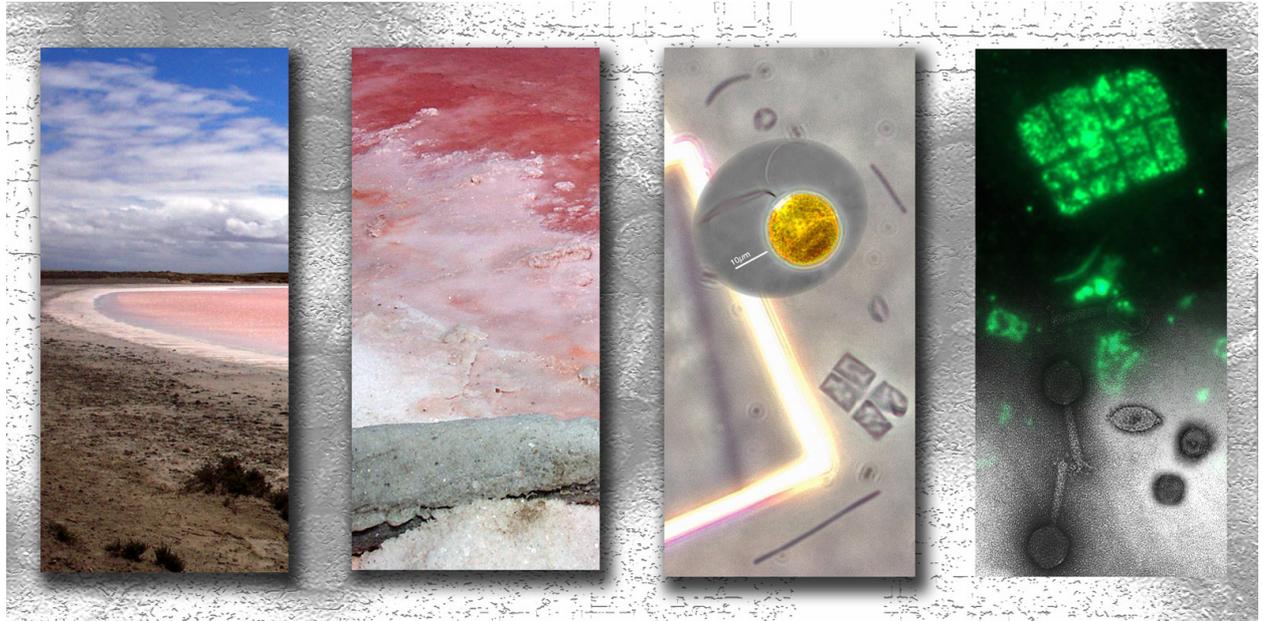


The Halohandbook



Protocols for haloarchaeal genetics[©]

Don't Panic. This document has a wide range of methods for haloarchaea and their viruses.

Version 7.2, March 2009

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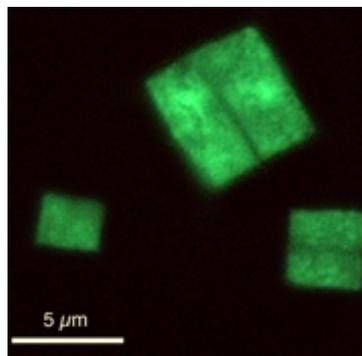


A typical salt lake in southern Australia. MDS, 2004

Contributors

Below are the people who have helped make this book so useful. Sources are quoted where possible. Please join this list by contributing your favourite method ! Address details of some contributors are just before the references, but google searches should get many anyway.

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Any comments or suggestions welcome!

*Square haloarchaeon of Walsby (SHOW organism, strain C23), grown for the first time in 2004, 25 years after their discovery by Prof. Tony Walsby (Walsby, 1980). The name of the type strain is *Haloquadratum walsbyi* C23^T*

TABLE OF CONTENTS

TABLE OF CONTENTS	3
1.0 Introduction to halobacteria	6
<i>Haloarchaeal Journals:</i>	10
2.0 Media Formulae and Cultivation of Haloarchaea	11
2.1 Artificial Seawater (3.6% salt)	11
2.2 Concentrated Salt Water (SW) stock solution - 30% (w/v).....	12
2.3 Modified Growth Medium (MGM) for Haloarchaea.....	14
2.4 CDM - Minimal Medium for Haloarchaea (Kauri et al., 1990).....	16
2.5 OTHER PUBLISHED (or unpublished!) MEDIA FORMULATIONS.....	18
2.7 Solutions for Media.....	33
2.8 Cultivation of Haloarchaea	36
3.0 Transformation Methods	41
3.1 Preparation of Competent Frozen Haloarchaeal cells	41
3.2 Transformation Protocols (MDS Lab).....	43
SUMMARY OF HALOARCHAEAL TRANSFORMATION PROTOCOL.....	46
3.2.4 Freeze-Thaw transformation method.....	51
3.2.5 Selectable markers and plasmid replicons:	52
3.3 Transformation Solutions - MDS Lab.....	53
3.3.7 Transformation Top Agar	57
3.3.8 Minimal Medium (MM) Transformation-Top Agar for plating transformation mixtures	58
3.4 <i>Haloferax volcanii</i> Transformation - Thorsten Allers Laboratory	59
3.5 Transposon Mutagenesis of <i>Haloarcula hispanica</i>	63
3.6 Natural Genetic Transfer (method of Tchelet and Mevarech, 1994).	64
4.0 DNA and RNA Extraction Procedures	65
4.1 Plasmid DNA	65
4.2.1 <i>Hfx. volcanii</i> Genomic DNA Miniprep by DNA Spooling.....	67
4.2.2 Chromosomal DNA isolation (rapid, small scale method)	69
4.2.3 Alternative rapid chromosomal DNA isolation	70
4.2.3 <i>Haloferax volcanii</i> DNA Prep in Agarose Plugs for PFGE	71
4.2.4 PCR of haloarchaeal DNA. Recommended enzymes and conditions	73
4.3 RNA isolation and northern blotting.....	74
4.3.1 RNA isolation using a commercial kit	74
4.3.2 RNA isolation using a manual method.....	74
5.0 Haloarchaeal Viruses (Haloviruses, Halophages)	78
5.2 HF1 and HF2 - examples of lytic, head-tail haloviruses.....	79

5.2.1 Haloviruses HF1 and HF2: large scale growth and purification	80
5.3 Salterproviruses His1 and His2: examples of spindle-shaped haloviruses	83
5.3.3 His1 and His2: Large scale growth and purification	83
5.4.3 Purification of halovirus SH1	86
5.5 Titrations of Haloviruses	86
5.5.1 Simple Method of titrating virus.	86
5.5.2 Standard plate titrations.	87
5.6 Virus DNA Preparation	88
5.6.1 SOLUTIONS	89
5.6.2 HVD. Used for dilution and storage of haloviruses	90
5.7 Notes on plaque assays and virus growth in halobacteria.	90
6.0 Whole Cell Methods	91
6.1 Measuring cell growth using Absorbance (optical density)	91
6.2.1 Making a cell lysate of <i>Hfx. volcanii</i> for enzyme assays.....	92
6.2.2 Cell Free Extract (CFE) of <i>Hbt. salinarum</i>	93
6.3 Making Gene Knock-Out Mutants of <i>Hfx. volcanii</i>	95
6.3.1 Characteristics and requirements of <i>Hfx. volcanii</i> mutants	96
6.3.2 Plasmids used with <i>Hfx. volcanii</i> mutants	96
6.3.3 Diagram of plasmid-chromosome recombination and selection method for creating mutants.....	97
6.4 Restriction Enzyme Activity of <i>Hbt. salinarum</i>	98
.....	98
6.5 Reporter genes in haloarchaea.....	99
6.5.0 Measuring haloarchaeal β -galactosidase activity.....	99
6.5.1 Protocol for β -galactosidase assays in Halobacteria.....	99
6.5.2 Strain purification and growth of <i>Hfx. volcanii</i> DS52 transformants (<i>bgaH</i> ⁺)	99
6.5.3 Assay for <i>BgaH</i> specific activity in <i>Hfx. volcanii</i> strain DS52 (<i>radA</i> ⁻)	100
6.6 Pulse-chase and Immunoprecipitations with <i>Hfx. volcanii</i>	105
6.6.1 Purification of tagged <i>Haloferax volcanii</i> proteins	109
6.6.2 Purification of His-tagged membrane proteins by Ni-NTA metal affinity chromatography	109
6.6.3 Purification of His-tagged cytoplasmic proteins by Ni-NTA metal affinity chromatography	110
6.7 EMS mutagenesis of <i>Hfx. volcanii</i>	112
6.8.1 Concanavalin A-Sepharose binding	113
6.8.2 Preparation of inverted membrane vesicles (IMVs)	114
6.83 Menadione-dependent NADH dehydrogenase activity	115
6.9 Storage of halobacteria.....	117
6.9.2 Storage at -80°C	118
6.9.3 L-Drying (liquid drying)	118
7.0 Reagents, Solutions, Drugs	120
7.1 Purification of Polyethylene Glycol PEG600 for transformations.....	120
7.2 DRUGS for use media.....	122
7.3 Molecular Weights of salts and buffers commonly used in media etc.	123

7.4 Codon usage for halobacteria	124
8.0 Microscopy	125
8.1.1 Fixing and Staining halobacteria for light microscopic examination	125
8.1.2 Phase contrast light microscopic examination on agarose coated slides.	126
8.2 Fluorescent in-situ hybridisation (FISH).....	128
8.2.1 FISH ON FILTERS	128
8.2.2 FISH ON SLIDES	132
8.2.3 Reagents, solutions, etc. for FISH	133
8.3 Acridine Orange Fluorescent staining of halobacteria	134
8.4 Direct counts of halobacteria using a standard (Neubauer) haemocytometer.....	135
8.4 Electronmicroscopy of halobacteria and halovirus particles.....	136
8.4.1 A simple negative-staining method.	137
9.0Contributors, Phylogenetic Tree, and References.....	138
Phylogenetic reconstruction of Halobacteriaceae	139
10. REFERENCES	140

1.0 Introduction to halobacteria

Natural habitats. The red colour of salt lakes is mainly due to the red carotenoid pigments of haloarchaea (i.e. bacterioruberins).

This is also very evident in the crystallizer ponds of solar salterns, where seawater is evaporated to make salt. At near saturating salinities (~ 35% w/v), these organisms grow to high densities (~ 10⁸ cells/ml) but have slow growth rates (doubling times > 2 days)



(Pedros-Alio et al., 2000). Red algae, like *Dunaliella salina*, usually only provide a small amount of colour (Oren et al., 1992). Few other organisms can survive at such high salt concentrations, which typically range from 15% w/v total salt, up to saturation (approximately 35%), and few (if any) protist grazers are found at the highest concentrations. Seawater is about 3.5% total salt, mainly sodium chloride, but halobacteria can only grow from about 12% salt, and most require 20 – 25% for good growth (i.e. about 6 – 7 fold concentrated seawater).

[picture above right: My son Adrian, on a pink lake in South Australia, 2001.]

Moderately halophilic haloarchaea have been detected by 16S rRNA sequencing in saltern ponds of around 8% salt (F. Rodriguez-Valera, personal communication), and recently a study has isolated haloarchaea that grow optimally around 10% salt (Purdy et al., 2004). As of early 2006 they have not been formally classified or their metabolic properties published.

Most genera are found in neutral salt lakes and salterns but salt lakes exist with low and high pH, or with high or low Mg or Ca. Haloalkiliphilic haloarchaea are found in highly alkaline soda lakes (e.g. Wadi Natrun in Egypt, which has a pH around 10)(Grant et al., 1999; Kamekura et al., 1997). Some halobacteria have been isolated from beach sand (*Natrialba*), salty soils (*Htg. distributum*) or deep, and essentially pristine, salt mines (*Hsx. carlsbadense*). Most laboratory studies have used members of only three genera, *Halobacterium*, *Haloferax*, and *Haloarcula*, and so most of the methods in this manual relate to these organisms. There are likely to be many more genera discovered as more sites are examined. For a good review of extremely halophilic *Archaea* and *Bacteria*, see Oren (Oren, 2002).

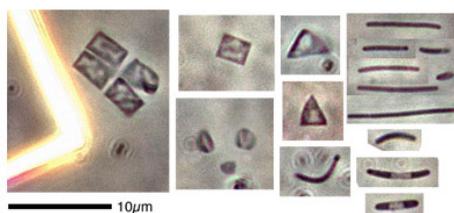
Taxonomy For the latest review of this topic, see Bergey's Manual, 2nd ed (2001) (Grant et al., 2001) and Oren (Oren, 2001). For the most up-to-date account of taxa, see the on-line web site (<http://www.the-icsp.org/taxa/halobacterlist.htm>). The names 'halobacteria' and 'haloarchaea' are generic epithets applied to members of the Class *Halomebacteria*, Order *Halobacteriales*, Family *Halobacteriaceae*. All are extremely halophilic Archaea. *Halobacteriaceae* now includes about 20 genera (see the tree at the end of this manual).

3-letter Genus abbreviations were decided in 1999 (Oren and Ventosa, 2000) and up-to-date descriptions can be found at the following URL (<http://www.bacterio.cict.fr/abbreviations.html>). The current abbreviations are: '*Haloalkilicoccus*' (*Hac.*), *Halogeometricum* (*Hgm.*), *Haloterrigena* (*Htg.*),

Natronomonas (Nmn.), *Haloarcula* (Har.), *Halomicrobium* (Hmc.), *Halovivax* (Hvx), *Natronorubrum* (Nrr.), *Halobacterium* (Hbt.), 'Haloquadratum' (Hqr.), *Natrialba* (Nab.), *Halobaculum* (Hbl.), *Halorhabdus* (Hrd.), *Natrinema* (Nnm.), *Halobiforma* (Hbf.), *Halorubrum* (Hrr.), *Natronobacterium* (Nbt.), *Halococcus* (Hcc.), *Halosimplex* (Hsx.), *Natronococcus* (Ncc.), *Haloferax* (Hfx.), *Halostagnicola* (Hst), *Natronolimnobius* (Nln.),

Physiology. All haloarchaea are either strict aerobes or facultative anaerobes, and have relatively long generation times in laboratory media, e.g. 3 – 4 hours for *Haloferax volcanii*; 8 – 12 hours for *Halobacterium* spp. (MGM, 37°C). They are chemoorganotrophs and grow on complex media (peptone or yeast extract). Many species can grow in defined media with a simple sugar as a carbon/energy source (e.g. *Haloferax* and *Haloarcula*). Some species can produce bacteriorhodopsin and use sunlight as an energy source, but expression of this protein is usually limited to high light and low oxygen conditions. Haloarchaea are true halophiles, and are completely adapted to high salinities. They are one of only a very few known organisms that accumulate K⁺ intracellularly (as KCl) to balance the external Na⁺ concentration (yes, molar concentrations!), and this is maintained against a large concentration gradient as the external K⁺ concentration is normally much lower in natural environments (e.g. 0.05M) than Na⁺.

