (at 20°C)



10 M NH_4OAc – 1 liter

- Dissolve 770.8 g ammonium acetate (NH_4OAc, MW = 77.08) in 800 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

1 M $CaCl_2$ – 1 liter

- Dissolve 219.08 g calcium chloride – $6H_2O$ ($CaCl_2 – 6H_2O$, MW = 219.08) in 800 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

250 x Denhardt Solution – 1 liter

- Dissolve 50 g Ficoll 400, 50 g polyvinylpyrrolidone and 50 g bovine serum albumin (BSA*) in 600 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Divide in aliquots of 25 ml and store at –20°C.

1 M DTT* – 20 ml

- Dissolve 3.085 g 1,4-dithio-DL-threitol (DTT, MW = 154.25) in 20 ml 10 mM sodium acetate (pH 5.2).
- Divide in aliquots of 1 ml and sterilize by filtration.
- Store at –20°C.

* Products available from Roche Applied Science

0.5 M Na_2EDTA (pH 8.0) – 1 liter	➢ Dissolve 186.12 g disodium ethylenediaminetetraacetate – $2H_2O$ (Na_2 EDTA – $2H_2O$, MW = 372.24)) in 800 ml H_2O; stir vigorously on a magnetic stirrer. ➢ Adjust to pH 8.0 with NaOH (~20 g NaOH pellets) and adjust volume to 1 liter with H_2O. ➢ Divide into aliquots and sterilize by autoclaving. ➢ Store at room temperature. ➢ *Note:* the disodium salt of EDTA will only solute when the pH of the solution is adjusted to 8.0 by the addition of NaOH.
0.1 M IPTG* – 50 ml	➢ Dissolve 1.19 g isopropyl ß-D-thiogalactopyranoside (IPTG, MW = 238.3) in 40 ml H_2O. ➢ Adjust volume to 50 ml with H_2O. ➢ Divide in 5 ml aliquots and sterilize by filtration. ➢ Store at –20°C, this solution is stable for 2 – 4 months.
1 M $MgCl_2$* – 1 liter	➢ Dissolve 203.31 g magnesium chloride – $6H_2O$ ($MgCl_2$ – $6H_2O$, MW = 203.31) in 800 ml H_2O. ➢ Adjust volume to 1 liter with H_2O. ➢ Divide in aliquots of 100 ml and sterilize by autoclaving. ➢ Store at room temperature.

10 x MOPS* – 1 liter

- Add 41.85 g 4-morpholinopropanesulfonic acid (MOPS – free acid, MW = 209.27) and 6.80 g sodium acetate – $3H_2O$ (NaOAc – $3H_2O$, MW = 136.08) to 800 ml DEPC treated H_2O and stir until completely dissolved.
- Add 20 ml of a DEPC treated 0.5 M Na_2EDTA solution and adjust pH to 7.0 with 10 M NaOH.
- Adjust volume to 1 liter with DEPC treated H_2O.
- Divide into 200 ml aliquots and store – protected from light – at 4°C.
- If the solution turns yellow, use a new aliquot.

3 M NaOAc – 1 liter

- Dissolve 408.24 g sodium acetate – $3H_2O$ (NaOAc – $3H_2O$, MW = 136.08) in 800 ml H_2O.
- Adjust to pH 5.2 with glacial acetic acid or to pH 7.0 with diluted acetic acid.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

5 M NaCl – 1 liter

- Dissolve 292.2 g sodium chloride (NaCl, MW = 58.44) in 800 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

* Products available from Roche Applied Science

Recipes for stock solutions (continued)

10% SDS* – 1 liter

- Dissolve 100 g sodium dodecyl sulfate crystals (SDS) in 900 ml H_2O.
- Heat to 68°C to solute the crystals.
- Adjust pH to 7.2 with HCl (~50 µl).
- Adjust volume to 1 liter with H_2O.
- Dispense into aliquots and store at room temperature.
- *Note:* the fine crystals of SDS disperse easily, wear a mask when weighing SDS and clean the weighing area and balance after use.
- When SDS crystals precipitate (*e.g.*, due to cold temperature), redissolve by warming the solution at 37°C.

100% (w/v) TCA

- Add 500 g trichloroacetic acid (TCA) to 227 ml H_2O.
- This solution contains 100% (w/v) TCA.

1 M Tris* – 1 liter

- Dissolve 121.14 g tris(hydroxymethyl)aminomethane (Tris, MW = 121.14) in 800 ml H_2O.
- Adjust pH to the desired value by adding concentrated HCl:
  - pH 7.4: ~70 ml
  - pH 7.6: ~60 ml
  - pH 8.0: ~42 ml
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

X-gal* (20 mg/ml) 20 ml	➢ Dissolve 400 mg 5-Bromo-4-Chloro-3-Indolyl-β-D-galactoside (X-gal) in 20 ml N,N'-dimethyl formamide. ➢ Divide into 500 µl aliquots and store in a glass or polypropylene tube protected from light at –20°C. This stock solution is stable for 2 – 4 months.

* Products available from Roche Applied Science

5.1. *Buffers*

Recipes for buffers

10 x PBS* – 1 liter	➢ Dissolve 80 g NaCl, 2 g KCl, 26.8 g Na_2HPO_4 – $7H_2O$ and 2.4 g KH_2PO_4 in 800 ml H_2O. ➢ Adjust to pH 7.4 with HCl. ➢ Adjust volume to 1 liter with H_2O. ➢ Divide in aliquots and sterilize by autoclaving. ➢ Store at room temperature.
20 x SSC* – 1 liter	➢ Dissolve 175.3 g NaCl and 88.2 g sodium citrate – $2H_2O$ in 800 ml H_2O. ➢ Adjust pH to 7.0 with HCl. ➢ Adjust volume to 1 liter with H_2O. ➢ Divide in aliquots and sterilize by autoclaving. ➢ Store at room temperature.
20 x SSPE – 1 liter	➢ Dissolve 175.3 g NaCl, 27.6 g NaH_2PO4 – $1H_2O$ and 7.4 g Na_2EDTA in 800 ml H_2O. ➢ Adjust pH to 7.4 with NaOH (~6.5 ml of a 10 M solution). ➢ Adjust volume to 1 liter with H_2O. ➢ Divide in aliquots and sterilize by autoclaving. ➢ Store at room temperature.

1 x TE – 1 liter

- Add 10 ml 1 M Tris (pH 8.0, 7.6 or 7.4) and 2 ml 0.5 M Na_2EDTA** (pH 8.0) to 800 ml H_2O.
- Mix and adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving.
- Store at room temperature.