Cell shape and cell wall structure Halobacteria are prokaryotic and, excepting halococci, are unusual in

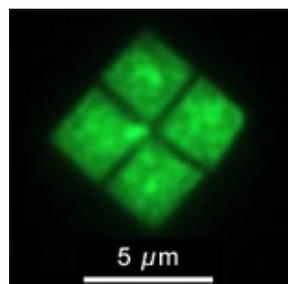


not having a rigid cell wall but only a single layer of (glyco)protein; the so called surface-layer or S-layer. The protein subunits are held together by divalent cations (probably Mg²⁺), and the layer can be completely removed by treating cells with chelating agents, such as EDTA. Due to the fragile nature of their

S-layer cell walls, haloarchaea are easily lysed by trace amounts of detergents so all glassware should be thoroughly rinsed in distilled water before use in preparing media. S-layers do not provide a rigid shape to cells, and they can vary considerably – even in a pure culture! Many are rod shaped, but discs, cocci, triangles (and flat 'geometric' shapes), long flat rods, and thin squares are common. (Grant et al., 2001)

Collage of cell morphological types found in salt lake water. Squares, discs, triangles, and rods are seen. MDS.

The **dominant cell type** found in crystallizer ponds is the square haloarchaeon of Walsby (SHOW group,



see picture at left), and has recently been cultured. The first publication was from this laboratory (Burns et al., 2004b) and then by (Bolhuis et al., 2004). *Haloarcula* spp. are often geometrically shaped with square or triangular forms. *Haloferax* spp. are often a thin, cup-shape (described as like a 'thick potato chip'). The cell shape can vary depending on the growth conditions. Gram staining should be done after first fixing the cells (e.g. in 2% acetic acid; see Microscopy section), otherwise they end up looking like misshapen balloons.

Those haloarchaea with only S-layers will be weakly Gram negative, while the halococci will stain Gram positive, because of the thick polysaccharide cell wall. Flagella are found on some species (e.g. *Halobacterium salinarum*, *Haloarcula hispanica*) but it should be remembered that archaeal flagella are

not biochemically similar to the flagella of bacteria. They might look and act similarly but at the molecular level they are completely different (Cohen-Krausz and Trachtenberg, 2002).

Growth conditions (see also sections below on growth media, particularly **section 2.6.5**)

Salt. The optimum salt concentration for growth varies between different halobacteria (ranging from about 15% to 25%), but most will grow at 23% (w/v, total salt). Typically, strains with low optima will grow quite well at higher salt, but strains with high optima will not grow at concentrations below 20% salt. For example, *Hfx. volcanii* grows well at even 15% (opt. around 18%), whereas *Har. hispanica* needs at least 23% for good growth and grows very poorly at 18%. Using a compromise concentration is convenient where a number of strains are being used in the same laboratory, and we have found 23% to be suitable for most. Since all media have high concentrations of sodium chloride and magnesium salts, bulk quantities of these should be obtained, preventing batch variations and inconvenience when supplies run out. We use AR grade salts. In addition, autoclaving mineral salts media may result in precipitation of some components so special care must be taken.

Temperature. An excellent study has recently examined the temperature optima of 14 type species of haloarchaea (Robinson et al., 2005). Given that many haloarchaea have been isolated from natural salt lakes where they are exposed to high temperatures, it is not surprising that **maximum growth rates for most species occurred at temperatures between 49 – 58°C**. Even the Antarctic isolate, *Halorubrum lacusprofundi*, grows optimally at about 30°C. Interestingly, the maximum survival temperature for *Hfx. volcanii*, is quite high, around 60°C (and is usually grown at 45°C for routing, rapid growth)

While most laboratories have access to a 37°C incubator, and this can conveniently be used for both *E. coli* and most haloarchaeal strains, there is a great advantage in using higher temperatures for haloarchaea, as 42–45°C may provide double the growth rate. Practically speaking, this can give you colonies in 2 days instead of 4–5. Drying of agar plates must be avoided, as the high salt, raised temperature and extended incubation times can rapidly cause evaporation to the point of salt crystallization. As the plates dry, the salt concentration increases, which usually slows the growth of haloarchaea. To prevent this, plates should be wrapped in plastic, or kept in sealed plastic containers. The supermarket is a good place to get these. See also the temperature curves of Thorsten Allers, section **2.6.1** (below). Make sure plates are incubated in the conventional upside-down position; expressed water then falls and collects on the lids rather than smearing over the agar surface.

Aeration. Oxygen supply is a problem in high salt solutions, as oxygen solubility decreases with increasing salt, especially at raised temperature. This is offset by the relatively slow growth rates of halobacteria, and their efficient terminal oxidase. For routine growth in liquid culture use a rotary shaker at around 200 rpm, and a ratio of liquid volume to the container volume of at least 1/3 (e.g. 50ml in a 150 ml flask) or better.

Anaerobic growth. Some strains are capable of fermentative growth (e.g. supposedly *Har. vallismortis*) and some can also use nitrate as an alternative electron acceptor (Wanner and Soppa, 1999). Arginine fermentation by *Hbt. salinarum* has been studied by (Ruepp and Soppa, 1996).

pH. Seawater is about pH 8, and the concentrated seawater in saltern crystallizer ponds is about the same. A neutral to slightly alkaline pH seems best for the growth of non-alkaliphilic haloarchaea (e.g. pH 7.2 – 7.5 is recommended). Buffering is usually not a problem for rich media (where the peptone has good buffering capacity) but is an issue for defined media, where some carbon sources can lead to acid production.

For alkaliphilic halobacteria, their pH optima range from 8.5 – 9.5, but be aware that at high pH many salts precipitate. This is why the Mg concentration is so low in soda lakes, and why haloalkaliphilic media can be so difficult to prepare.

Substrates. Many members of the genus *Halobacterium* require a complex growth medium or a defined medium with many amino acids. Other genera are far better for physiological or genetic studies because they can be grown in defined media on simple carbon sources. *Haloarcula* and *Haloferax* spp. can grow in a simple salts medium with lactate, pyruvate, glucose, or glycerol as sole carbon sources. (*see minimal media formulations, section 2*). All haloarchaea grow more slowly than *E. coli*. You can speed things up by using an incubator at 42-45°C; *Haloferax volcanii* produces visible colonies in about 5 days at 37°C but this can be reduced to about 2 days at 42-45°C (on 18% MGM). *Hbt. salinarum* can take 2 weeks or more at 37°C. I have not tried it at 45°C but I expect it would grow much faster.

Genetic stability. If you need to work with strains of *Halobacterium salinarum* then be aware that this organism has the biological peculiarity of being highly genetically unstable. It has a number of very active insertion sequences and transposons that are constantly jumping around the genome (Pfeifer, 1986; Pfeifer and Blaseio, 1990). This is easily recognized by the rate at which colour mutants are observed among colonies on plates. This issue, coupled with their slower growth rates (compared to *Haloferax* and *Haloarcula* spp.) and greater sensitivity to lysis during PEG-mediated transformation make it ‘difficult’ to work with.

REVIEWS & BOOKS on haloarchaea (see also references section). There are now some excellent on-line references and reviews on haloarchaea.

1. Life at high salt concentrations. A. Oren. *In*, the Prokaryotes. This is an excellent introduction and review of haloarchaea and other extremely halophilic microorganisms. Find it for free (and download it while you can!): [release year, 2000]

<http://141.150.157.117:8080/prokPUB/chaprender/jsp/showchap.jsp?chapnum=242>

2. Correct names of taxa within the family *Halobacteriaceae* - August 2005. As the name says, it is the most up-to-date reference on haloarchaeal nomenclature. All the names and abbreviations.

<http://www.the-icsp.org/taxa/halobacterlist.htm>

3. Bergey's Manual of Systematic Bacteriology, 2nd Ed, Volume 1 The *Archaea* and the Deeply Branching and Phototrophic *Bacteria*. D.R. Boone and R.W. Calstenholz (Eds). Springer, 2001.
** essential, but expensive to buy. Check out your libraries.
4. Molecular ecology of extremely halophilic Archaea and Bacteria. Oren, A., 2002. FEMS Microbiol Ecol 39, 1-7.
5. Archaeal genetics - the third way. Allers, T., Mevarech, M., 2005. Nat. Rev. Genet. 6, 58-73.

Older reference books: These may be difficult to access unless you have a good library.

1. New Comprehensive Biochemistry [The Biochemistry of Archaea] vol 26 (eds. M. Kates, D. Kushner & A.T. Matheson). Elsevier/North Holland Biomedical Press, Amsterdam. (1993)
2. The biology of halophilic bacteria. 1992. Vreeland, RH & Hochstein, LI (eds.)
3. Halophilic bacteria, 1988. Rodriguez-Valera (ed.)
4. Systematic and Applied Microbiology, 16(4):501-767, 1994 (the whole issue)
5. Halophilic Microorganisms, A. Ventosa (ed.), Springer, Berlin. (whole book)

Haloarchaeal Journals:

1. Extremophiles (Springer publn):
[http://www.springerlink.com/\(zhj5m550oe55b4540qxoq3u\)/app/home/journal.asp?referrer=parent&backto=linkingpublicationresults,1:100494,1](http://www.springerlink.com/(zhj5m550oe55b4540qxoq3u)/app/home/journal.asp?referrer=parent&backto=linkingpublicationresults,1:100494,1)
2. Archaea (open access): <http://www.hindawi.com/journals/archaea/>
3. Saline Systems. An open access e-journal: <http://www.salinesystems.org/>

Web sites:

1. My web site sports some rather nice pictures of salt lakes, halobacteria, haloviruses, *Salinibacter* and *Dunaliella*, as well as a links page: www.haloarchaea.com
 2. International Society for Salt Lake Research: <http://isslr.org/>
 4. Haloarchaeal genome sequences (manually annotated) at www.halolex.mpg.de (or Genbank of course).
- A new archaeal genomics site (2006) is available at : <http://archaea.ucsc.edu/>
5. *Hfx. volcanii* genetic discussion list: haloferax@jiscmail.ac.uk

2.0 Media Formulae and Cultivation of Haloarchaea

Make sure that all glassware is thoroughly rinsed with pure water to avoid any traces of detergent that will inhibit growth or lyse the cells. Certain brands of peptone (e.g. Bacto-Peptone from Difco) are contaminated with bile salts, which are also detergents and will lyse haloarchaeal cells (Kamekura et al., 1988). While not wishing to push any one brand, Oxoid media has give consistently good results in my laboratory. Remember to add peptone/yeast extract/tryptone powders on top of water and not the other way round, as it is harder to dissolve these when they form a gelatinous mass stuck to the bottom of the glassware! Use a calibrated pH meter that can handle Tris buffers and high salt (sodium) concentrations, and if in doubt check with multi-coloured pH papers (we always double-check this way). Molecular weights of the common drugs (section 7.2) salts and buffers (section 7.3) are given later.

2.1 Artificial Seawater (3.6% salt)

Given below are two formulae for synthetic seawater as given in the excellent ASM publication 'Manual of Methods for General Bacteriology' (published by the American Society for Microbiology). These are useful to compare with the formulations devised by others to mimic the concentrated brines where halobacteria are found.

2.1.1 Artificial Seawater Compositions

These give a final salt concentration of 3.6% w/v

SALT	grams per Litre	
	<i>(Pelczar, M.J., Jr., 1957) (Zobell, C.E., 1946)^a</i>	
NaCl	27.5	24.32
MgCl ₂	5.0	5.14
MgSO ₄	2.0	
Na ₂ SO ₄		4.06
NaHCO ₃		0.20
H ₃ BO ₃		0.027
KBr		0.10
KCl	1.0	0.69
CaCl ₂	0.5	1.14
SrCl ₂		0.026
NaF		0.003
NaSiO ₃		0.002
FePO ₄		0.001
FeSO ₄	0.001	

^a These references are cited in the ASM Manual, and are found below in section 10.

2.2 Concentrated Salt Water (SW) stock solution - 30% (w/v)

This formulation is based upon that described by Rodriguez-Valera et al. (Rodriguez-Valera et al., 1980) and Torreblanca et al. (Torreblanca et al., 1986) and contains salts in approximately the same proportions as found in sea water (see formulae above) but at a much higher concentration of total salt. Most halobacteria will grow in 25% SW with 0.5% yeast extract: the exceptions being the alkaliphilic halobacteria (Natronobacterium and Natronococcus spp.) which need very low Mg^{2+} ; Hrr. sodomense, which apparently needs starch/clay minerals; and certain haloarchaea that prefer specific carbon sources e.g., Halosimplex (Vreeland et al., 2002), ADL group and Haloquadratum (Burns et al., to be published).

NOTES:

- The original formula of Rodriguez-Valera also contains $NaHCO_3$ and $NaBr$, but we have found these to be unnecessary.
- I have slightly adjusted the masses given in Juez et al. (6) to more closely give a total of 30% total salt, and to make weighing out simpler.
- When calculating % w/v salts, the masses of the hydrated salts (e.g. $MgSO_4 \cdot 7H_2O$) need to be adjusted down to allow for the water content.
- You can't use this salt formulation for alkaliphiles; there is massive precipitation above ~ pH 8.5.

1. Add the following salts to a large beaker.

SALT	g per 1 Litre	g per 5 L	g per 10 L
NaCl	240	1200	2,400
^a $MgCl_2 \cdot 6H_2O$	30	150	300
^a $MgSO_4 \cdot 7H_2O$	35	175	350
KCl	7	35	70
1M Tris.Cl, pH7.5	5 ml	15 ml	50 ml
*NaBr	0.8	4.0	8
* $NaHCO_3$	0.2	1.0	2

^aNote the hydrated salts: adjust if using anhydrous salts

*we do not include these in our normal media, although you should consider if they may be important for your work.

2. ADD pure water to near the final required volume and dissolve the salts completely using a glass stirring rod or a magnetic stirrer (warming may help).
3. ADD $CaCl_2 \cdot 2H_2O$ slowly from a 1M sterile stock solution:

(final concn = 0.5 g/L) 5 ml (for 1L) 25 ml (for 5L) 50 ml (for 10L)

note: a 1M CaCl₂.2H₂O solution = 147g/L (= 11% w/v of CaCl₂)

4. ADJUST the pH, if necessary, up to 7.5 with a minimum volume of 1M Tris base.

In some cases, the pH may go down after storage for several weeks, or after autoclaving.

5. Transfer to a large, graduated cylinder, then top up with water to the exact final volume.

Do not trust graduated beakers, as they can be very inaccurate.

6. Dispense into convenient volumes in autoclavable glassware (e.g. 100 ml of artificial salt water in 200 ml bottles)

7. AUTOCLAVE at 101kPa (15lb) for 15 min. Store at room temperature.

There should be no precipitate after autoclaving. If this occurs, try adding the CaCl₂ solution after autoclaving and when the solution has cooled to room temperature.

OR, alternatively, the whole solution can be stored in a large flask at 4°C without sterilization for at least 6 months (cover the top with plastic film).

NOTES:

2.3 Modified Growth Medium (MGM): a general medium for Haloarchaea

Developed in my laboratory from that described by Rodriguez-Valera et al. (Rodriguez-Valera et al., 1983; Rodriguez-Valera et al., 1980). I found it gave faster growth of *Haloferax* species than the original recipe that uses only yeast extract (0.5%). Be careful about the brand of peptone, they are not all the same, and one contains bile salts that will lyse halobacteria (Difco Bacto-Peptone). We routinely use 23% (SW) MGM for a variety of halobacteria, but *Haloferax* spp. will grow faster on 18% MGM, and *Halobacterium* spp. may grow faster on 25% MGM. *Haloarcula hispanica* prefers 23% and grows only poorly at 20%. Note that the salt concentration of the medium is that of the total salt concentration, not just the sodium chloride.

per Litre:

1. Add the following ingredients (in a large beaker):

	12% MGM	18% MGM	23% MGM	25% MGM
Salt Water (30% stock)	400	600	767	833
Pure Water	567	367	200	134
Peptone (Oxoid)*	5	5	5	5
Yeast Extract	1	1	1	1

*Don't use Difco Bacto-Peptone, as it was reported in 1988 that it contained bile salts that lyse halobacteria (Kamekura et al., 1988). This was still the case in 2001, when tested by Thorsten Allers!

2. Stir to dissolve (you may have to warm slightly).

Adjust the pH up to 7.5 with 1M Tris.Cl, pH 7.5, using 5 ml per Litre. If this is not sufficient, then use 1M Tris base, drop-wise, to reach the final pH.

In Melbourne, Australia, I had no problems adjusting and maintaining the pH, but in Germany the pH would drop to 5.5 after autoclaving, unless I used at least 10 mM Tris. Note that Tris.Cl is inhibitory if > 45mM.

3. Adjust volume to 1000 ml with pure water (about another 30 ml water)

For solid medium: Plates, add 15g Difco Bacto-agar; Top Layer Agar (for virus plaquing or transformations), add 7 g. It is better if the agar is largely dissolved before autoclaving, i.e. by heating to 100°C for 10-20 min. However with large volumes this can be difficult. The longer autoclaving time (30 min) should overcome this problem. The brand of agar is important, see note below at the end of section 2.4.

4. Sterilize (e.g. in a large, wide-mouthed 2 – 3 L flask) for 30 min, 101 kPa (15 lb).

Cool to 55 – 60°C and pour into Petri dishes (see note below on pouring plates).

5. After autoclaving, make sure the agar is mixed before pouring as it tends to settle at the bottom.

Pour relatively hot (about 60°C) as the high salt concentration makes the agar set easily. Bubbles are more difficult to get rid of (i.e. by flaming with a bunsen burner) than in normal media so be careful.

6. Dry plates before use (30 – 60 min, 37°C, upside-down, with lids removed; or more conveniently, overnight at RT on the bench). Store plates at 4°C, wrapped in plastic film.

Thorsten Allers (see below) prefers to store plates at 4°C immediately upon setting, then dry at 60°C for 10 min just before use.

2.4 CDM - Minimal Medium for Haloarchaea (Kauri et al., 1990)

Chemically Defined Medium (CDM) of Kauri, Wallace and Kushner, 1990 (Kauri et al., 1990). Used for Haloferax volcanii, and introduced into the lab. by Juan Serrano (University of Alicante, Spain) who visited here in 1997. It is very simple, easy to prepare, and works well! This is the MDS preferred minimal medium.

1. Make up 18% Salt Water (for *Hfx. volcanii*; or use the opt. % SW for other haloarchaea)

NaCl	125	g
MgCl ₂ .6H ₂ O	50	g
K ₂ SO ₄	5	g
CaCl ₂ .2H ₂ O	0.26	g

2. Add pure water to 1000 ml. For solid medium add 15 g of Difco Bacto-Agar. Check the pH and adjust if necessary with 1M Tris.Cl, pH 7.5 (~ 1- 5 ml).
3. Autoclave, in a large, wide-mouthed 2 – 3 L flask, for 30 min, 101 kPa (15 lb).
4. When cooled to 55 – 60°C, add the following (all sterilized by filtration).

For 1 liter (1000 ml) add:

1M NH ₄ Cl	5 ml
Carbon Source*	See below
K ₂ HPO ₄	2 ml ^a
trace elements ^b	1 ml
1 mg/ml thiamine	0.8 ml
1 mg/ml biotin	0.1 ml

^asee recipes in sections 2.5.2.4 or 2.7.2; 0.5 M, pH 7.5

^bSee sections 2.5.5.2 and 2.8.2.2 for trace elements solutions

4. Check the pH (again), but it should be about pH 7.5. If not, use a small volume of 1M Tris base.
5. Pour plates, dry and store as described above. (*see the notes at end of this section*)

****see notes on various carbon sources for this medium on the next page.**

* **Carbon sources:** *The amount of acid produced varies depending on the carbon source, and those that produce more acid need to be present in lower concentrations (or extra buffering must be maintained).*

Na Lactate	0.5%	(excellent growth substrate, as is pyruvate ; the next in the pathway)
Na Acetate	0.1%	(poor growth substrate, but possible if the glyoxylate pathway is present)
Na Succinate	0.5%	
Glucose*	0.02% - 0.5%	
Glycerol*	0.02% - 0.5%	
Galactose*	0.1 - 0.5%	(can be difficult to grow some strains on this substrate)

*Add 5 ml/L of 1M NaHCO₃ (as a buffer) when glucose, glycerol or galactose is used as a C-source. (5 mM final)

Trace elements solution: see section 2.7.2

***Notes:**

1. **Agar volume.** As Wan Lam once said to me (MDS) regarding plates when I was working in Ford Doolittle's lab in 1991, "*pour them thick*". A thicker agar plate seems to give better growth of *Hfx. volcanii*. Perhaps a reflection of pH control (?).
2. **Drying plates.** This can be done by:-
 - a) leaving plates on the bench overnight at room temperature (very easy)
 - b) incubating 30 – 40 min at 37°C, upside-down, with lids removed.
 - c) incubating at 60°C for 20 – 30 min, upside-down, with lids removed (time this carefully or they will dry too much). Thorsten Allers prefers this method.
3. The choice of **agar concentration** is determined by the brand and type - different ones will have different gelling strengths. It also depends on the purpose, for example if you only want to use the plates for support of top agar layers then 15 g/L is sufficient, but if you are replica-plating then a stiffer gel (20 g/L) may be less likely to get distorted or crushed. We prefer Difco Bacto-Agar, but have also used Oxoid Bacteriological Agar.

2.5 OTHER PUBLISHED (or unpublished!) MEDIA FORMULATIONS

2.5.1 Media Recipes from the Thorsten Allers Lab

2.5.1.1 Hv-YPC medium (Complete Medium) (18% SW)

Contributed by Thorsten Allers, University of Nottingham, UK. He has looked at the ingredients carefully and assessed their importance for his studies. Growth rates are good, as reported below in **section 2.6.1**.

1. Make up **30% SW (Salt Water)** stock solution. The CaCl₂ is not added until just before pouring plates, and some Tris.HCl pH 7.5 is added.

Ingredients	For 1 (ONE) litre	For 5 (FIVE) litres
pure H ₂ O (warm)	850 ml	~4 litres
NaCl	240 g	1200 g
MgCl ₂ ·6H ₂ O	30 g	150 g
MgSO ₄ ·7H ₂ O	35 g	175 g
KCl	7 g	35 g
1 M Tris.HCl pH 7.5	20 ml	100 ml

* Warm water on hot plate to dissolve salts.

2. Make up **10× nutrient stock (YPC)**. The final concentrations are 0.5% yeast extract (Difco), 0.1% peptone (Oxoid) and 0.1% casamino acids (Difco). The sources are important.

10x YPC (Yeast Extract (Y), Peptone (P) & Casamino Acids):

Ingredients	170 ml (1L medium)	340 ml (5L medium)
pure H ₂ O (warm)	~130 ml	~250 ml
Yeast Extract (Difco)	8.5 g	17 g
Peptone (Oxoid)	1.7 g	3.4 g
Casamino Acids	1.7 g	3.4 g
Adjust pH to 7.5 with 1 M KOH	(~3 ml)*	(~6 ml)*

* Slowly add ~ 1 M KOH while stirring.

Finally, add enough pure H₂O to get the correct final volume of 170 or 340 ml

Use directly (i.e. do not store, autoclave or filter sterilize)

3. For Hv-YPC AGAR [see below for liquid Hv-YPC]

a) Combine in a 500 ml volume Duran bottle:

pure H ₂ O	100 ml	
30% SW	200 ml	
Agar (Difco)	5 g	(Difco Bacto-Agar is best)

b) Put a stir bar in the bottle and microwave until agar has dissolved. It tends to heat at the sides (and crackles a bit), so shake bottles between heating – the solution should become clear, and without particles of agar

For 5 bottles of 300 ml agar, microwave for 10 minutes on High, 10 minutes on Medium, and twice for 5 minutes on Medium.

** Do not have a screw lid on this bottle! Anything blocking the exit of steam can produce the most amazingly dangerous explosion, with flying glass tearing out of the door (and possibly the door with it). Wear gloves.*

4. Add **10x YPC** nutrients (this prevents the yeast extract etc. from being caramelised during microwaving):

To each 300 ml batch of Hv-YPC agar add:

10x YPC 30 ml

5. Autoclave as soon as possible.

6. After autoclaving, allow to cool to about 57°C, and slowly add 2 ml of 0.5 M CaCl₂ to each bottle (for a total volume of 333 ml, or 1/3 of a litre)

Use a magnetic stirrer to avoid bubbles (i.e. do NOT shake)

7. Pour thick plates (i.e. 8 – 9 plates per 300 ml).

8. Once plates have set, store in plastic bags at 4°C as soon as possible.

Before use, dry plates upside-down in 60°C oven for 20 – 30 minutes (this only dries the surface of the plate, so that the water stored in the depth of the thick plate will keep the cells adequately hydrated)

6. Incubate plates at 45°C in plastic bags (e.g. the ones the Petri dishes come in).
Single colonies are visible after 3 days, and can be picked after 4 days.

For Liquid Hv-YPC Medium (18% SW final)

1. For 333ml of medium, add the following into a clean, 500 ml volume Duran bottle:

Distilled H ₂ O	100 ml
30% SW	200 ml
10X YPC	33 ml

2. Autoclave in benchtop autoclave.
3. When cool, add 2 ml of 0.5 M CaCl₂ to each 500 ml bottle.

Store liquid media at room temperature in a dark cupboard.

2.5.1.2 Casamino Acids Media (Hv-Ca)

For 5 or 10 bottles of 333 ml Hv-Ca Agar

1. Make up 30% SW [For details see page 9, above]
2. Make up 10x Ca

	5 bottles	10 bottles
<i>Distilled H₂O</i>	~130 ml	~250
<i>Casamino Acids</i>	8.5 g	17g

3. Slowly add 4 ml 1 M KOH while stirring. Add pure H₂O to final volume of 170 ml.
4. Do not autoclave, but use the same day.

Hv-Ca Agar

1. Into 500 ml Duran bottles measure:

Agar	5 g
Distilled H ₂ O	100 ml
30% SW	200 ml

3. Microwave* (or boil/low autoclave) until agar has dissolved. [** see p. 16 above for details*]
4. Then add to each 500 ml bottle:

10× Ca	33 ml
--------	-------

5. Add a large stir bar to each 500 ml bottle. Autoclave as soon as possible in benchtop autoclave.
6. After cooling to ~57°C, add slowly to each 500 ml bottle:

0.5 M CaCl ₂	2	ml
Thiamine & Biotin	300	µl

To avoid bubbles in agar, use a magnetic stirrer and pour the additives so they run down the inside glass surface of the bottle.

7. If additives are required, add at this point as well. We use colour coding of supplements to avoid mistakes. We add per 500 ml bottle (333 ml agar):

<i>Additive (stock solution)</i>	<i>Volume</i>	<i>Final concn.</i>
<i>Thymidine & Hypoxanthine</i> <i>(4 mg/ml each in 50 mM NaOH)</i>	<i>3.4 ml</i>	<i>(40 µg/ml) (Red)</i>
<i>Tryptophan</i> <i>(10 mg/ml in pure H₂O)</i>	<i>1.7 ml</i>	<i>(50 µg/ml) (Brown)</i>
<i>Uracil (50 mg/ml in DMSO)</i>	<i>340 µl</i>	<i>(50 µg/ml) (Pink)</i>
<i>5-FOA* (50 mg/ml in DMSO)</i>	<i>340 µl</i>	<i>(50 µg/ml) (2x Pink)</i>

**Note: Hv-Ca + 5-FOA agar normally requires addition of 67 µl uracil (50 mg/ml) to 10 µg/ml*

8. Pour thick plates (i.e. ~9 plates per 333 ml) as soon as possible.
9. Mark the plates with a red stripe using the thick marker pen. If additives are included, indicate on the plate and add a thin stripe according to the key (above). Once the plates have set, seal in plastic bags and store in the cold room in a cardboard box.

NOTES:

2.5.1.3 Minimal Medium (Hv-Min) - Thorsten Allers Lab

For 5 or 10 bottles of 333 ml **Hv-Min** Agar

1. **Make up 30% SW** [For details see above]

2. Hv-Min Agar. *Into 500 ml Duran bottles measure:*

Agar	5 g
Distilled H ₂ O	110 ml
30% SW	200 ml

3. Microwave* (or boil/low autoclave) until agar has dissolved. [* see p. 16 above for details]

4. Add to each 500 ml bottle:

1 M Tris.HCl pH 7.5 10 ml

Add a large stir bar to each 500 ml bottle.

5. Autoclave as soon as possible in benchtop autoclave.

6. After autoclaving, add slowly to each 500 ml bottle:

Hv-Min Carbon Source	8.5 ml
Hv-Min Salts	4 ml
0.5 M KPO ₄ buffer (pH 7.5)	650 µl
Thiamine & Biotin	300 µl

To avoid bubbles in agar, use a magnetic stirrer and pour the additives so they run down the inside glass surface of the bottle.

7. If supplements are required, add at this point as well. We use colour coding of supplements to avoid mistakes.

i.e. per 500 ml bottle (333 ml agar) add:

Additive (stock solution)	Volume added	Final concentration
Histidine (10 mg/ml in pure H ₂ O)	2.2 ml	(65 µg/ml) (Blue)
Leucine (10 mg/ml in pure H ₂ O)	1.7 ml	(50 µg/ml) (Green)
Methionine (10 mg/ml in pure H ₂ O)	1.7 ml	(50 µg/ml) (Orange)
Uracil (50 mg/ml in DMSO)	340 µl	(50 µg/ml) (Pink)
Tryptophan (10 mg/ml in pure H ₂ O)	1.7 ml	(50 µg/ml) (Brown)
Thymidine & Hypoxanthine* (4 mg/ml each in 50 mM NaOH)	3.4 ml	(40 µg/ml) (Red)

*Note: *Hv-Min + Thy&Hyp agar* requires addition of 1.7 ml of methionine & glycine & pantothenic acid (10 mg/ml each in pure H₂O) to 50 µg/ml each

8. Pour thick plates (i.e. ~9 plates per 333 ml) as soon as possible.
9. Mark the plates with a Green stripe using the thick marker pen. If supplements are included, indicate on the plate and add a thin stripe according to the key (above). Once the plates have set, seal in plastic bags and store in the cold room in a cardboard box.