* Products available from Roche Applied Science

** For certain applications (*e.g.,* storage of DNA that will be used in PCR or other enzymatic reactions), add 200 µl 0.5 M Na_2EDTA instead of 2 ml.

5.1. *Buffers*

Recipes for buffers with desired pH

0.1 M NaOAc – 100 ml

- **0.2 M acetic acid:**
  - mix 11.55 ml glacial acetic acid in 500 ml H_2O and adjust to 1 liter with H_2O.
- **0.2 M sodium acetate:**
  - dissolve 27.21 g sodium acetate – $3H_2O$ (NaOAc – $3H_2O$, MW = 136.08) in 800 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed to obtain a 100 ml solution of 0.1 M NaOAc with a specific desired pH.

0.1 M KOAc – 100 ml

- **0.2 M acetic acid:**
  - mix 11.55 ml glacial acetic acid in 500 ml H_2O and adjust to 1 liter with H_2O
- **0.2 M potassium acetate:**
  - dissolve 19.62 g potassium acetate (KOAc, MW = 98.14) in 800 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed to obtain a 100 ml solution of 0.1 M KOAc with a specific desired pH.

pH table for acetate buffers[a]

Desired pH[a]	0.2 M acetic acid solution (ml)	0.2 M sodium or potassium acetate solution (ml)	H_2O (ml)
3.6	46.3	3.7	50
3.8	44.0	6.0	50
4.0	41.0	9.0	50
4.2	36.8	13.2	50
4.4	30.5	19.5	50
4.6	25.5	24.5	50
4.8	20.0	30.0	50
5.0	14.8	35.2	50
5.2	10.5	39.5	50
5.4	8.8	41.2	50
5.6	4.8	45.2	50

[a]It is strongly recommended to check the final pH with a pH meter.

0.1 M Na Phosphate 200 ml

- **0.2 M sodium phosphate, mono-sodium salt:**
  - dissolve 27.6 g $NaH_2PO_4 - 1H_2O$ (MW = 138) in 500 ml H_2O and adjust to 1 liter with H_2O
- **0.2 M sodium phosphate, di-sodium salt:**
  - dissolve 53.62 g $Na_2HPO_4 - 7H_2O$ (MW = 268.1) in 500 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed together to obtain a 200 ml solution of 0.1 M Na-phosphate with a specific desired pH.

0.1 M K Phosphate 200 ml

- **0.2 M potassium phosphate, mono-potassium salt:**
  - dissolve 27.2 g KH_2PO_4 (MW = 136.09) in 500 ml H_2O and adjust to 1 liter with H_2O
- **0.2 M potassium phosphate, di-potassium salt:**
  - dissolve 34.8 g K_2HPO_4 (MW = 174.18) in 500 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed together to obtain a 200 ml solution of 0.1 M K-phosphate with a specific desired pH.

pH table for phosphate buffers[a]:

Desired pH[a]	sodium or potassium, phosphate mono salt solution (ml)	sodium or potassium, phosphate di salt solution (ml)	H_2O (ml)
5.7	93.5	6.5	100
5.8	92.0	8.0	100
5.9	90.0	10.0	100
6.0	87.7	12.3	100
6.1	85.0	15.0	100
6.2	81.5	18.5	100
6.3	77.5	22.5	100
6.4	73.5	26.5	100
6.5	68.5	31.5	100
6.6	62.5	37.5	100
6.7	56.5	43.5	100
6.8	51.0	49.0	100

Desired pH[a]	sodium or potassium, phosphate mono salt solution (ml)	sodium or potassium, phosphate di salt solution (ml)	H_2O (ml)
6.9	45.0	55.0	100
7.0	39.0	61.0	100
7.1	33.0	67.0	100
7.2	28.0	72.0	100
7.3	23.0	77.0	100
7.4	19.0	81.0	100
7.5	16.0	84.0	100
7.6	13.0	87.0	100
7.7	10.5	90.5	100
7.8	8.5	91.5	100
7.9	7.0	93.0	100
8.0	5.3	94.7	100

[a] It is strongly recommended to check the final pH with a pH meter.

0.05 M Tris – 100 ml

➢ The table below gives the volumes of 0.1 M Tris buffer, 0.1 M HCl and H_2O that should be mixed together to obtain a 100 ml solution of 0.05 M Tris with a specific desired pH[a]:

pH table for Tris buffers

Desired pH[a]	0.1 M HCl (ml)	0.1 M Tris (ml)	H_2O (ml)
7.3	43.4	50	6.6
7.4	42.0	50	8.0
7.5	40.3	50	9.7
7.6	38.5	50	11.5
7.7	36.6	50	13.4
7.8	34.5	50	15.5
7.9	32.0	50	18.0
8.0	29.2	50	20.8
8.1	26.2	50	23.8
8.2	22.9	50	27.1
8.3	19.9	50	30.1
8.4	17.2	50	32.8
8.5	14.7	50	35.3
8.6	12.4	50	37.6

[a] It is strongly recommended to check the final pH with a pH meter.

10 x agarose gel sample buffer 100 ml	➢ Dissolve 250 mg bromophenol blue and/or 250 mg xylene cyanol in 33 ml 150 mM Tris pH 7.6. ➢ Add 60 ml glycerol and 7 ml H_2O. ➢ Store at room temperature.
10 x TBE* (Tris-borate) 1 liter	➢ Dissolve 108 g Tris and 55 g Boric acid in 900 ml H_2O. ➢ Add 40 ml 0.5 M Na_2EDTA (pH 8.0) and adjust volume to 1 liter with H_2O. ➢ Store at room temperature.
50 x TAE* (Tris-acetate) 1 liter	➢ Dissolve 242 g Tris in 500 ml H_2O. ➢ Add 100 ml 0.5 M Na_2EDTA (pH 8.0) and 57.1 ml glacial acetic acid . ➢ Adjust volume to 1 liter with H_2O. ➢ Store at room temperature.
10 x TPE (Tris-phosphate) 1 liter	➢ Dissolve 108 g Tris in 700 ml H_2O ➢ Add 15.5 ml 85% Phosphoric acid (1.679 g/ml) and 40 ml 0.5 M Na_2EDTA (pH 8.0). ➢ Adjust volume to 1 liter with H_2O ➢ Store at room temperature

* Products available from Roche Applied Science as convenient ready-to-use solutions

5.1. *Buffers*

Electrophoresis of RNA

RNA Sample buffer

- Mix 10 ml deionized formamide, 3.5 ml 37% formaldehyde and 2 ml 5 x MOPS.
- Divide into 500 µl aliquots and store at –20°C.
- The buffer is stable for 6 months.
- Use 2 parts sample buffer for each part of RNA.
- *Note:* Formamide is a teratigen and formaldehyde is a carcinogen. Work in a fumehood and follow laboratory safety procedures.