2.5.1.4 Hv-Min Medium Solutions and Components

2.5.1.5 Hv-Min Carbon Source (10% Lactic Acid, 9% Succinic Acid, 1% Glycerol)

1. For 250 ml Hv-Min Carbon Source

Pure H ₂ O	~150	ml	
60% dl-lactic acid, Na salt	41.7	ml	(Sigma L-4263)
Succinic acid, Na ₂ salt, .6H ₂ O	37.5	g	(Sigma S-9637)
80% Glycerol	3.15	ml	

2. Adjust to pH 6.5 with 5 M NaOH (~2 ml), then carefully add 1 M NaOH to adjust to pH 7.5
3. Add pure H₂O to final volume of 250 ml and filter

2.5.1.6 Trace Elements (*this is the original formulation of Thorsten, but Iain Duggin found better growth of *Hfx. volcanii* by replacing this with a more complex formula published in 2014 – and this is given later in section 2.5.5.2)

1. To **100** ml water add a few drops of concentrated HCl acid (*be careful*)

Dissolve the following *one-by-one*

MnCl ₂ ·4H ₂ O	36	mg
ZnSO ₄ ·7H ₂ O	44	mg
FeSO ₄ ·7H ₂ O	230	mg
CuSO ₄ ·5H ₂ O	5	mg

2. Filter sterilize, store at 4°C

2.5.1.7 Hv-Min Salts

1. For 72 ml Hv-Min salts combine

1 M NH ₄ Cl	30	ml
0.5 M CaCl ₂	36	ml
Trace elements	6	ml

2. Store at 4°C. Use 3.6 ml for 300 ml Hv-Min media

2.5.1.8 Thiamine & Biotin for Hv-Min

1. For 10.8 ml combine

Thiamine (1 mg/ml)	9.6 ml
Biotin (1 mg/ml)	1.2 ml
2. Use 270 μ l in 300 ml Hv-Min media

2.5.1.9 0.5 M KPO₄ Buffer pH 7.5

1. For 200 ml 0.5 M KPO₄ buffer (pH 7.5)

1 M K ₂ HPO ₄	83.4 ml
1 M KH ₂ PO ₄	16.6 ml
2. Check pH = 7.5
3. Add equal volume (100 ml) of pure H₂O
4. Autoclave

80% Glycerol, 6% SW

1. For 100 ml mix

Glycerol	80 ml
30% SW	20 ml
2. Autoclave. When cool add 200 μ l of 0.5 M CaCl₂

18% SW (from 30% SW stock)

1. For 333 ml mix

30% SW	200 ml
Pure H ₂ O	133 ml
2. Autoclave. When cool add 2 ml of 0.5 M CaCl₂

2.5.2.0 Enhanced Casamino Broth (Hv-Ca⁺)

Contributed by Thorsten Allers, Nottingham University

For: 1 bottle of 333 ml Hv-Ca⁺ Broth

1. Make up 30% SW

For details see recipe above, section 2.5.2.1.

2. Make up 33 ml 10x Ca

Distilled H₂O ~25 ml

Casamino Acids 1.7 g

When dissolved, slowly add 0.8 ml 1 M KOH while stirring.

3. Add distilled H₂O to final volume of 33 ml.

Filter sterilize through 0.45 µm filter.

4. Autoclave SW

5. Into a 500 ml volume Duran bottle measure:

30% SW 200 ml

1 M Tris.HCl pH 7.5 10 ml

Distilled H₂O 75 ml

6. Autoclave in benchtop autoclave.

For liquid medium: Hv-Ca⁺ Broth

7. When cool, add to diluted SW (from step 5):

10× Ca (from step 2) 33 ml

Hv-Min Carbon Source 8.5 ml

Hv-Min Salts 4 ml

0.5 M KPO₄ buffer (pH 7.5) 650 µl

Thiamine & Biotin 300 µl

Store liquid media at room temperature in a dark cupboard.

NOTES:

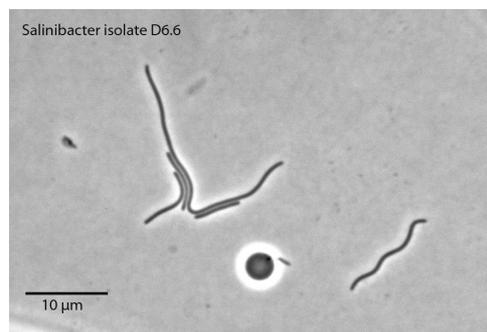
2.5.3 Rich media published by other groups.

Comments:	reference	composition
Extreme halophiles NOT for <i>Haloferax</i> or <i>H. sodomense</i> as they require high magnesium and low total salt concn. NOT for bacterio- rhodopsin production unless trace salts added.	(Hochstein et al., 1987) (about 27%salts)	add to 800 ml water in the following order: Difco Yeast Extract 5 g Hy-Case SF ^a 5g NaCl 250 g Tris base 12.1 g MgCl ₂ .6H ₂ O 20 g KCl 2 g CaCl ₂ .2H ₂ O 0.2 g glycerol 10 ml <i>adjust to 1000 ml and pH to 7.2</i> <i>autoclave. Any precipitate should</i> <i>disappear in 2-3 days.</i>
Extreme halophiles such as <i>Hbt. salinarum</i> . (NOT <i>Haloferax spp.</i> as the Mg conc. is too low)	(Cline et al., 1989a) (about 27%salts)	per Litre: NaCl 250g Tris.Cl (1M, pH7.4) 50ml MgSO ₄ .7H ₂ O 20g KCl 2g CaCl ₂ .2H ₂ O 0.2 g Na ₃ Citrate.2H ₂ O 3g Bacto-Yeast Extract 3g Bacto-Tryptone 5 g <i>adjust to 1000ml and pH 7.4. Autoclave.</i>
Extreme halophiles such as <i>Hbt. salinarum</i> (NOT <i>Haloferax spp.</i> as the Mg conc. is too low)	(Hackett & DasSarma, 1989) (about 27% salts)	per litre: NaCl 250 g MgSO ₄ .7H ₂ O 20 g peptone (Oxoid) 10 g Na ₃ Citrate 3 g KCl 2 g <i>adjust to 1000ml and pH 7.4.</i> <i>Autoclave.</i>

^aAn acid-hydrolysed casein (Humko-Sheffield Chemical Co).

2.5.4 Media for *Salinibacter ruber*.

Contributed by Kate Porter and Aharon Oren, University of Melbourne.



The DSMZ medium for this strain is below, but we can grow it easily on 23% MGM solid or liquid medium. It grows poorly at 18% salt. Changes in salinity or medium often cause morphological changes in these cells, with strange ‘match-stick’ like forms instead of the normal population of uniformly curved rods (picture left). It appears that the cell wall is weakened, particularly at the cell ends, and the membrane-bound cytoplasm

extrudes to form a sphere (one is visible in the middle of the picture, showing the vestigial cell ‘tail’.

Colonies are slow growing and bright red; very similar to haloarchaeal colonies.

DSMZ Medium for *Salinibacter ruber* strain M31^T

per L

NaCl	195 g
MgCl ₂ .6H ₂ O	16.3 g
MgSO ₄ .7H ₂ O	25 g
CaCl ₂ .2H ₂ O	1.25 g
KCl	5 g
NaHCO ₃	0.25 g
NaBr	0.62 g
Yeast Extract	1 g (<i>higher levels may inhibit growth</i>)

pH to 6.8-7.2 with NaOH, Autoclave. Grow cells in liquid medium with shaking. Improved growth may be achieved by the addition of 1 g/L glycerol or glucose (added after autoclaving, from a sterile stock).

2.5.5 Alkaliphile Medium

This protocol was kindly provided by Peter Jablonski who, at the time (around 2001), was working at Integrated Genomics, Inc., Chicago. He has since left and I am unsure of his current address. I have had great difficulty in making up alkaliphile media that do not precipitate, but this medium of Pete's is very *simple* and gives a clear, stable solution.

Per Litre:

1. **Solution 1:** Dissolve in 900 ml of pure water

NaCl	200 g
KH ₂ PO ₄	1.0 g
MgSO ₄ ·7H ₂ O	0.2 g
Casamino Acids	5 g
Trace metals	1 ml (see recipe SL-6, next page)
(Agar)	(20 g per L for solid media)

2. **Solution 2:** Dissolve 18 g of Na₂CO₃ in 100 ml of pure water
3. Autoclave the two solutions, 15 min at 101 kPa (15 psi)
4. Let both solutions cool to room temperature and add solution 2 (Na₂CO₃) to solution 1 (basal salt medium). Mix and use. There should be no precipitation when solutions 1 and 2 are mixed.

Pour the plates around 65°C. There is almost no chance of contaminants growing on this medium (except of course haloalkaliphiles!). Strains grown on this medium include: Nbt. gregoryi, Natrialba (formerly Natronobacterium) magadii, Natronomonas (formerly Natronobacterium) pharaonis. Halorubrum (formerly Natronobacterium) vacuolatum grows VERY slowly and probably requires additional components (perhaps not surprising now that we know this strain is genetically very different from the others).

2.5.5.1 Trace Metal solution (SL-6)

Note: SL-6 is usually a component of SL-4 trace elements solution (which includes Fe)

Per Liter

ZnSO ₄ .7 H ₂ O	0.1 g
MnCl ₂ .4H ₂ O	0.03 g
H ₃ BO ₃	0.3 g
CoCl ₂ .6H ₂ O	0.2 g
CuCl ₂ .2H ₂ O	0.01 g
NiCl ₂ .6H ₂ O	0.02 g
Na ₂ MoO ₄ .H ₂ O	0.03 g

Adjust final volume to 1000 ml with pure water

Adjust the pH of SL-6 solution to 3–4 with HCl (to prevent precipitation of metal salts).

Store 4°C.

2.5.5.2 Trace Elements solution for *Haloferax volcanii* (Iain Duggan, for Hv-Ca medium)

Recommended by Iain Duggan, who stated that it “improved growth and cell-shape uniformity” of Hfx. volcanii when used in Hv-Ca medium (when compared to the original trace elements solution. Recipe from doi:10.1038/nature13983. This is similar in composition to the classical SL-4 or SL-10 trace elements solution (and SL-10 is given later in section 2.8.2.2).

Per Litre:

EDTA	5 g
FeCl ₃	0.8 g
ZnCl ₂	0.05 g
CuCl ₂	0.01 g
CoCl ₂	0.01 g
H ₃ BO ₃	0.01g
MnCl ₂	1.6 g
Ni ₂ SO ₄	0.01g
H ₂ MoO ₄	0.01 g

Adjust pH to 7.0 with NaOH, filter sterilize (0.2 µm), and store at room temperature. This is a 100 x stock

solution, so for use it is diluted 100-fold in media to obtain the working concentration.

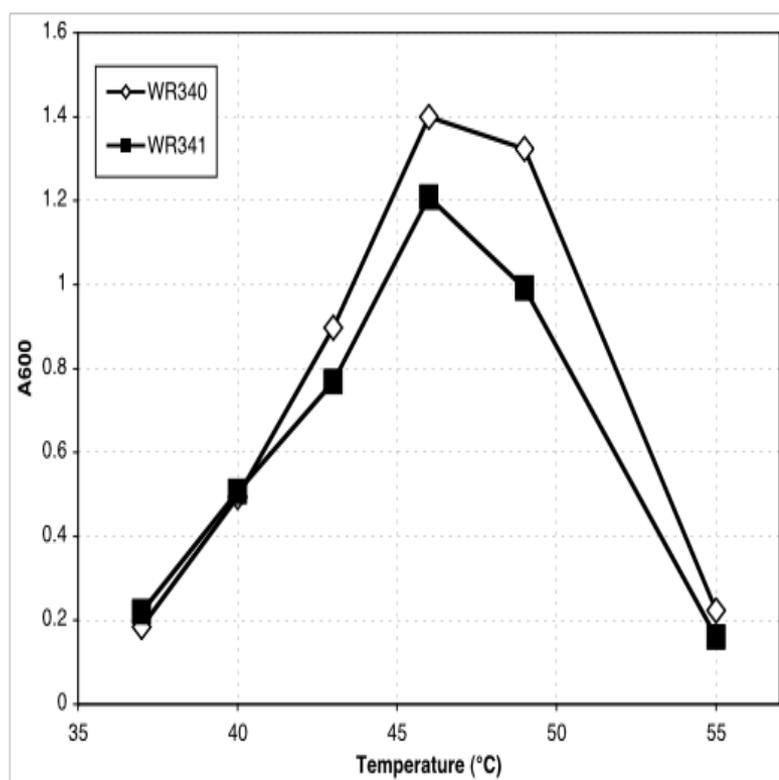
2.6.1 Growth studies with *Haloferax volcanii*

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These were studies performed by Thorsten while setting up to study haloarchaeal genetics. He looked at temperature and media and pH control, and different molecular methods (e.g. chDNA extraction method). He writes in the first person below.

Effect of **temperature** on growth of *Hfx. volcanii*. The optimum is around 47°C. At 49°C there are a small percentage of damaged cells (bloated spheres) and these are dominant at 55°C. He recommends 45°C to give a margin of safety (unless you are actually studying heat-shock responses!). The viable counts parallel the growth curves (the peak is around 4×10^8 cfu/ml).



Legend: Cultures of *Hfx. volcanii* strains WR340 (*his*⁻) and WR341 (*met/cys*⁻) with A₆₀₀ of 0.8 were diluted 1/250 in 5 ml of 18% SW + 5 g/litre Difco Yeast extract, and grown for 17 hours at the temperatures shown, shaking at 200 rpm. Viable cell count data corroborates these OD values as being representative of the ideal growth temperature (i.e. 45 – 47°C).

What buffering agent to use (particularly in minimal media, as the protein in complex media is usually

sufficient for buffering)? Some use MOPS, some use Tris.Cl, some use carbonate (see sections 2.4 – 2.5 above). Tris is cheap and easy to use, but is it toxic?

Minimal media: Tris vs HEPES. I tested growth of liquid cultures in minimal media (recipe of **2.5.1**) (Kauri et al., 1990), but with either 40 mM Tris.HCl pH 7.5 or 40 mM HEPES pH 7.5 as a buffer. Although the difference wasn't huge, the Tris was better than the HEPES. I also noticed that there was precipitate in the HEPES cultures, which may account for the lower growth. In any case, HEPES is much more expensive than Tris.

Tris concentration: I measured the growth of *Hfx. volcanii* in complex media (18% salt + 0.5% yeast extract etc.) with different concentrations of Tris-HCl (pH 7.5), ranging from 5 mM to 45 mM. I measured the OD₆₅₀ at different points in the growth, and the pH of the medium. The pH didn't change significantly from the starting value in any case, and the growth wasn't affected at any concentration of Tris except 45 mM, where it dropped from 1.15 to 1.05, so **40mM** would seem to be a good compromise for minimal media, as buffering is adequate and effects on growth are minimal.

Carbon sources in minimal media: (recipe of **2.5.1**) I compared 0.5% lactate (made up from a 20% stock that had been adjusted to pH 7.5) and either 0.45% succinate/0.05% glycerol or 0.05% succinate/0.45% glycerol. The lactate and 0.45% succinate/0.05% glycerol were about equally good, but the 0.05%succinate/0.45% glycerol was terrible

2.6.2 Optimum Temperature and Growth condtions for *Hfx. mediterranei* and other haloarchaea

The growth of Hfx. mediterranei was studied in detail by Lillo and Rodriguez-Valera (Lillo and Rodríguez-Valera, 1990). They found the following optima using a defined medium in batch culture:

pH	7.2
marine salts	25%
temperature	45°C
NH ₄ Cl	0.2%
OTR (O ₂ transfer rate)	0.4 (the maximum tried)

The **temperature optima** of many different haloarchaea were elegantly determined by (Robinson et al., 2005), who found that the preferred growth temperature was around 45°C. At their optimal temperatures, generation times ranged from 1.5 h (*Haloterrigena turkmenica*) to 3.0 h (*Haloarcula vallismortis* and *Halorubrum saccharovororum*). The Arrhenius plots produced in the Robinson *et al.* study are superb, cover 14 different haloarchaea, and set a high standard for future work on this aspect.

2.7 Solutions for Media

2.7.0 Glycerol/Succinate solution (for defined media)

For 100 ml of this stock solution (50% glycerol v/v, 5% succinate w/v).