RNA Loading Buffer

- Prepare in DEPC-treated H_2O: 50% glycerol, 1 mM Na_2EDTA, and 0.4% Bromophenol Blue.
- Use the highest possible grade of glycerol to avoid ribonuclease contamination.
- Divide into 500 µl aliquots and store at –20°C.
- Use 2 µl loading buffer per 10 – 20 µl RNA sample (RNA plus sample buffer).

DEPC-treatment per 100 ml solution

- Add 0.1–0.2 ml diethylpyrocarbonate (DEPC) to 100 ml of a solution (*e.g.*, H_2O).
- Shake vigorously and incubate overnight in a fume hood.
- Autoclave the solution to inactivate the remaining DEPC.
- Store treated solution at room temperature.
- *Note:* Wear gloves and use a fume hood when using DEPC (suspected carcinogen).
- All chemical substances containing amino groups (*e.g.*, Tris, MOPS, EDTA, HEPES, etc.) cannot be treated directly with DEPC. Prepare these solutions in DEPC-treated H_2O.

Ethidium Bromide 100 ml

- Prepare a stock solution of 10 mg/ml by adding 1 g ethidium bromide to 100 ml H_2O. Stir until the dye has complete dissolved.
- Store in the dark at 4°C.
- Staining:
  - During electrophoresis: add 0.5 – 1 µg per ml agarose solution
  - After electrophoresis: add 0.5 – 2 µg per ml staining solution
- *Caution:* Ethidium bromide is a mutagen and is toxic. Wear gloves when working with the solution and wear a mask when dissolving the powder.

SYBR Green I Nucleic Acid Gel Stain*

- Supplied* as a stock solution in DMSO, stable at –20°C for 6 –12 months.
- Working solution: dilute the stock solution 1:10,000 in TE, TBE or TAE buffer.
- Sensitivity: as low as 80 pg per band dsDNA using 312 nm transillumination with the Lumi-Imager F1 System.

Phenol* – 1 liter

- Dissolve 500 g phenol in 500 ml 1 M Tris (pH 8.0).
- Once dissolved, let phases separate and remove upper aqueous phase.
- Add 500 ml 100 mM Tris (pH 8.0); stir to emulsify and let phases separate.

- Repeat procedure with TE until pH of upper, aqueous phase is less than pH 7.2.
- Store at 4°C protected from light.
- Discard when the solution turns red/brown.

Phenol* (acid) – 1 liter

For RNA only!

- Dissolve 500 g phenol in 500 ml 50 mM sodium acetate (pH 4.0).
- Once dissolved, let phases separate and remove upper aqueous phase.
- Add 500 ml 50 mM sodium acetate (pH 4.0); stir to emulsify and let phases separate.
- Repeat procedure until the pH of upper, aqueous phase is less than pH 4.1.
- Store at 4°C protected from light.

Caution: Phenol is highly corrosive and can cause severe burns. Always wear gloves, protective clothing and safety glasses when working with phenol.
If skin comes in contact with phenol, immediately wash with a large volume of water and soap.

* Products available from Roche Applied Science

5.1. *Buffers*

Electrophoresis of Proteins

10% Ammonium persulfate – 10 ml	➢ Dissolve 1 g ammonium persulfate (APS) in 8 ml H_2O. ➢ Adjust volume to 10 ml with H_2O. ➢ Solution is stable at 4°C for two weeks.
30% acryl-bisacryl-amide mix – 100 ml	➢ Dissolve 29 g acrylamide and 1 g N,N'-methylenebisacrylamide in 60 ml H_2O. ➢ Heat the solution to 37°C to dissolve the chemicals. ➢ Adjust volume to 100 ml with H_2O. ➢ Store at 4°C protected from light. ➢ *Caution:* Acrylamide is a neurotoxin and is absorbed through the skin. Always wear gloves and a mask when preparing the solutions.
2 x SDS PAGE sample buffer 100 ml	➢ Mix 10 ml 1.5 M Tris (pH 6.8), 6 ml 20% SDS, 30 ml glycerol, 15 ml β-mercaptoethanol and 1.8 mg bromophenol blue. ➢ Adjust volume to 100 ml with H_2O. ➢ Aliquot in 10 ml stock solution and store at –20°C. ➢ Store working solution at 4°C.

10 x SDS PAGE Running buffer 1 liter	➢ Dissolve 10 g SDS, 30.3 g Tris and 144.1 g glycin in 800 ml H_2O. ➢ Adjust volume to 1 liter with H_2O. ➢ Store at room temperature.
Coomassie Blue staining solution 1 liter	➢ Dissolve 2.5 g "Coomassie Brilliant Blue R-250" in a mixture of 450 ml methanol, 100 ml acetic acid and 400 ml H_2O. ➢ Adjust volume to 1 liter with H_2O. ➢ Store at room temperature.
Coomassie Blue destaining solution 1 liter	➢ Mix 450 ml methanol, 100 ml acetic acid and 400 ml H_2O. ➢ Adjust volume to 1 liter with H_2O. ➢ Store at room temperature.

5.1. *Buffers*

Resolving gels for denaturing SDS – PAGE

% Gel	Components	Volume of components (ml) per gel mold volume of							
		5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
6%	H_2O	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.50
	30% acryl-bisacrylamide mix	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.00
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8%	H_2O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.20
	30% acryl-bisacrylamide mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.30
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	1.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10%	H_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.80
	30% acryl-bisacrylamide mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.70
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12%	H_2O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.50
	30% acryl-bisacrylamide mix	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.00
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15%	H_2O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.50
	30% acryl-bisacrylamide mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.00
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

5% stacking gels for denaturing SDS-PAGE

Components	Volume of components (ml) per gel mold volume of							
	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acryl-bisacrylamide mix	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.5 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium persulfate	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

5.1. *Buffers*

Western Blotting

1x Transfer Buffer for wet blots – 1 liter

- Dissolve 2.9 g Glycine, 5.8 g Tris and 0.37 g SDS in 200 ml methanol.
- Adjust volume to 1 liter with H_2O.
- Store at 4°C.

1x Transfer Buffer for semi-dry blots

- **Scheme:**

Layer	Content and Buffer
5 (top)	3 x 3 MM papers soaked in 300 mM Tris, pH 10.4
4	membrane
3	gel
2	3 x 3 MM papers soaked in 25 mM Tris, pH 9.4 and 40 mM amino hexane acid
1 (bottom)	3 x 3 MM papers soaked in 25 mM Tris, pH 10.4

Ponceau S Solution – 100 ml

- Mix 2 g Ponceau S, 30 g trichloracetic acid and 30 g sulfosalicylic acid in 80 ml H_2O.
- Adjust volume to 100 ml with H_2O.
- Store at room temperature.

1 x TBS (Tris Buffered Saline) – 1 liter	➢ Dissolve 6.05 g Tris (50 mM) and 8.76 g NaCl (150 mM) in 800 ml H_2O. ➢ Adjust pH to 7.5 with 1 M HCl (~9.5 ml). ➢ Adjust volume to 1 liter with H_2O. ➢ TBS is stable at 4°C for three months. ➢ *Note:* Since sodium azide inhibits peroxidase activity, it is not recommended for use as an antimicrobial reagent.
1 x TBST (Tris Buffered Saline Tween) – 1 liter	➢ Dissolve 1 ml Tween 20* in 1 liter TBS buffer. ➢ TBST is stable at 4°C for three months. ➢ Note: Tween 20 is suitable for most applications but depending on the type of membrane and antibodies used, different detergents (like SDS*, Triton X100* or Nonidet P40*) and concentrations between 0.01 – 1% may lead to better results.
Blocking solution*	➢ A wide variety of different blocking solutions are described in the literature. ➢ A solution of 2.5 to 5% (w/v) nonfat dried milk in TBS or PBS is sufficient for most blots. ➢ If background is high, use alternative blocking solutions* containing 1% of *e.g.*, BSA, gelatine or casein.

* Products available from Roche Applied Science

5.2. *Antibiotics*

Selection of prokaryotic cells

Antibiotic	Mode of Action	Mechanism of Resistance	Working Concentration[a]	Stock Solution[b]
Ampicillin *(Amp)*	Inhibits cell wall synthesis by inhibiting formation of the peptidoglycan cross-link.	The resistance gene *(bla)* specifies an enzyme, β-lactamase, which cleaves the β-lactam ring of the antibiotic.	20 – 100 µg/ml (50 µg/ml)	100 mg/ml in H_2O Store at –20°C.
Chloramphenicol *(Cm)*	Prevents peptide bond formation by binding to the 50S subunit of ribosomes.	The resistance gene *(cat)* specifies an acetyltransferase that acetylates and thereby inactivates the antibiotic.	25 – 170 µg/ml (100 µg/ml)	34 mg/ml in Ethanol Store at –20°C.
Kanamycin *(Kan)*	Causes misreading of mRNA by binding to 70S ribosomes.	The resistance gene *(kan)* specifies an aminoglycoside phosphotransferase that inactivates the antibiotic.	10 – 50 µg/ml (30 µg/ml)	30 mg/ml in H_2O Store at –20°C.
Streptomycin *(Sm)*	Causes misreading of mRNA by binding to 30S subunit of ribosomes.	The resistance gene *(str)* specifies an enzyme that modifies the antibiotic and inhibits its binding to the ribosomes.	10 – 125 µg/ml (50 µg/ml)	50 mg/ml in H_2O Store at –20°C.
Tetracycline *(Tet)*	Prevents protein synthesis by preventing binding of the aminoacyl tRNA to the ribosome A site.	The resistance gene *(tet)* specifies a protein that modifies the bacterial membrane and prevents transport of the antibiotic into the cell.	10 – 50 µg/ml (10 µg/ml in liquid culture – 12.5 µg/ml in plates)	12.5 mg/ml in Ethanol Store at –20°C.

[a] lower concentrations: for the selection of plasmids with a low copy number, higher concentrations: for the selection of plasmids with a high copy number, values in brackets indicate commonly used concentrations.
[b] stock solutions in H_2O: sterilize by filtration and store protected from light. Stock solutions in ethanol should not be sterilized.

Selection of eukaryotic cells

Antibiotic	Mode of Action	Mechanism of Resistance	Working Concentration	Stock Solution[a]
Geneticin *(G 418)*	Interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells.	The resistance gene *(neo)* encodes a bacterial aminoglycoside phosphotransferase that inactivates the antibiotic.	50 – 1000 µg/ml; optimal concentration is to be tested experimentally[b]	5 – 50 mg/ml in culture medium or physiological buffers Store at –20°C
Gentamycin	Inhibits protein synthesis by binding to the L6 protein of the 50S ribosomal subunit.	The resistance gene specifies an aminoglycoside phosphotransferase that inactivates the antibiotic.	100 µg/ml	10 – 50 mg/ml solution in H_2O. Store at 4°C
Hygromycin B	Inhibits protein synthesis of bacteria, fungi and eukaryotic cells by interfering with translocation and causing mistranslation.	The resistance gene *(hyg or hph)* codes for a kinase that inactivates Hygromycin B through phosphorylation.	50 – 1000 µg/ml; optimal concentration is to be tested experimentally[b]	50 mg/ml in PBS Store at 4°C

[a] stock solutions in H_2O, culture medium, physiological buffers or PBS: sterilize by filtration, store protected from light. Stock solutions in ethanol should not be sterilized.
[b] a table with optimal cell-line specific concentrations is available from Roche Applied Science.

5.3. *Media for Bacteria*

Recipes for 1 liter

LB

- Mix 10 g Bacto-Tryptone, 5 g Bacto-Yeast extract and 10 g NaCl in 900 ml H_2O.
- Adjust the pH to 7.0 with 10 M NaOH (~ 200 µl).
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

M9 minimal

- Mix 12.8 g Na_2HPO_4 – $7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl in 750 ml H_2O.
- Adjust the pH to 7.4 with 10 M NaOH (~ 100 µl).
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and cool to room temperature.
- Add 2 ml 1 M $MgSO_4$ – $7H_2O$, 0.1 ml 1 M $CaCl_2$ and 10 ml 20% glucose.
- Sterilize by filtration and store at room temperature.

SOB

- Mix 20 g Bacto-Tryptone, 5 g Bacto-Yeast extract, 0.5 g NaCl and 2.5 ml 1 M KCl in 900 ml H_2O.
- Adjust pH to 7.0 with 10 M NaOH (~ 100 µl) and add H_2O to 990 ml.
- Sterilize by autoclaving and store at room temperature.
- Before use, add 10 ml sterile 1 M $MgCl_2$.