1. To 50 ml of pure water add:

glycerol	50 ml
sodium succinate	5 g

2. Mix and adjust pH to 7.5
3. Sterilize at 10 psi, 20 min. Store at room temperature.

2.7.1 0.5 M Potassium Phosphate buffer for defined media

1. Firstly, make 0.5M stocks of K_2HPO_4 and KH_2PO_4 .

For 100ml of each:

- a) Add K_2HPO_4 8.7 g to 100ml of pure water
- b) Add KH_2PO_4 6.8 g to 100ml of pure water

2. To the 100ml of K_2HPO_4 solution, add 30ml of the KH_2PO_4 solution and then slowly add 2ml volumes until the pH is close to 7.5.
3. Sterilize at 101kPa (15lb), for 15min. Store at room temperature.

MW of K_2HPO_4 = 174.18 (then for 100ml of 0.05M = 0.87 g)

MW of KH_2PO_4 = 136.09 (then for 100ml of 0.05M = 0.68 g)

2.7.2 Trace Elements Solution (*but see sections 2.5.5.1 – 2.5.5.2, above)

Per 100 ml:

Weigh out the following and add to 100 ml of pure water that has had a few drops of concentrated HCl added so as to lower the pH (this will help to keep the salts in solution). Stir vigorously while adding each powder separately. When each has dissolved, add the next one. As autoclaving tends to precipitate the salts, filter sterilize, and store at room temperature.

MnCl ₂ .4H ₂ O	36mg
ZnSO ₄ .7H ₂ O	44mg
FeSO ₄ .7H ₂ O	230mg
CuSO ₄ .5H ₂ O	5mg

2.7.3 Drugs added to Media: (add to solid media just before pouring plates)

Drug	Stock Solution	Final Concentration ^a
Anisomycin	10 mg/ml in 70% ethanol	10 µg/ml (<i>Hfx. volcanii</i>)
Bleomycin	1 mg/ml in water	4 µg/ml (<i>Hfx. volcanii</i>)
Fusidic acid	10 mg/ml in 70% ethanol	15 µg/ml (<i>Hfx. volcanii</i>)
Mevinolin (MW = 341) ^b (or Simvastatin MW = 404)	20 mg/ml or 10mg/ml in ethanol Store at -20°C	4-5µg/ml (<i>Hfx. volcanii</i>) 10µg/ml (<i>Hbt. salinarum</i>) 2µg/ml (<i>Har. hispanica</i>) ^d
Novobiocin (Sodium salt, MW = 634)	1 mg/ml in water, filter sterilized, stored -20°C.	0.3µg/ml (<i>Hfx. volcanii</i>) 0.1µg/ml (<i>Har. hispanica</i>)
Pseudomonic acid ^c	10 mg/ml in water Store at -20°C	5µg/ml (<i>Hfx. volcanii</i>) 2-3µg/ml (<i>Har. hispanica</i>) ^c
Thiostrepton		3 µg/ml
Trimethoprim	20 mg/ml in water	2µg/ml (<i>Hfx. volcanii</i>) ^e

^a based on selection of transformants in top agar.

^b **Note:** a 10mM solution of Simvastatin = 4.04 mg/ml (but 3.41 mg/ml for Mevinolin).

^c MIC of pseudomonic acid for wild-type *Hfx. volcanii* is approx. 0.1µg/ml

^d MIC of Simvastatin for wild-type *Har. hispanica* is approx. 1 µg/ml

^e MIC of trimethoprim is 0.1 µg/ml for *Hfx. volcanii* (in yeast extract media without added thymidine). A trimethoprim-resistance gene has been developed by Rosenshine *et al.* (Rosenshine *et al.*, 1987). Many halobacteria are insensitive to trimethoprim so its applicability is limited (but it also works for *Hfx. mediterranei*). See, Ortenberg *et al.* (Ortenberg *et al.*, 2000) and references cited within.

2.7.4 Media Components – commercial sources

Component	Supplier	Code ^a
Peptone	Oxoid	LP0037 (L37)
Tryptone	Oxoid	LP0042 (L42)
Yeast Extract	Oxoid	LP0021 (L21)
Bacto [®] Agar	Difco	214010 (0140-01)
Agar No. 1	Oxoid	LP0011 (L13)

^aOxoid and Difco have changed their product codes . The old codes are in brackets. In fact both companies have changed hands one or more times in the recent past. I believe Fisher Scientific now owns both Oxoid and Difco.

2.7.5 NOTES on media

1. Pouring plates - a useful tip for large volumes (1 – 2 L).

Autoclave in a large (3 L) flask, cool to 60°C, and place on a heat-resistant surface. Remove the covering and with a gloved hand hold the top of the flask. Using the edge of the flask as a pivot, tip the vessel over enough to pour the required amount of liquid into a Petri dish. At the beginning, when the level is high, the Petri dishes can be stacked so that the top one is close to the lip of the flask when tipped. Later, when the level in the flask is low, the flask can be raised by placing it on top of some stable, heat resistant object. Then it can be tipped on its edge to a greater angle; the Petri dishes being at bench level. Always wear a lab coat to avoid hot spills in delicate places.

2. Flaming to eliminate bubbles in high salt plates is difficult. Try not to make bubbles in the liquid to start with (no shaking the flask – just gentle swirling when necessary), and pour the plates in a smooth manner to reduce turbulence forming bubbles in the plates. Otherwise, lightly mix the plate by moving in a circle, trying to get the bubbles to the edge . The higher the salt concentration, the worse the problem.

3. Note Thorsten Allers' tips for preparing and storing agar plates (**section 2.5.1.2**). He uses a magnetic stirrer to mix in additives after autoclaving.

i.e. Once plates have set, store in plastic bags at 4°C as soon as possible. Before use, dry plates upside-down in 60°C oven for 20 – 30 minutes (this only dries the surface of the plate, so that the water stored in the depth of the thick plate will keep the cells adequately hydrated)

2.8 Cultivation of Haloarchaea

In a study published in 2004 we found culture conditions that would grow all the dominant groups of haloarchaea in a saltern crystallizer pond, except members of the square haloarchaea (SHOW group; see next section) (Burns et al., 2004a). While we tried many different media and conditions, it appeared that the best results were achieved by using our standard MGM medium (25% SW, washed agar), and **long incubation times** (8 – 12 weeks). It also helps to inoculate plates at dilutions that will have relatively few colony-forming units (CFU) per plate, of around 25 for a standard sized agar plate.

2.8.1 Cultivation from salt lakes

We recommend using Difco Bacto Agar, and for environmental isolations we also recommend washing it further, as described below. This removes any residual growth inhibitors .

2.8.1.1 Washed Difco-Bacto agar

1. Add 16.5 g Difco-Bacto agar to distilled water.

The extra is a 10% allowance for loss during washing

2. Mix well for 5 min then allow the agar to settle for 30 min. Decant the supernatant.
3. Repeat washing as in the previous step until the supernatant is clear

This normally takes 2 - 4 rinses

4. Make the volume to 50 ml with water, mix to suspend the agar, then add to the desired medium, e.g. adding all 50 ml to 950 ml of medium will give a 1.5% final concentration.

The agar suspension will dilute the salt concentration of the medium, so take this into account.

Possibly agarose or gellan (Gelrite®, Phytigel™) could be used instead. We have not tried agarose, and our experience with Gellan is that sets almost instantly when mixed with solutions of moderate to high salinity. It is possible to make gellan plates, but it isn't easy! Also note that it is full of carbon compounds that can be used as growth substrates.

After isolation, and a few passages, it is likely that isolates will tolerate higher nutrient levels and unwashed agar (i.e. standard media). They may even grow better. This is a common phenomenon in environmental microbiology.

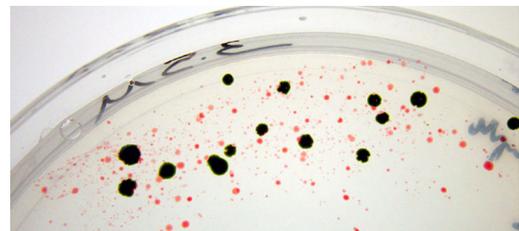
Plating and Incubation

After determining the direct counts of your samples, an appropriate range of dilutions should be made (in isolation medium) and plated. The plated dilutions should cover two ranges. Firstly, a statistically accurate viable count should be obtained (30-300 colonies/plate). Secondly, for isolates, you want to pick from plates with minimal crowding of colonies (10 to 50 colonies/plate). The latter is important as growth of environmental organisms is often more efficient under less crowded conditions (as seen by a higher viable count).

Plates are incubated, in the dark, at 37°C, for at least 8 weeks in sealed plastic containers, to prevent desiccation. Humidity can be maintained by including some exposed water agar plates or an open vessel of water (but be careful about knocking the container)

Check growth on plates every 2 weeks, and remove excess moisture from lids if necessary (either flick off the water from the lids, or replace with new lids).

*If you wish to culture isolation plates under lights (e.g. looking for bacteriorhodopsin-positive organisms), be aware that algae, such as *Dunaliella salina*, may also form colonies (green or orange, depending on the salt level) that overgrow haloarchaea.*

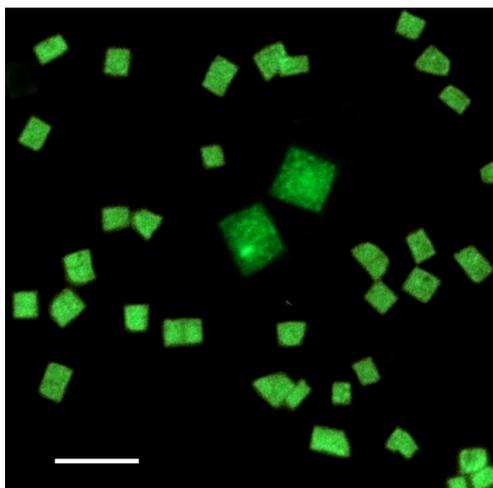


Dunaliella salina (dark green) and haloarchaea (pink) colonies growing on a high salt agar medium (under light)

When cultures are passaged from plates into liquid media, some strains grow poorly if shaken at rates > 150 rpm. Using 50 – 120 rpm should overcome this.

2.8.2 Cultivation of the Square Haloarchaea of Walsby (SHOW)

Contributed by David Burns, 2006, who first cultivated a member of this group (Burns et al., 2004b). These cells are one of the hardest of the haloarchaea to grow. Doubling times are relatively long, and they prefer cultivation in liquid media. Growth on plates is often very slow, taking 4 – 12 weeks for small colonies.



The beautiful cells of the C23^T isolate of the ‘Square haloarchaeon of Walsby’, *Haloquadratum walsbyi*. This is the strain isolated in the MDS laboratory. Cultures have been sent to several labs around the world, where it grows well. (Fluorescence micrograph, acridine orange stain).

2.8.2.1 Medium DBCM2, for square haloarchaea of Walsby (“*Haloquadratum walsbyi*”)

1. To 1 L of autoclaved 25% SW (ie. 833 ml 30% SW + 167ml pure water)
2. Add the following volumes of filter sterilized solutions

1 M Tris.Cl, pH 7.4	10 ml
1 M NH ₄ Cl, (53.4g/L)	5 ml
K ₂ HPO ₄ buffer, pH 7.4	2 ml (see below)
SL10 Trace Elements	1 ml
Vit10 vitamin solution	3 ml
Carbon source: For SHOW cells,	~10 mM pyruvate, or 0.44 ml per 100 ml of a filter-sterilized, 25% w/v refrigerated stock solution.

The pH should be around 7 – 7.5

Check the pH after making up the complete medium. Remove 5-10 ml and check the pH accurately. I had a pH problem when working at the MPI in Germany. The medium would go down to pH 5.5 after autoclaving the SW and adding all the components. I think this happened because the water used for producing the steam during autoclaving had a low pH. The solution (now incorporated into the medium composition above) was to increase the level of Tris buffer, so there is now 10mM Tris.Cl, pH7.4 (10 ml, 1M Tris solution, as above). I had no problems with pH in Melbourne, Australia (which has excellent water).

If using an acid-producing or acidic substrate, extra buffering may be required. I have found that pyruvate does not substantially alter the pH. If extra buffering is necessary, NaHCO₃ could be added (at 5 ml/L), but we have not tested this.

The addition of a small quantity of 23% MGM (i.e. 23% SW, 5 g/L Oxoid peptone, 1 g/L yeast extract,

Tris buffer – see section 2.3 above) to the medium above, in the range of 5 – 50 ml per litre, generally improves growth. Currently, we use 50ml/L. Pure MGM does not support good growth, probably due to the high concentrations of peptone and YE. I find best growth of SHOW isolates with 50 ml of 23% MGM added per litre of the minimal medium above.

You may be able to leave out the trace elements and vitamins - they seem to grow acceptably without them, but I've never passaged from these cultures so I don't know how they go long-term without the supplements. I have found that the SHOW cells grow better **either still or preferably shaken at low rpm** (≤ 120). Growth is definitely worse at shaker speeds ~ 200 rpm.

I passage with heavy inoculum (at least 10%), usually into 10 ml of fresh medium in glass tubes.

Growth also may be a bit better at $> 37^\circ\text{C}$, but the warmest incubator I have easy access to is only 40°C , and any difference above 37°C doesn't seem significant, at least for unshaken cultures.

K₂HPO₄ buffer

This is described at item 2.7.2 of The Halohandbook; briefly:

Mix 30 ml of 0.5 M KH₂PO₄ solution with 100 ml of 0.5 M K₂HPO₄, then add more of the second solution slowly, in 2 ml volumes, until the pH is close to 7.5. Autoclave at 101kPa for 15 min, store at RT.

2.8.2.2 Trace element solution SL10

The components of the trace element solutions SL10 (Widdel et al., 1983) are added and dissolved in the order listed.

SL10		
25% HCl	10	ml
FeCl ₂ ·4H ₂ O	1.5	g
H ₂ O	1.0	Liter
CoCl ₂ ·6H ₂ O	190	mg
MnCl ₂ ·4H ₂ O	100	mg
ZnCl ₂	70	mg
H ₃ BO ₃	6	mg
Na ₂ MoO ₄ ·2H ₂ O	36	mg
NiCl ₂ ·6H ₂ O	24	mg
CuCl ₂ ·2H ₂ O	2	mg

The trace element solution is autoclaved under air, in 25 ml aliquots in 50 ml screw-capped bottles. Large stocks do not need to be sterilised for storage. If you are storing this for very long periods, do not add the FeCl₂ until required.

Vitamin 10 stock

H ₂ O	1000	ml	
4-aminobenzoate	13	mg	
d-(+)-biotin	3	mg	(store biotin at 4°C)
nicotinic acid	33	mg	
hemicalcium D-(+)-pantothenate	17	mg	(store pantothenate at 4°C)
pyridoxamine hydrochloride	50	mg	(store pyridoxamine at 4°C)
thiamine chloride hydrochloride	33	mg	(store thiamine at 4°C)
cyanocobalamin	17	mg	(store cyanocobalamin at 4°C)
D,L-6,8-thioctic acid	10	mg	(store thioctic acid at 4°C)
riboflavin	10	mg	(store riboflavin at 4°C)
folic acid	4	mg	(store folic acid at 4°C)

Vitamin 10 solution is sterilized by filtration into sterile bottles through sterile 0.2 µm pore-size cellulose acetate filters. The bottles are wrapped in aluminium foil to protect against light, and stored at 4°C.

Vitamin 10 is a combination of the two vitamin solutions described in (Janssen *et al.*, 1997). *Note that solubility is not an issue with these ingredients; you could make up a more concentrated solution and then use less in formulating the final medium, eg. the same masses in 330 ml, and then use 1ml per L.*

References:

- Janssen, P.H., Schuhmann, A., Morchel, E., and Rainey, F.A. (1997)** Novel anaerobic ultramicrobacteria belonging to the *Verrucomicrobiales* lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. *Applied and Environmental Microbiology*: 1382-1388.
- Kauri, T., Wallace, R. and Kushner, D.J. (1990)** Nutrition of the halophilic archaeobacterium *Haloferax volcanii*. *Syst. Appl. Microbiol.* 13:14-18.
- Widdell, F., Kohring, G., and Mayer, F. (1983)** Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acid III. Characterisation of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. *Archives of Microbiology*: 286-294.

3.0 Transformation Methods

3.1 Preparation of Competent Frozen Haloarchaeal cells

This works well for both *Hfx. volcanii* and *Har. hispanica*, and can be recommended as a ready source of competent cells for transformations. I have stored cells for at least a year (-70°C) without any great loss in competence. The first method below is for one or more 50 ml cultures. Each 50ml culture will provide 5 ml of competent cells, and as the usual volume used for transformation is 100 µl, this should last quite a while. Alternatively, the second method is scaled down to a 5-10 ml fresh culture, where only a few transformation reactions are required.

1. To make sure the stocks are pure, plate out the laboratory strain on plates and pick off a single colony into 10ml of MGM. Incubate at 37°C, overnight (shaking, 180 rpm), and use 5ml of this as an inoculum for a 50ml culture in a 150ml flask. Again an overnight incubation (shaking, 180rpm) should provide a late exponential culture which can either be used immediately or split into larger cultures for more cells. At late exponential the culture is quite turbid, and has a pink tinge (A_{600} of 0.8 – 1.0).

Do not use cells that have entered stationary phase, as they lyse more easily. For example Hfx. volcanii cells go distinctly red after entering stationary phase.

2. Spin the cells down in a Sorvall SS34 rotor in 30ml centrifuge tubes at 6,000 rpm for 15min, at room temperature. Do not be concerned if the supernatant is slightly turbid, but be careful not to disturb the pellet when removing it, as it has a nasty habit of being sucked up the pipette.
3. Resuspend the pellet in a smaller (e.g. 10 ml) volume per tube, of buffered spheroplasting solution, and spin 5k, 10min. This washes the cells of residual Mg^{2+} ions, in preparation for EDTA treatment later.
4. Remove the supernatant **carefully** again, and resuspend in a final volume of 1/10th in relation to the starting culture volume (e.g. 5 ml for 50 ml of original culture), in buffered spheroplasting solution WITH 15% GLYCEROL. *Make sure the suspension is even*, but do not vortex vigorously, try to gently mix with a wide-mouthed sterile Pasteur pipette or equivalent.

The suspension should look opalescent and not be stringy.

5. Use the cells immediately, or freeze convenient lots (0.5 - 1 ml) in labeled 1.5 ml microcentrifuge tubes (or cryotubes). Snap freeze on dry ice, or liquid nitrogen, and store at -70°C or lower. To use, thaw out at room temperature, with occasional tapping of the tube to mix. I have not tried to re-freeze and use such cells again. They seem to last at least 6 months frozen.

For small scale production of competent cells for immediate use

1. Spin down a 10 ml culture (mid to late-exponential phase) in a 10 ml plastic centrifuge tube, 6K rpm, 15 min.
2. Resuspend the pellet in (e.g. 1 ml of buffered spheroplasting solution and transfer to a 1.5 ml microfuge tube.
3. Spin 6k, 10 min. This washes the cells of residual Mg^{2+} ions, in preparation for EDTA treatment later. This step can be left out if high transformation rates are not required.
4. Remove the supernatant **carefully** again, and resuspend in a final volume of 1ml buffered spheroplasting solution. *Make sure the suspension is even*, but do not vortex vigorously. Try to gently mix with a 1ml glass pipette or equivalent.
5. Use immediately for preparing competent cells. Follow the method described on the next page.

You can vary the format of this enormously. If you are transforming several different cultures of halobacteria you can do individual lots of 1.5 ml in microfuge tubes (for a single transformation), e.g. spin down 1.5ml 6k rpm, 10min (RT), resuspend in 300ul of BSS, spin again and remove the supernatant (carefully), resuspend cells in 150 μ l BSS and add 15 μ l 0.5M EDTA, etc. Wan Lam said it can be done in 96 well microtitre trays (he did it !), where you spin the tray (in a special centrifuge adaptor).

NOTES

3.2 Transformation Protocols (MDS Lab)

3.2.1 Standard PEG-mediated transformation of haloarchaea

This method works well for *Hfx. volcanii* or *Har. hispanica*, and will probably work for other halobacteria that are fairly robust compared to *Hbt. salinarum* strains. The latter organisms need higher salt (2M NaCl) in the spheroplasting solutions, and some labs use a lower PEG concentration (e.g. 30% instead of 60%: but see separate section, 3.2.2). For *Hfx. volcanii* and *Ha hispanica* the expected transformation efficiencies are about 10^6 transformants per μg (plasmid DNA). All procedures are at room temperature.

1. To 1 ml of concentrated cell suspension (fresh or freshly thawed and at RT; see previous section) in buffered spheroplast solution, add 100 μl of 0.5M EDTA (pH 8.0), and mix gently. Leave for 10 min at RT to form spheroplasts.

The EDTA causes the S-layer to fall off, so the cells round up. If new strains are being attempted, or difficulties are being experienced, check cells during EDTA treatment to see that they have all formed spheroplasts, i.e. individual, well-rounded cells, and that no lysis has occurred. If necessary incubate longer and/or raise the temperature to 37°C. and/or lower the salt concentration in the Spheroplast Dilution Solution.

2. While the cells are spheroplasting, pipette the DNA samples (1-10 μl) into the bottom of 1.5ml plastic microfuge tubes (sterile). Do not put more than 1-2 μg per transformation as the DNA will all precipitate if there is too much present when the PEG is added later.

You can add an equal volume of BSSG if you are worried about cells lysing when added to the DNA preparation, but this is usually not a problem.

3. Add 100 μl of spheroplasts to the tubes containing DNA and mix well by tapping the tube. Leave for 2 – 5min at RT.
4. Add an equal volume (i.e. 100 μl) of 60% PEG₆₀₀ solution, and mix by tapping the tube until a homogeneous opalescent suspension is obtained. This is a critical step and the PEG solution should be accurately prepared, ie. for 1 ml of 60% PEG₆₀₀ solution mix:

600 μl of pure PEG₆₀₀

400 μl of Unbuffered Spheroplast Solution (sectn. 3.3)

This is the step at which some cell preparations can lyse, producing a viscous, stringy solution. Transformation efficiencies will be so low it is not worth continuing. Try to find the reason for the lysis (detergent, old cells, wrong solutions, bad karma, etc.)

5. Incubate the mixture for 20 – 30 min at RT.

**The cells at this stage are somewhat aggregated by the PEG, and this can affect the apparent viable count.*

PLATING Transformation Mixtures

6. There are a number of ways of treating the mixtures at this stage to remove the PEG and allow the cells to regenerate; the choice being determined by the type of experiment, and cells. *Hfx. volcanii*, and *Har. hispanica* cells are quite hardy and can be centrifuged to remove the PEG, then resuspended in rich medium and immediately plated. I prefer to recover for 3 hours (to overnight) in rich medium before plating. For transposon mutagenesis, or where auxotrophic mutants are being studied, there must be sufficient nutrients for mutant cells to grow (i.e. not into minimal medium!)

- e.g.
- i. Add 1 ml of MGM and mix well by inversion.
 - ii. Centrifuge 5 min at 6,500 rpm (in a microfuge) to pellet cells.
 - iii. Remove the supernatant carefully, and resuspend cells completely in 1 ml of MGM.

The cells are sticky but can be teased apart by gently pipetting up and down in a 1ml tip of a micropipette

- iv. Allow to recover for 2 hr to overnight at 37°C.
- v. Plate onto selective media (below)

Before plating, the cells can be checked by light microscopy for their condition and degree of aggregation. If the cells have regained their shape then they should be healthy enough to plate. Lots of variations are possible for the transformation protocol. It can be done in a microtitre tray format (but you will need to have a centrifuge that can handle these trays). You can scale it up or down (I have used 50 µl up to 400 µl competent cells per transformation).

7. Plate in selective medium either by **spreading on plates** OR by incorporating cells into **overlays** (as described below).

- e.g.
- i. have a 100 ml lot of Top Agar (see 3.3.7) molten and stored at 55 – 60°C.
 - ii. Allow your base plates to warm up to RT and label them !
 - iii. Warm up a series of 10 ml plastic tubes (one for each plate) to 50°C.

If the same selective drug is to be used throughout, then add it to the top agar at the required final concentration. If several drugs or concentrations are to be used, then have stock solutions ready and at a concentration that can readily be dispensed into 4 ml volumes of top agar.

- iv. Dispense 3 – 4 ml volumes of top agar into the plastic tubes about 5 min before plating.

Keep the remainder of the top agar at 55-60°C

NOW, if everything is ready:-

8. Take 5 – 200 μ l of cells (depending on how many transformants per plate you expect; for normal plasmids you expect good efficiencies and 10 μ l may be plated, whereas for transposons you should plate 100 μ l).
9. Add this to a top agar tube (and if necessary, immediately add the selective drug). Mix quickly by hand or on a vortex mixer and pour onto a base plate (MGM or Defined Medium). Leave a couple of minutes on a level area to set.

We have used both methods successfully. The overlay method is an advantage when high concentrations of cells need to be plated (e.g. for transposon mutagenesis), as it gives a better selection. You can also incorporate all your selective drugs into the top agar and use normal plates as the base (so saving drug use). In our hands there seems to be little difference in transformation.

10. Incubate in plastic containers at 37 – 42°C for 5 – 15 days.

*I check progress at 3 days as colonies should be visible either by eye or with a 10x hand lens. Colonies should be large enough to subculture by 10 days (*Hfx. volcanii*), 12 – 15 days (*Har. hispanica*), or 20 days (*Halobacterium*). * (Note: as of 2005 we have started incubating *Hfx. volcanii* transformants at 45°C – see section 2.6.5. Transformants are ready for sub-culture within 5 days!). Make sure expressed water does not collect on the lid as this can splatter bacteria all over the plate.*

Cut the protocol out below and stick over your bench for a quick reference

SUMMARY OF HALOARCHAEAL TRANSFORMATION PROTOCOL

1. Thaw frozen cells in the hands and place on ice (use as soon as possible)
2. To 1 ml of frozen cells add 100 μ l of 0.5M EDTA pH 8.0, and mix quickly by tapping tube.
3. Leave 10min at RT to form spheroplasts
4. Add 1-10 μ l of DNA solution to a fresh tube, then add 100 μ l spheroplasts, mix well by tapping the tube.
5. Incubate cells and DNA for 2-5 mins.
6. Add an equal volume (100 μ l) of 60% PEG solution, mix well by tapping the tube (don't vortex)
7. Incubate 20min at RT
8. Add 1ml of growth medium and spin 5 min at 6,500 rpm (microcentrifuge) to pellet cells.
9. Resuspend cell pellet in 1ml of fresh growth medium and allow recovery (not really necessary) by incubating 2-4 hr 37°C.
10. Plate out on selective media

3.2.2 PEG-mediated Transformation of *Halobacterium salinarum*

This is a more difficult strain to transform as it lyses easily, and grows slowly. It requires a complex medium (doesn't grow on single carbon sources). The method below is a modification of that described by (Cline *et al.*, 1989a), and works well for *Hbt. salinarum* strains R1M1 and NCIMB 763. Valery Tarasov supplied the protocol below. Matthias Pfeiffer also recommends the use of 60% PEG.

1. Grow cells in good aerobic conditions: e.g. 50 ml in 100 ml flask with cotton-wool stopper, shaken 180 rpm.
2. Centrifuge cells in 1.5 ml volumes, 1 min at 10,000 g (in a microfuge).
3. Remove the supernatant and resuspend cells completely in 150 µl of spheroplasting solution (see section 3.3 below).
4. Add 15 µl of 0.5M EDTA (pH 8.0), mix well, and leave for 10 min at RT to form spheroplasts.
5. Add 10 µl of DNA (5 µl of DNA in water + 5 µl of spheroplasting solution), mix and leave for 5 minutes. (for recombination experiments, 5 µg is sufficient)
6. Add an equal volume (i.e. 175 µl) of 60% PEG₆₀₀ solution and mix by vigorously shaking by hand (3 – 4 times, 2 – 3 sec). NOT by tapping tube. (NOTE: vigorous shaking is necessary to avoid cell lysis).
7. Incubate the mixture for 20-30 min at RT.

Recovery of cells (two methods)

First method (a):

- 8a. Add 1ml of growth medium containing 15% sucrose.
- 9a. Centrifuge 2 min at 10,000 rpm to pellet cells.
- 10a. Remove the supernatant and resuspend carefully in 1ml of growth medium containing 15% sucrose.

Allow cells to recover overnight at 37°C, with shaking. (Note: the sucrose is necessary for recovery).

Second method (b):

8b. Add the mixture to a flask containing 15 ml of growth medium with 15% sucrose. Allow cells to recover overnight at 37°C, with shaking.

Finally:

11. Plate in selective media by spreading, and incubate 37°C. Colonies are visible to the eye in 8 – 10 days.

3.2.3 Comments and tips on PEG transformation of haloarchaea

Cell preparation and use in transformation. A general rule is to stick to standard regimes of cell preparation, and if in any doubt, look under the phase-contrast microscope to make sure the cells have formed spheroplasts. For long-term use, make a large batch of frozen cell stocks. *Halobacterium salinarum* strains are generally more delicate and demanding than *Haloferax* and *Haloarcula* spp. While Cline et al. (Cline et al., 1989a; Cline et al., 1989b) found 60% PEG was satisfactory for transformation of *Hbt. salinarum* R1, others have found lowered PEG concentrations are necessary, e.g. 30% for 30 min; Hackett and DasSarma (Hackett and DasSarma, 1989). Cline et al. (1989) noted that if *Hbt. salinarum* R1 cells were harvested too early in the growth phase they resisted spheroplasting. We found spheroplast formation was difficult in one strain; *Hrr. coriense* (previously Ch2, a close relative of *Hrr. saccharovororum*), and needed 30min at 37°C (compared to 10 min at RT for *Hfx. volcanii*). In our labs experience, some species can only be transformed when the cultures are at very early exponential phase ($OD_{600} = 0.2$)

Valery Tarasov (currently at Max-Planck, Munich) recommends only handling small volumes of *Hbt. salinarum*, i.e. just a 1.5 microfuge tube – larger volumes are too prone to lysis when handled for transformation. He also thought growth was faster (for *Hbt. salinarum*) when the medium began at an initial pH of 5.5 (i.e. without initial pH adjustment). He believes Tris buffer is inhibitory.

John Marsh of NCSU has also told me that 50% glycerol:50% spheroplasting solution also makes halobacteria competent (not as good as PEG). I've not heard more of this observation.