SOC	➢ Identical to SOB medium, except that it additionally contains 20 ml sterile 1 M glucose.
TB	➢ Mix 12 g Bacto-Tryptone, 24 g Bacto-Yeast extract and 4 ml glycerol in 900 ml H_2O. ➢ Sterilize by autoclaving and cool to < 60°C. ➢ Add 100 ml of sterile 10 x TB phosphate* and store at room temperature.
YT	➢ Mix 8 g Bacto-Tryptone, 5 g Bacto-Yeast extract and 2.5 g NaCl in 900 ml H_2O. ➢ Adjust pH to 7.0 with 10 M NaOH (~ 200 µl). ➢ Adjust volume to 1 liter with H_2O. ➢ Sterilize by autoclaving and store at room temperature.

* Made by dissolving 2.31 g KH_2PO_4 (= 0.17 M) and 12.54 K_2HPO_4 (= 0.72 M) in 90 ml H_2O. Adjust volume to 100 ml with H_2O and sterilize by autoclaving. Store at room temperature.

5.3. *Media for Bacteria*

Recipes for 1 liter (for ~ 40 plates of 90 mm, ~ 25 ml per plate)

Agar plates

- Prepare liquid media according to the recipes given on page 161.
- Add 15 g Bacto-Agar to 1 liter liquid medium and sterilize by autoclaving.
- Allow the medium to cool to 50°C and add the appropriate amount of antibiotic (see chapter 5.2., page 160, for the best concentration of antibiotic).
- Gently mix the medium by swirling and pour into sterile plates.
- Flame the surface of the medium in the plates with a bunsen burner to remove air bubbles.
- Allow the medium to solidify and store the dishes at 4°C in the inverted position.
- Store tetracycline plates in the dark.
- Use a color code to indicate the type of antibiotic in the plate (*e.g.*, black stripe for ampicilin plates, blue stripe for kanamycin plates, …).
- Before use, incubate plates at 37°C in the inverted position for 1 hour to remove condensation within the plate.

X-gal/IPTG indicator plates

- Before pouring the plates, add:
  - 2 ml of IPTG stock solution (0.1 M) – final concentration: 0.2 mM
  - 2 ml of X-gal stock solution (20 mg/ml) – final concentration: 40 µg/ml

Top agar overlay

- Prepare liquid media according to the recipes given on page 161.
- Add 7 g Bacto-Agar to 1 liter liquid medium and sterilize by autoclaving.

5.4. *References*

1. Ausubel, F. M. *et al.*, (1991) In: Current Protocols in Molecular Biology. John Wiley and Sons, New York
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3. Fluka (1998), MicroSelect: Basics for Biochemistry and Molecular Biology
4. Good, N. E. , Izawa, S. (1972) In: *Methods in Enzymology*, **24**, 53 – 68
5. Roche Applied Science, Technical Data Sheets
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Conversion Tables and Formulas

Chapter 6

6.1. *Nucleotide ambiguity code*

Code	Represents	Complement
A	Adenosine	T
G	Guanine	C
C	Cytosine	G
T	Thymidine	A
Y	Pyrimidine (C & T)	R
R	Purine (A & G)	Y
W	weak (A & T)	W
S	strong (G & C)	S
K	keto (T & G)	M
M	amino (C & A)	K
D	not C	H
V	not T	B
H	not G	D
B	not A	V
X/N	unknown	X/N

6.2. *Formulas to calculate melting temperature T_m*

System[a]	Formula[b]
DNA – DNA hybrids (see reference 6)	➢ $T_m = 81.5°C + 16.6 \log[Na^+] + 0.41$ (%GC) – 0.61 (%for) – 500/N
DNA – RNA hybrids (see reference 7)	➢ $T_m = 79.8°C + 18.5 \log[Na^+] + 0.58$ (%GC) + 11.8 $(\%GC)^2$ – 0.50 (%for) – 820/N
RNA – RNA hybrids (see reference 8)	➢ $T_m = 79.8°C + 18.5 \log[Na^+] + 0.58$ (%GC) + 11.8 $(\%GC)^2$ – 0.35 (%for) – 820/N
Oligonucleotides (see reference 9)	➢ For oligonucleotides 14 – 25 nucleotides in length: ● T_m = [2°C x (number of A and T bases)] + [4°C x (number of G and C bases)] ➢ For Oligonucleotides longer than 25 nucleotides: see formula for DNA/DNA hybrids

[a] The proposed formula is valid for Na^+ concentrations between 0.01 – 0.4 M and %GC values between 30 – 75%
[b] $[Na^+]$ = concentration of Na^+ ions, %GC = percentage of G and C nucleotides in the nucleic acid, %for = percentage of formamide in the hybridisation solution, N = length of the duplex in base pairs

6.3. *%GC content of different genomes*

Organism	%GC content
Phages	
T2	34.6
T3	49.6
T7	47.4
Lambda	48.6
Prokaryotes	
Agrobacterium tumefaciens	58 – 59.7
Bacillus subtilis	42.6
Escherichia coli	51
Mycobacterium tuberculosis	65
Staphylococcus aureus	32.4 – 37.7

Organism	%GC content
Vertebrates	
Homo Sapiens	40.3
Xenopus laevis	40.9
Mus musculus	40.3
Rattus species	41.8

6.4. *Metric prefixes*

Factor	Prefix	Symbol
10^{18}	exa	E
10^{15}	peta	P
10^{12}	tetra	T
10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{-3}	milli	m
10^{-6}	micro	µ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a

1 Å* = 10^{-10} m
10^{-8} cm
10^{-4} µm
10^{-1} nm

* Named after Anders J. Ångström (1814 – 1874), a famous Swedish spectroscopist

Examples: 1 ng (nanogram) = 10^{-9} gram
5 pmol (picomole) = 10^{-12} mol

6.5. *Greek alphabet*

Α	α	alpha
Β	β	beta
Γ	γ	gamma
Δ	δ	delta
Ε	ϵ	epsilon
Ζ	ζ	zeta
Η	η	eta
Θ	ϑ	theta
Ι	ι	iota
Κ	κ	kappa
Λ	λ	lambda
Μ	μ	mu
Ν	ν	nu
Χ	χ	xi
Ο	ο	omicron
Π	π	pi
Ρ	ρ	rho
Σ	σ	sigma
Τ	τ	tau
Υ	υ	upsilon
Φ	ϕ	phi
Χ	χ	chi
Ψ	ψ	psi
Ω	ω	omega

6.6. *Properties of radioisotopes*

Isotope		Half-life[a]	Decay[b]	Maximum range in air (cm)	Protection	Detection[c]	Autoradiography cpm needed overnight	Fluorography cpm needed overnight	Fluorography method
Calcium-45	^{45}Ca	164 d	β	52	acrylic shield	GM, LS			
Carbon-14	^{14}C	5730 y	β	24	acrylic shield	LS,P	2,000	200	PPO impregnated gel (–70°C)
Chromium-51	^{51}Cr	27.7 d	EC		lead shield	LS,P			
Iodine-125	^{125}I	59.6 d	EC		lead shield	GM, LS	100	10	screen (–70°C)
Phosphor-32	^{32}P	14.3 d	β	790	acrylic shield	GM, LS	50	10	screen (–70°C)
Phosphor-33	^{33}P	25.3 d	β	46	acrylic shield	GM, LS	1,000	100 – 200	PPO impregnated gel (–70°C)
Sulphur-35	^{35}S	87.4 d	β	30	acrylic shield	GM	1,000	100 – 200	PPO impregnated gel (–70°C)
Tritium	^{3}H	12.4 y	β	0.6		LS	$>10^7$	3,000	PPO impregnated gel (–70°C)

Units: 1 µCi = 2.2 x 10^6 desintegrations/minute
= 3.7 x 10^4 becquerels (Bq)
1 Becquerel = 1 desintegration/second
Dosis: 1 Gray (Gy) = 100 rad
1 Sievert (Sv) = 100 rem

[a] d = days, y = years
[b] β = beta radiation
EC = electron capture
[c] GM = Geiger-Müller detector
LS = liquid scintillation
P = End window

6.7. *Temperatures and Pressure*

From Centigrade to Fahrenheit	From Fahrenheit to Centigrade
$°F = 32 + (°C \times \frac{9}{5})$	$°C = \frac{5}{9} \times (°F - 32)$

From millibars (mbar) to	Multiply by
➢ Millimeters of mercury (mm Hg)	0.750000
➢ Inches of mercury (inch Hg)	0.039400
➢ Pounds per square inch (psi)	0.014500
➢ Atmospheres (atm)	0.000987
➢ Kilopascals (kPa)	0.100000
➢ Torrs (Torr)	0.750000

6.8. *Centrifugal forces*

link between revolutions/minute, centrifugal force and rotor radius

Mathematical:

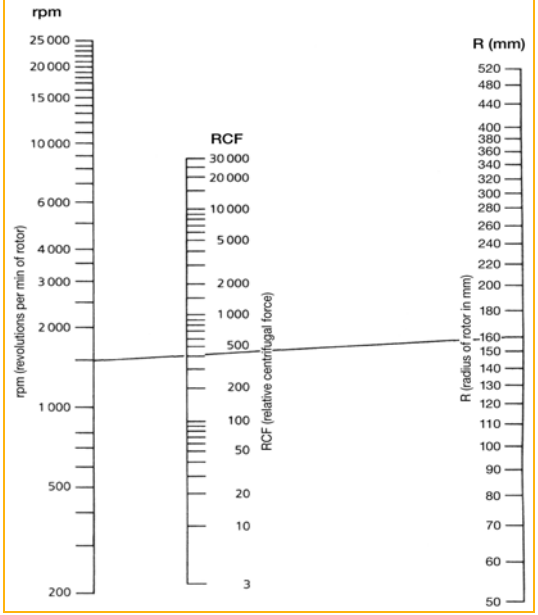
$$rpm = 1000\sqrt{\frac{RCF}{1.12 \times R}}$$

$$RCF = 1.12 \times R\left(\frac{rpm}{1000}\right)^2$$

where
rpm = revolutions per minute of rotor
RCF = relative centrifugal force
R = radius of rotor in mm
at room temperature

Align a straight line through known values in two columns and read desired value in third column

Nomogram:



6.9. *Periodical System of Elements*

I	II											III	IV	V	VI	VII	VIII
1																	2
H																	He
1.0079																	4.0026
3	4											5	6	7	8	9	10
Li	Be											B	C	N	O	F	Ne
6.941	9.0122											10.811	12.011	14.007	15.999	18.998	20.180
11	12											13	14	15	16	17	18
Na	Mg											Al	Si	P	S	Cl	Ar
22.990	24.305	3	4	5	6	7	8	9	10	11	12	26.982	28.086	30.974	32.066	35.453	39.948
19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	Ge	As	Se	Br	Kr
39.098	40.078	44.956	47.88	50.942	51.996	54.938	55.847	58.933	58.69	63.546	65.39	69.723	72.61	74.922	78.96	79.904	83.80
37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54
Rb	Sr	Y	Zr	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd	In	Sn	Sb	Te	I	Xe
85.468	87.62	88.906	91.224	92.906	95.94	[98]	101.07	102.91	105.42	107.87	112.41	114.82	118.71	121.75	127.60	126.90	131.29
55	56	57	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86
Cs	Ba	La	Hf	Ta	W	Re	Os	Ir	Pt	Au	Hg	Tl	Pb	Bi	Po	At	Rn
132.91	137.33	138.91	178.49	180.95	183.85	186.21	190.2	192.22	195.08	196.97	200.59	204.38	207.2	208.98	[209]	[210]	[222]

Transition Metals

Atomic weights are based on $^{12}C = 12$ and conform to the 1987 IUPAC report values rounded to 5 significant digits. Numbers in [ ] indicate the most stable isotope.

6.10. *Hazard Symbols and Risk Phrases*

Symbol	Hazard	Caution
Flammable 	Spontaneously flammable substances. Chemicals igniting in air.	Avoid contact with air.
	Gases, gas mixtures (also liquefied ones) which have an ignition range with air at normal pressure.	Avoid formation of flammable gas-air mixture.
	Substances sensitive to moisture. Chemicals which readily from flammable gases on contact with water.	Avoid contact with moisture water.
	Liquids with flash point below 21°C.	Keep away from open fires, sources of heat and sparks
	Solid substances which ignite easily after a short term effect of a source of ignition.	Avoid all contact with all sources of ignition.
Oxidizing 	Oxidizing substances can ignite combustible material or worsen existing fires and thus make fire-fighting more difficult.	Keep away from combustible material.
Explosive 	This symbol designates substances which may explode under definite conditions.	Avoid shock, friction, sparks, and heat.

Symbol	Hazard	Caution
Harmful or Irritant 	Inhalation and ingestion of, or skin penetration by these substances is harmful to one's health. Non-recurring, recurring, or lengthy exposure to these substances may result in irreversible damage.	Avoid contact with the human body, including inhalation of the vapors and in cases of malaise, consult a doctor.
	This symbol designates substances which may have an irritant effect on skin, eyes, and respiratory organs.	Do not breathe vapors and avoid contact with skin and eyes.
Corrosive 	Living tissue as well as equipment are destroyed on contact with these chemicals.	Do not breathe vapors and avoid contact with skin, eyes, and clothing.
Toxic 	The substances are very hazardous to health when breathed, swallowed, or in contact with the skin and may even lead to death. Non-recurring, recurring, or lengthy exposure to these substances may result in irreversible damage.	Avoid contact with the human body and immediately consult a doctor in cases of malaise.