Regeneration of spheroplasts before plating. *Haloferax* and *Haloarcula* cells are quite rugged and can be centrifuged and washed of PEG, then resuspended in medium and plated in top agar immediately. *Halobacterium* species are too delicate for this and should be diluted in medium with 15% sucrose (10-100 fold) to lower the PEG concentration, then allowed to recover before plating (but see note below for *Haloferax*). The time necessary for recovery can be ascertained by looking at the cell morphology (i.e. have the cells regained their normal shape).

Methods of plating of transformation mixtures. The PEG will inhibit growth and must be either diluted or washed away from the cells. The use of sucrose (15%) in top agar overlay seems to be inhibitory. Older recipes used it but recent tests in our lab and Thorsten Allers (2003-4) indicate it is not useful, and often inhibitory. For *Hfx. volcanii*, spread plating is just as good as using top agar layers. Maybe it is useful in other, more delicate haloarchaea, but for *Haloferax* it seems better to leave it out.

Restriction barriers. *Haloferax volcanii* has at least two restriction systems. One degrades methylated DNA, specifically methylated A residues (at GATC sites), and can be avoided by using non-methylated DNA derived from *E. coli* strains with the *dam* genotype (e.g. JM110). The difference in transformation rates is about 1000-fold. The other system recognizes CTAG sites (Allers and Mevarech, 2005).

Restriction systems abound in most halobacteria, and have been detected in *Halobacterium* spp. using haloviruses (Patterson and Pauling, 1985), and in *Haloterrigena* (D. Wendoloski, unpublished). One restriction enzyme has been purified and studied in some detail by Schinzel and Burger (Schinzel and Burger, 1986), and although the specificity was not known when they published this, we recognized it to be CTCCT, and that it is not a type II enzyme (in 1986, personal communication to the authors).

A few strains seem to have little restriction of foreign DNA, among them *Haloarcula hispanica*, *Halobacterium* GRB, *Halobacterium salinarum* R1. *Hfx. lucentense* (= "*Hfx. lucentensis*", "*Hfx. alicantei*" or phenon K Aa 2.2) seems to have high nuclease activity, making plasmid preparations and DNA extractions tricky (Darrow Wendoloski, unpublished).

Types and size of DNA used to transform halobacteria. Plasmid, chromosomal, and phage DNA have all been used to transform halobacteria by the PEG mediated method of Cline et al. (Cline et al., 1989a). The size of the DNA fragments used does not seem to greatly affect transformation rate, with very large fragments (70 – 150 kb) being superior to smaller fragments (12 kb). Recombination rates of incoming DNA are estimated to be very good.

Types of transformants. Drug-resistant transformants are the easiest to select, and this has been done for *Hbt. salinarum*, *Hfx. volcanii*, and *Har. hispanica*. Transfection of Φ H DNA has been done in *Hbt. salinarum* and *Hfx. volcanii*, and more recently in this lab using other haloviruses and hosts (unpublished data). Gene replacement via recombination at chromosomal loci has been done in *Hfx. volcanii* and *Hbt. salinarum*, and this has produced both auxotrophic and deletion mutants (with or without a drug resistance gene).

If you are selecting for transformants that have LOST an enzymic function, then you may need to allow a longer (e.g. 24 hr) period for recovery after the PEG step to allow phenotypic expression.

Plasmid/genetic instability due to recombination: can be reduced or eliminated by using *radA*⁻ strains. First derived in this lab in 1997 for *Hfx. volcanii* (Woods and Dyall Smith, 1997) but I hear that other labs have generated similar mutants of *Hbt. salinarum*. These strains grow more slowly but plasmid stability is greatly enhanced (e.g. highly expressing beta-galactosidase plasmids). The strain name of our *radA* strain of *Hfx. volcanii* is DS52.

NOTES:

3.2.4 Freeze-Thaw transformation method

A method for transforming *Hbt. salinarum* with plasmid DNA was published by Arne Zibat in Biotechniques (Zibat, 2001). While the efficiency is very low (120 Tf/ μ g) compared to PEG method, it may be of use for large-scale screens where transformation efficiency is not a problem. The method presented was rather sketchy and further optimization could well improve it. It may also have possibilities for transforming strains recalcitrant to the PEG method. I have not tested this method, and since this publication I have not seen it used by others (*so please email me if you use it successfully*).

1. *Hbt. salinarum* cells were grown at 40°C in liquid complex medium, shaken, until an OD₅₅₀ of 0.9 – 1.0
2. Cells were spun down 3000× g at 4°C
3. Wash once with ice-cold FT buffer (4.3M NaCl, 27 mM KCl, 100 mM CaCl₂, 10 mM PIPES, pH 6.8)
4. Resuspend in 1 ml of ice-cold FT buffer.

If frozen stocks of competent cells are to be prepared, add glycerol to 10% (v/v) and store frozen at -70°C in 100 μ l lots. Cells were then thawed when required and processed the same as fresh cells

5. A quantity of 1-2 μ g plasmid DNA (in 2-5 μ l FT buffer) was added to 100 μ l of competent cells, and immediately mixed by tapping with a finger.
6. Leave on ice for 15 min, then store at -70°C for 30 min (to freeze).
7. Mixture thawed by incubation for 80 sec in 40°C waterbath, then kept on ice.
8. Add 900 μ l of ice-cold complex medium, and incubate 60min at 40°C without shaking.
9. Plate 200 μ l in 3ml of molten top agar (0.7% agarose, complex medium, kept at 60°C), and pour on top of support plates with selective drug (e.g. 10 μ g/ml mevinolin/simvastatin)
10. Incubate 40°C until colonies arise.

NOTES:

3.2.5 Selectable markers and plasmid replicons:

Below is a table that gives an idea of which plasmids and (drug) selectable markers work in different hosts. (Please tell me if you have any additional data!). A recent reviews on gene transfer system in haloarchaea is (1).

Plasmid	Marker/Replicon	Hosts (+/-)	Comment/Reference
pMDS20	NovR(Hfx.)/pHK2	<i>Hfx. volc.</i> , <i>Hb. sal.</i> (+) <i>Har. hisp</i> (-), <i>Har.vall</i> (-)	Not sure why it doesn't work in <i>Haloarcula hispanica</i> . (Holmes et al., 1994)
pMDS30	as for pMDS20	as for pMDS20	Blue/White selection in <i>E.coli</i> . (Kamekura et al., 1996)
pMDS99	MevR(Har.)/pHV2	<i>Hfx. volcanii</i>	<i>Har. hispanica</i> derived MevR gene, does not recombine with <i>hmgA</i> in <i>Hfx. volcanii</i> . (Wendoloski et al., 2001).
pWL102	MevR(Hfx.)/pHV2	<i>Hfx.volc.</i> , <i>Hfx. vall.</i> , <i>Har. hisp.</i> <i>Hb. sal.</i> (all +)	Will not replicate in <i>radA</i> host. (Lam and Doolittle, 1992)
pUBP2	MevR(Hfx.)/pHH1	<i>Hfx. volc.</i> , <i>vall.</i> , <i>Har. hisp.</i> (all +)	(Cline and Doolittle, 1992) (Blaseio and Pfeifer, 1990)
TmR (dfr)	TmR(Hfx.)/pHV2	<i>Hfx.volc.</i> (+)	Only works in <i>Hfx. volcanii</i> . (Zusman et al., 1989)
pGRB1 pMPK series	MevR(Hfx.)/pGRB	<i>Hbt.sal.</i> (+)	(Krebs et al., 1991; Krebs et al., 1993)
pMLH32	NovR(Hfx.)/pHK2	<i>Hfx. volc</i> (+)	(Holmes and Dyal-Smith, 2000)
pUS- MEV ^a	MevR(Hfx) / non	<i>Hbt. sal.</i> (+)	Suicide vector. Positive clones only by integration in the genome; (Pfeiffer et al., 1999). See below

Abbreviations: *Hfx.* = *Haloferax*. *Har.* = *Haloarcula*. *vall.* = *Haloarcula vallismortis*. *Hbt.sal.*=*Hbt.salinarum*.

NovR = novobiocin resistance (*gyrB* gene). MevR = mevinolin resistance (*hmgA* gene)

TmR = trimethoprim resistance (*dfr* gene, coding for dihydrofolate reductase)

Note: Novobiocin is said to affect motility in *Halobacterium salinarum*, so mevinolin-resistance may be better if studying this aspect. (M. Alam, personal communication)

^a pUS-MEV: for details see Matthias Pfeiffer: Max-Planck-Institut für Biochemie, Dept. Membrane Biochemistry, 82152 Martinsried, Germany. fax +49-(0)89-8578-2370; email: pfeiffer@biochem.mpg.de; web: www.biochem.mpg.de

See section 7.2 for more details on drugs and resistance.

3.3 Transformation Solutions - MDS Lab

3.3.1 Unbuffered Spheroplasting Solution - Low Salt (UBSS-LS)

For Haloferax and Haloarcula (1M NaCl, 27mM KCl, 15% (w/v) sucrose)

For 1 Litre: Made up from sterile stock solutions or powders as follows:

1. Add the following components to a 1 or 2 L beaker or graduated cylinder.

COMPOUND*	VOLUME (Stock)	OR	Mass (g)
(final concn)			
NaCl (1M)	200 ml (5M)		58.5 g
KCl (27mM)	27 ml (1M)		2.01 g
Sucrose (15%)	300 ml (50% w/v)		150.0 g

(MW NaCl= 58.5, MW KCl = 74.5)

2. Add pure water to 900 ml, and mix to dissolve most of the salt and sucrose.
3. Check the pH is around neutral, adjust with a small amount of dilute acid or base.

Pour into a graduated cylinder and add water to exactly 1000 ml.

4. Dispense 100 ml volumes into suitable bottles and autoclave 10lb, 30 min.
- *S. Cline changed to 0.8M NaCl in his later publications and I suspect there is little difference.*

3.3.2 Buffered Spheroplasting Solution - Low Salt with Glycerol (BSS-LS+G)

For *Haloferax* and *Haloarcula* (1M NaCl, 27mM KCl, 50mM Tris.Cl(pH 8.2), 15% (w/v) sucrose, 15% glycerol)

For 1 Litre: Made up from sterile stock solutions or powders as follows.

1. Add the following components to a 1 or 2L beaker or graduated cylinder.

Compound (concn)	Volume (Stock)	or	Volume/Mass (Stock)
(final concn)			
NaCl (1M)	200 ml (5M)		58.5 g
KCl (27mM)	27 ml (1M)		2.01 g
Tris.Cl (50mM, pH 8.2)	50 ml (1M)		50 ml (1M)*
Sucrose (15%)	300 ml (50% w/v)		150 g
Glycerol (15%)	180 ml (80% v/v)		150 ml (100%)

(MW NaCl= 58.5, MW KCl = 74.5)

*Adding the Tris buffer after autoclaving may help reduce the possibility of caramelisation of the sucrose.

2. Add pure water to 1000 ml, mix to dissolve all components and dispense into 100ml volumes in suitable bottles and sterilize by autoclaving (10 p.s.i. , 30 min). Store at RT.

3.3.3 Unbuffered Spheroplasting Solution - High Salt (BSS-HS)

For *Halobacterium salinarum* - **NOTE** the higher NaCl concentration.

2M NaCl, 27mM KCl, 15% (w/v) sucrose

For 1 Litre: Made up from sterile stock solutions or powders as follows:

1. Add the following components to a 1 or 2L beaker or graduated cylinder.

COMPOUND	VOLUME (Stock)	OR	MASS
(final concn)			
NaCl (2M)	400 ml (5M)		117 g
KCl (27mM)	27 ml (1M)		2.01 g
Sucrose (15%)	300 ml (50% w/v)		150 g

(MW NaCl= 58.5, MW KCl = 74.5)

2. Add pure water to 900 ml, and mix to dissolve most of the salt and sucrose.
3. Check the pH is around neutral, adjust with a small amount of dilute acid or base.

Pour into a graduated cylinder and add water to exactly 1000 ml.

4. Dispense 100 ml volumes into suitable bottles and autoclave 10 p.s.i., 30 min.

3.3.4 Buffered Spheroplasting Solution - High Salt with Glycerol (BSS-HS+G)

For *Halobacterium salinarum* - Note the higher NaCl concentration.

(2M NaCl, 27mM KCl, 50mM Tris.Cl(pH 8.75), 15% (w/v) sucrose, 15% glycerol)

For 1 Litre: Made up from sterile stock solutions or powders as follows.

1. Add the following components to a 1 or 2L beaker or graduated cylinder.

Compound	Volume (Stock soln.) or	Volume/Mass (Stocks)
		(final concn.)
NaCl (2M)	400 ml (5M)	117 g
KCl (27mM)	27 ml (1M)	2.01 g
Tris.Cl (50mM, pH 8.2)	50 ml (1M)	50 ml (1M)
Sucrose (15%)	300 ml (50% w/v)	150 g
Glycerol (15%)	180 ml (80% v/v)	150 ml (100%)

(MW NaCl= 58.5, MW KCl = 74.5)

2. Add pure water to 1000ml, mix to dissolve all components and dispense into 100ml volumes in suitable bottles and sterilize by autoclaving (10 p.s.i., 30 min). Store at RT.

3.3.5 Spheroplast Dilution Solution (23% SW)

For Haloferax and Haloarcula

23% salt water, 10mM Tris.Cl (pH 7.5), 15% (w/v) sucrose, 0.2%(w/v) peptone.

Modified from the original recipe of Cline et al. (1989).

We now use **23% MGM** instead of this for the recovery of spheroplasts.

For 1 Litre: *Made up from sterile stock solutions or powders as follows:*

1. Add the following components to a 1 or 2L beaker or graduated cylinder.

30% SW (-> 23% final)	766	ml
Pure Water	150	ml
1M Tris.Cl (10 mM, pH 7.5)	10	ml
sucrose (15%)	150	g
peptone (0.2%)	2	g

2. Mix until dissolved. Check pH. Adjust volume with pure water to 1000 ml.
3. Dispense into 100 ml volumes in suitable glassware
4. Autoclave 10 p.s.i., 20 min.

3.3.6 60% PEG₆₀₀ solution

We make this up fresh for each transformation (though it could probably be stored at -20°C). Make sure the volumes are accurate, as correct PEG concentration is very important for high transformation rates. The PEG₆₀₀ was shown to perform best if it was freshly purified, but the method is not trivial and uses hazardous organic solvents. Most labs seem to get by using freshly bought PEG, but be aware there may be considerable batch-to-batch variation (S. Cline, personal communication).

i.e. for 1 ml (enough for 10 × 100 µl transformations).

In a 1.5ml microfuge tube add: 600 µl of purified PEG₆₀₀ and

400 µl of Unbuffered Spheroplast solution and mix thoroughly.

The PEG stock is viscous, so use a wide bore micropipette tip to pick up the correct volume. The correct concentration is important for high transformation rates.

3.3.7 Transformation Top Agar

For plating transformation mixtures. This is identical to MGM except that it has reduced agar (6 g/L). It can also have 15% (w/v) sucrose (i.e. 15 g per 100 ml MGM) if necessary (see * below). If using sucrose, dissolve the sucrose before adding the agar, then autoclave at reduced pressure (10 lb, 20 min) to avoid caramelisation (browning) of the sucrose, which will inhibit the growth of transformants. Adding the Tris after sterilization can also reduce caramelisation. Mix when cooling so as to dissolve all the agar (which tends to settle to the bottom).