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Addresses, Abbreviations and Index

Chapter 7

7.1. *Selection of useful addresses**

Name	Internet address	Contains information on
Arabidopsis	http://arabi4.agr.hokudai.ac.jp/Links/Links.html#At_genome	➢ Genomic and related data, physical and genetic maps of Arabidopsis thaliana
ATCC	http://www.atcc.org	➢ American type culture collection
Biochemical Pathways	http://www.expasy.ch/tools/pathways/	➢ The internet version of the traditional Boehringer Mannheim wall chart.
BioCyc	http://biocyc.org/	➢ BioCyc is a collection of 260 Pathway/Genome Databases.
CATH	http://www.biochem.ucl.ac.uk/bsm/cath	➢ Hierarchical domain classification of protein structures
dbEST	http://www.ncbi.nlm.nih.gov/dbEST/index.html	➢ Sequence data and other information on single pass cDNA sequences or expressed sequence tags from a number of organisms
EcoCyc	http://ecocyc.org/	➢ Encyclopedia of E.coli genes and metabolism
ENTREZ	http://www.ncbi.nlm.nih.gov/Entrez	➢ PubMed/MEDLINE ➢ Protein DB ➢ Nucleotide DB ➢ NM3D (data from crystallographic and NMR structural determinations)
Flybase	http://flybase.bio.indiana.edu/	➢ Database of the Drosophila Genome

Name	Internet address	Contains information on
GDB	http://www.gdb.org	➢ Location of human genes, DNA fragments, fragile sites and breakpoints ➢ Populations, polymorphisms, maps and mutations
Genbank	http://www.ncbi.nlm.nih.gov/genbank	➢ Genetic sequence database automatically collected of publicly available sequences
KEGG/ LIGAND	http://www.genome.ad.jp/kegg/kegg.html http://www.genome.ad.jp/dbget/ligand.html	➢ Enzyme reactions ➢ Chemical compounds ➢ Metabolic pathways with links to gene catalogues.
MGD	http://www.informatics.jax.org	➢ Genetics of laboratory mouse.
Omim	http://www.ncbi.nlm.nih.gov/omim/	➢ Human genes and genetic disorders
PDB	http://www.rcsb.org/pdb/	➢ 3D coordinates of macromolecular structures.
REBASE	http://rebase.neb.com	➢ Restriction enzymes and methylases

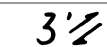
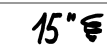
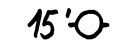
* Web sites continually appear, disappear and change addresses, quickly invalidating any list of “useful” sites. All of the web sites given here were operating in July, 2007.

7.1. *Selection of useful addresses** (continued)

Name	Internet address	Contains information on
Restriction Enzymes	http://www.restriction-enzymes.com	➢ Tools for Mapping & Cloning
Roche Applied Science	http://www.roche-applied-science.com	➢ total product portfolio, online technical support and e-shop available from Roche Applied Science.
SCOP	http://scop.mrc-lmb.cam.ac.uk/scop	➢ Structural classification of protein domains
SWIS-PROT	http://www.expasy.ch/sprot	➢ Protein sequences obtained or translated from nucleotide sequences, including descriptions of protein functions, domain structure, posttranslational modifications, …
Universal ProbeLibrary	http://www.universalprobelibrary.com	➢ Design real-time qPCR assays in seconds.
Yeast	http://mips.gsf.de/proj/yeast/	➢ Comprehensive Yeast Genome Database, annotated complete DNA sequence of Saccharomyces cerevisiae

For information on	Web address
Gateway to North American Libraries	http://lcweb.loc.gov/z3950/gateway.html
PCR and multiplex PCR: guide and troubleshooting	http://www.roche-applied-science.com

* Web sites continually appear, disappear and change addresses, quickly invalidating any list of “useful” sites. All of the web sites given here were operating in July, 2007.

Symbol	Explanation	Example	Translation
	centrifuge		spin at 13 000 g-force
	mix “over head”		mix 5–times “over head”
	shake		shake for 3 minutes
	vortex		vortex for 15 seconds
	rotate / mixing by turn over		rotate the sample for 15 minutes
	do for (time) at (number) °C		incubate for 5 min at 4°C
	next step		
	add		add 5 ml buffer A
	discard		
	divide into		
	digest		digest pUC19 with BamH I

Symbol	Explanation	Example	Translation
ppt	precipitate / pelletate		
PPT	pellet	PPT +5ml (A)	redisolve pellet in 5 ml buffer A
Sup	supernatant		
○	buffer / solution	(B)	buffer B
⊗	interrupt		
●	stop		
Ab. mAb	(monoclonal) antibody	Ab ∝ HIS	antibody against HIS
∝	anti	2nd ∝ POD	2nd anti mouse POD
	mouse		rabbit anti mouse
	rabbit	sheep ∝	sheep anti rabbit
∅ ⊕	negative/positive	Rx5 ∅	reaction #5 is negative

7.3. *Abbreviations*

A

A	adenine or adenosine
A_{260} unit	Absorbance unit (≈ 50 µg for double-stranded DNA)
AAS	Atomic absorption spectrophotometry
Ab	antibody
ABTS	2,2-Azino-di-[3-ethylbenzthiazoline sulfonate (6)]
Acetyl-CoA	Acetyl-coenzyme A
ADA	Adenosine deaminase
ADH	Alcohol dehydrogenase
ADP	Adenosine-5'-diphosphate
Agarose LE	Agarose low electroendosmosis
Agarose LM-MP	Low melting point multi purpose agarose
Agarose MP	Multi purpose agarose
AIDS	Acquired immunodeficiency syndrome
Ala	L-Alanine
AMP	adenosine monophosphate
AMV	Avian myeloblastosis virus
AOD	Amino acid oxidase
AP	Akaline phosphatase
APMSF	(4-Amidinophenyl)-methane-sulfonyl fluoride
Arg	L-Arginine
Arg-C	Endoproteinase, arginine-specific
Asn	L-Asparagine
Asp	L-Aspartic acid
ATP	Adenosine-5'-triphosphate

B

BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
bp	Base pair(s)
Bq	Becquerel
Br-UTP	5-Bromo-uridine-5'-triphosphate
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumin

C

C	cytosine or cytidine
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate
Ci	Curie
CK	Creatine kinase
CL	Citrate lyase
CMC	Critical micellar concentration
CMP	Cyclic monophosphate
CoA	Coenzyme A
cpm	Counts per minute
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)-tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate
CTP	Cytidine-5'-triphosphate
Cys	L-Cysteine
cyt-c	Cytochrome c

D

dADP	2'-Deoxyadenosine-5'-diphosphate
dAMP	2'-Deoxyadenosine-5'-monophosphate
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride
dATP	2'-Deoxyadenosine-5'-triphosphate
dCDP	2'-Deoxycytidine-5'-diphosphate
dCTP	2'-Deoxycytidine-5'-triphosphate
ddATP	2',3'-Dideoxyadenosine-5'-triphosphate
ddCTP	2',3'-Dideoxycytidine-5'-triphosphate
ddGTP	2',3'-Dideoxyguanosine-5'-triphosphate
ddNTP	Dideoxynucleoside triphosphate
ddTTP	2',3'-Dideoxythymidine-5'-triphosphate
ddUTP	2',3'-Dideoxyuridine-5'-triphosphate
dCDP	2'-Deoxyguanosine-5'-diphosphate
dGMP	2'-Deoxyguanosine-5'-monophosphate
dGTP	2'-Deoxyguanosine-5'-triphosphate
DIG	Digoxigenin
DIN	German standards
dITP	2'-Deoxyinosine-5'-triphosphate
DL	Dosis lethal
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
dpm	Decays per minute
ds	Double-stranded (DNA)
dTDP	Thymidine-5'-diphosphate
DTE	1,4-Dithioerythritol
dTMP	Thymidine-5'-monophosphate
DTT	1,4-Dithiothreitol
dTTP	Thymidine-5'-triphosphate
dU	Deoxy-uridine
dUTP	2'-Deoxy-uridine-5'-triphosphate

E

ϵ	Absorptivity (absorption coefficient)
E. coli	*Escherichia coli*
EC	Enzyme classification (enzyme commission) number
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol-bis-(2-aminoethylether)-N,N,N',N' tetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunoabsorbent assay
EPO	Erythropoietin

F

$F(ab')_2$	Variable sequence fragment of immunoglobulin, generated by pepsin
Fab	Variable sequence fragment of immunoglobulin, generated by papain
FACS	Fluorescence-activated cell sorting
FAD	Flavin adenine dinucleotide

FC	Flow cytometry
FITC	Fluorescein isothiocyanate
FLUOS	5(6)-Carboxyfluorescein-N-hydroxysuccinimide ester
FMN	Flavin mononudeotide
fmol	femto mol
$FRET^{+}$	Fluorescence Resonance Energy Transfer

G

G	guanine or guanosine
β-Gal	β-Galactosidase
GDP	Guanosine-5'-diphosphate
Gln	L-Glutamine
Glu	L-Glutamic acid
Glu-C	Endoproteinase, glutamic acid specific
Gly	L-Glycine
GMP	Guanosine-5'-monophosphate
GTP	Guanosine-5'-triphosphate

H

Hepes	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
Hg	Mercury
His	L-Histidine
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase

I

Ig	Immunoglobulin
IH	Immunohistochemistry
IHC/ICC	Immunohisto/cytochemistry
IL	Interleukin
IL-2R	Interleukin 2-receptor
Ile	L-Isoleucine
INT	2-(4-Iodophenyl)-5-(4-nitrophenyl)-3-phenyl-tetrazolium chloride
IR	Infrared spectroscopy
ISH	*In situ* hybridization
IU	International unit

K

kbp	Kilobase pair(s)
kBq	Kilobecquerel
kD	Kilo Dalton

L

LD	Lethal dosis
Leu	L-Leucine
Ile	L-Isoleucine
Lys	L-Lysine
Lys-C	Endoproteinase, lysine-specific

M

β-ME	β-Mercaptoethanol
MBq	Megabecquerel
Mes	4-Morpholineethanesulfonic acid
Met	L-Methionine
mg	milligram (10^{-3} g)
µg	microgramm (10^{-6} g)
µl	microliter (10^{-6} L)
µM	micromolar (10^{-6} M)

ml	milliliter (10^{-3} L)
mm	millimeter (10^{-3} m)
mM	millimolar (10^{-3} M)
mRNA	Messenger RNA
MTP	Microtiter plate
MW	molecular weight

N

NBT	4-Nitro blue tetrazolium chloride
NC	Nitrocellulose
nm	Nanometer
nmol	nanemole (10^{-9} mole)

O

ONPG	2-Nitrophenyl-β-O-galactopyranoside

P

PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PFGE	Pulsed field gel electrophoresis
pg	picogram (10^{-12} g)
Phe	L-Phenylalanine
pI	Isoelectric point
pmol	picomole (10^{-12} mole)
PMSF	Phenyl-methyl-sulfonyl fluoride
POD	Peroxidase
ppm	Parts per million
Pro	L-Proline
PVDF	Polyvinylidene-difluoride

R

RACE	Rapid amplification of cDNA ends
RARE	recA-assisted restriction endonuclease cleavage
RE	Restriction enzyme
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase

S

SDS-PAGE	Sodium dodecyl sulfate polyacrylamid gel electrophoresis
SDS	Sodium dodecyl sulfate
Ser	L-Serine
ss	Single-stranded (DNA)

T

T	thymine or thymidine
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBq	Terabecquerel
TCA	Trichloroacetic acid
TEA	Triethanolamine
Thr	L-Threonine

T_M	Melting temperature
TMB	Tetramythylbenzidine
TPCK	N-Tosyl-L-phenyl-alanine-chloromethyl ketone
Tris	Tris(hydroxymethyl)-amino-methane
tRNA	Transfer ribonucleic acid
Tyr	L-Tyrosine

U

U	Unit (of enzyme activity)
U	uracil or uridine
UDP	Uridine-5'-diphosphate
UTP	Uridine-5'-triphosphate
UV	Ultraviolet

V

v/v	Volume/volume
Val	L-Valine

W

w/v	Weight/volume
WB	Western (protein) blots

X

X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid
X-phosphate	5-Bromo-4-chloro-3-indolyl-phosphate
XTT	Sodium 3'-[1-(phenyl-amino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)-benzene solfonic acid hydrate

Y

YAC	Yeast Artificial Chromosome

Notes

7.4. *Alphabetical index*

The colors of the topics refer to the color code of the respective chapter.

A

B

C

D

E

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G

H

I

K

L

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R

S

T

U

W

X

Y

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