***NOTE:** it may not be necessary to have sucrose present when using *Hfx. volcanii*. Test it out and see for yourself. It was originally recommended by Steve Cline but members of my lab (and Thorsten Allers) have told me it doesn't affect transformation rates of *Hfx. volcanii* and *Har. hispanica*.

i.e. per 100ml:

1. Weigh out into a 100 ml graduated cylinder: 15 g of sucrose
0.6 g of Oxoid Agar (No.1)
2. Add (23%) MGM to 100 ml and stir to dissolve the sugar.
3. Sterilize at reduced temperature, e.g. 10 p.s.i (70 kPa) for 20 min.
4. Mix when removing from the autoclave and store at 55-60°C until needed.
5. Before use, make sure any selective drugs have been added.

Both novobiocin and Simvastatin/Mevinolin are relatively heat stable.

3.3.8 Minimal Medium (MM) Transformation-Top Agar for plating transformation mixtures

This is the same as minimal medium except that it also contains 15% (w/v) sucrose, and a lower agar concentration (6 g/L). It is convenient to add the sucrose to the salt water/Tris buffer solution and dissolve it before adding the agar. Autoclaving at reduced temperature (10lb, 20 min) avoids caramelisation of the sucrose. When molten, make sure to mix up the agar, which settles to the bottom, and store at 55-65°C. It can be stored at this temperature for 2-3 days (without drugs added).

**NOTE: as of 2003, we no longer believe sucrose is necessary, at least for *Haloferax* and *Haloarcula*. More sensitive strains may need it (e.g. *Halobacterium*).*

3.4 *Haloferax volcanii* Transformation - Thorsten Allers Laboratory

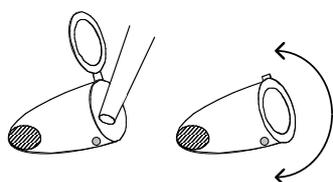
*Communicated by Thorsten Allers, Nottingham, UK, 2003. See the reference below for details:
Allers, T., Ngo, H., Mevarech, M., and Lloyd, R. G. (2004). Development of additional selectable markers for the halophilic archaeon Haloferax volcanii based on the leuB and trpA genes. Appl Environ Microbiol 70: 943-953*

Day 1

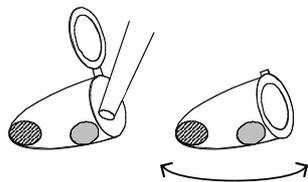
1. Ensure adequate quantities of transformation solutions are present, i.e. buffered and unbuffered spheroplasting solutions, spheroplast dilution solution and regeneration solution – see below
Also need **3 selective and 1 non-selective plates per transformation**
2. Set up 10 ml overnight culture(s) of strain in Hv-YPC (+additives), using 1–4 colonies
10 ml overnight culture enough for 3–4 transformation reactions
3. Leave PEG 600 at RT to thaw overnight
Warm to 37°C if necessary

Day 2

4. When 10 ml culture reaches $A_{650} \approx 0.8$, pellet at 6000 rpm for 8 min at 25°C.
4,500 × g. Use round-bottomed (e.g. 14 ml) tubes. All subsequent steps at RT unless indicated otherwise.
5. Resuspend pellet gently in 2 ml buffered spheroplasting solution.
Transfer to 2 ml round-bottomed tube and pellet cells at 6000 rpm, 8 min at 25°C.
6. Very gently resuspend in 600 µl of buffered spheroplasting solution.
For 4 transformations use 800 µl, i.e. 200 µl/transformation. Avoid air bubbles.
7. For each transformation, transfer 200 µl cells to clean 2 ml round-bottomed tube.
Add 20 µl drop of 0.5 M EDTA pH 8.0 on side of tube, then invert to mix.
Leave at RT for **10 min** to form spheroplasts



8. Meanwhile set up DNA samples in 30 μ l total:
- 10 μ l of *dam*⁻ DNA (~1–2 μ g, ultra-pure) or 10 μ l of dH₂O (control)
- 15 μ l of unbuffered spheroplasting solution
- 5 μ l of 0.5 M EDTA pH 8.0
6. After 10 minutes add DNA to spheroplasts in same manner as EDTA (see 4 above).
Leave at RT for **5 minutes**.
7. Meanwhile prepare 60% PEG 600 solution. For 3 transformation reactions use:
- 480 μ l of PEG 600
- 320 μ l of unbuffered spheroplasting solution.
8. After 5 minutes add 250 μ l (equal volume) of 60% PEG 600 to each transformation
Add in same manner as EDTA, but shake tube horizontally ~10 times to mix gently.
Leave at RT for **30 minutes**.
9. Add 1.5 ml spheroplast dilution solution, invert to mix and leave at RT for **2 min**.
Pellet in microfuge at 6000 rpm for 8 min at 25°C, and remove supernatant.
10. Add 1 ml regeneration solution (+ 60 μ g/ml thymidine, if required).
Use wide-bore blue tip to suck up and transfer whole pellet to 4 ml sterile tube.
Avoid resuspending pellet at this point, but use blue tip to scrape cells off wall of tube
11. Leave tube undisturbed at 45°C for **1.5 – 2 hr** to regenerate.
Resuspend pellet by tapping side of tube. Return to 45°C for **3 – 4 hr**, rotating.



12. Transfer cells to 2 ml round-bottomed tube, pellet at 6000 rpm for 8 min at 25°C.
Remove supernatant and resuspend gently in 1 ml transformant dilution solution
This step is not necessary if NovR or MevR selection is used. For Thy⁺ selection, regeneration solution can be used

13. Plate 100 μ l on Hv-YPC, Hv-Ca or Hv-Min plates. Use following dilutions:
Dilute in spheroplast regeneration solution for antibiotic selection, otherwise use transformant dilution solution

	Selective	Non-selective
+DNA	10 ⁰ , 10 ⁻¹ , 10 ⁻² , 10 ⁻³ or 10 ⁻⁴ use 3 dilutions, depending on transforming DNA ^{1*}	10 ⁻⁶
No DNA control	10 ⁰ , 10 ⁻¹ , or 10 ⁻² use 2 dilutions, depending on selection	10 ⁻⁶

* For integration of linear or circular DNA use 10⁰, 10⁻¹, 10⁻², for episomal DNA use 10⁻², 10⁻³ or 10⁻⁴, 100 \times more for dam⁺ DNA

14. Leave plates at 45°C for at least 5 days.

3.4.1 Transformation Solutions: (Thorsten Lab. recipes)

3.4.1.1 Buffered Spheroplasting Solution *(these are similar to the recipes described in 3.3)*

14.61 g NaCl (1 M)
0.5 g KCl (27 mM)
12.5 ml 1 M Tris.HCl pH 8.5 (50 mM)
37.5 g Sucrose (15%)
pure H₂O to 250 ml

Autoclave for 10 minutes

3.4.1.2 Unbuffered Spheroplasting Solution

5.84 g NaCl (1 M)
0.2 g KCl (27 mM)
15 g Sucrose (15%)
Pure H₂O to 100 ml

Adjust to pH 7.5 (~10 μ l 1 M NaOH). Autoclave for 10 minutes

3.4.1.3 Spheroplast Dilution Solution

76 ml 30% SW	(23%)
15 g Sucrose	(15%)
dH₂O	to 100 ml

Autoclave for 10 minutes. When cool add 0.75 ml of 0.5 M CaCl₂

3.4.1.4 Regeneration Solution

150 ml 30% SW	(18%)
25 ml 10X YPC	(1x)
37.5 g Sucrose	(15%)
dH₂O	to 250 ml

Autoclave for 10 minutes. When cool add 1.5 ml of 0.5 M CaCl₂

3.4.1.4 Transformant Dilution Solution

150 ml 30% SW	(18%)
37.5 g Sucrose	(15%)
dH₂O	to 250 ml

Autoclave for 10 minutes. When cool add 1.5 ml of 0.5 M CaCl₂

NOTES:

3.5 Transposon Mutagenesis of *Haloarcula hispanica*

Transposons for use in *Haloarcula hispanica* have been constructed (e.g. Thd76 for Transposon of halobacteria version d76) and are based on the ISH28 insertion sequence from *Halobacterium salinarum*, and the mevinolin resistance marker (*hmg* gene) from *Hfx. volcanii*. The sequence identities of these constructs to the genomes of *Hbt. salinarum* and *Hfx. volcanii* means they can only be used in hosts that contain neither ISH28 or a *hmg* gene of high similarity (otherwise they recombine homologously into the chromosome). Experiments to date have used *Har. hispanica* as it is an easily transformed and fairly robust strain. It grows a bit more slowly than *Hfx. volcanii*, requires 23% salt for growth, and is slightly more sensitive to Mevinolin/Simvastatin (MIC about 1 μ M) than *Hfx. volcanii*. One marvelous feature of *Har. hispanica* is that it differentiates into thick-walled cell clusters (and forms very pretty colonies) if left for a long time in liquid or solid media.

At the present time we know that insertion is not entirely random: there is a preference for AT-rich sequences, but they integrate at many different sites (including GC-rich genic DNA), and produce an 8 bp duplication of the target. The latest versions (as yet unpublished) only jump once and are easily recoverable into *E. coli*. We are currently testing completely new transposons for use in *Hfx. volcanii*. See, (Dyall-smith and Doolittle, 1994) and (Woods et al., 1999).

METHOD

1. Transform frozen cells with plasmid DNA using the normal PEG method for halobacteria.
2. Use 200 μ l of cell suspension and the highest concentration of DNA possible (about 1-2 μ g/100 μ l cells). The plasmid does not need to come from a *dam*⁻ *E.coli* strain for use in *Har. hispanica*
3. After the 20 min PEG treatment, add 1 ml of 23% MGM to the microfuge tube, mix and spin down the spheroplasts (5 min, 6,500 rpm) in a microfuge.
4. Discard the supernatant and resuspend the spheroplasts gently but thoroughly (with a 1 ml micropipette) in 1ml of 23% MGM.
5. Incubate 37°C for 2 hrs to overnight without shaking to allow the cells to recover.
6. Plate 100 μ l volumes in top agar (containing 2 μ g/ml Simvastatin).
7. Incubate 37°C making sure to remove any liquid that accumulates in the Petri lid. (e.g. use a tissue to absorb it from between the lid and base). Colonies will be visible (by hand lens at least) by 6 days, and can be picked by 10 – 15 days.

We routinely expect transformation efficiencies of about 10³ tf/ug plasmid, which for 1ug of input DNA should produce about 100 colonies per plate using the method above.

3.6 Natural Genetic Transfer (method of Tchelet and Mevarech, 1994).

The currently favoured mechanism of transfer is one of cell fusion, allowing both plasmid and chDNA to cross in both directions between cells. This process is blocked by restriction barriers and by halocins (e.g. Hfx. mediterranei has a potent inhibitor of Hfx. volcanii cells (see above paper for details). (Tchelet and Mevarech, 1994)

NOTE: transformation frequencies are low; of the order 10^{-6} .

1. Parental *Haloferax* strains are grown up in rich medium to an A₆₀₀ of 2.5
2. 0.5 ml of each culture was centrifuged and resuspended in SMT solution e.g. 6,500 rpm, 5 min, RT.
3. These were then mixed and filtered onto a nitrocellulose filter (BA85, 25 mm, Schleicher and Schuell Inc.)
4. The filter was incubated on a rich medium plate for 24 hr, 37°C
5. The bacteria were suspended in liquid medium by vortexing the filter in a test tube containing 5 ml of rich medium.
6. The culture was shaken for 24 hr at 37°C to allow phenotypic expression.
7. 1 ml of culture was centrifuged (6,500 rpm, 5 min) and the cells resuspended in SMT, and plated on the appropriate selective media to score transformants.

NOTES:

4.0 DNA and RNA Extraction Procedures

4.1 Plasmid DNA

4.1.1 Plasmid Minipreps

There are two modifications one needs to make to standard alkaline lysis plasmid miniprep protocols:

1. In the first centrifugation step, since haloarchaeal cells are usually smaller than *E.coli* they need to be centrifuged for at least 2min (not 30 sec) at 10,000 g. Remove the supernatant carefully with a micropipette so as not to disturb the pellet.
2. To resuspend the cell pellet, the buffer must have salt in it (1M for *Haloflex* spp; 2M for *Halobacterium salinarum*) or the cells will lyse immediately and the pellet will not be able to be evenly resuspended.

The rest is the same as normal, but read notes below.

TYPICAL PROTOCOL:

1. Spin down 1.5 ml of culture 2 min, 13,000 ×g, in a microfuge tube, discard supernatant carefully without disturbing the pellet.
2. Resuspend pellet in 50 µl of 1M NaCl.
3. Lyse with 200 µl SDS/OH solution (1%/0.2M). Mix gently by **INVERSION**. Leave 5 min at RT
4. Add 150 µl potassium acetate solution, mix by **inversion**, spin 3 min, 13,000 ×g.
5. Remove supernatant to fresh tube and precipitate with 2 vol ethanol. Leave 2min, spin 5 min, 13,000 ×g. Wash 2×.

NOTES:

a) You may need to double the volume of cells to get enough plasmid to see on a gel. Simply spin down another 1.5 ml of cells in the same tube.

b) Several halobacteria produce intracellular and/or extracellular polysaccharides (e.g. *Hfx. mediterranei* accumulates polyhydroxybutyrate). The amount of this material will vary from strain to strain and with medium and culture conditions, but if present it will often contaminate plasmid minipreps (e.g. using the alkaline lysis method), and cause poor resolution of bands in

agarose gel electrophoresis. Commercial plasmid purification kits (e.g. Wizard Preps) can overcome this problem (as can CsCl gradient purification).

c) Some lab strains of *Hfx. volcanii* WFD11 may not give as good a preparation of plasmid as the wt parent. Not sure why.

d) Some strains have a strong endonuclease activity making plasmid yields very low from stationary cultures. Darrow Wendoloski (my lab) found that you can often get round this by using cultures at very early exponential phase (e.g. $A_{600} = 0.1 - 0.2$). You need more culture (to get the same amount of cells), but the amount of plasmid you get is much better.

4.1.2 Large Scale Plasmid DNA Preparation

*As discussed above, the only important modification necessary for the standard alkaline-lysis method is to add NaCl (1M for *Haloflex* spp; 2M for *Halobacterium salinarum*) to the resuspension buffer so that after the initial centrifugation step a homogeneous cell suspension can be made which will allow even and complete lysis in the following step.*

4.2.1 *Hfx. volcanii* Genomic DNA Miniprep by DNA Spooling

Contributed by Thorsten Allers, University of Nottingham, UK, who adapted it from a method used in Moshe Mevarech's laboratory. Also works for Hbt. salinarum, but that needs a phenol-chloroform extraction before the final ethanol precipitation. The DNA cuts well for restriction analyses.

1. Set up 5 ml liquid culture in Hv-YPC using 1 – 4 colonies.
Grow overnight at 45°C until $A_{650} \approx 0.6 - 0.8$, i.e. dense but not saturated (not red)
2. Pellet 1 ml of culture at 6,000 rpm for 5 min in microcentrifuge (use a 2 ml, round-bottomed microfuge tube). Remove supernatant
3. Resuspend in 200 μ l of **ST buffer** (1 M NaCl, 20 mM Tris.HCl), scraping cells off wall of tube before pipetting
4. Add 200 μ l **lysis solution** (100 mM EDTA pH 8.0, 0.2% SDS) to each tube and invert to mix

At this point deal with each sample one at a time

5. Overlay aqueous layer with 1 ml ethanol
Spool DNA at interface onto capillary (gel-loading) tip (Glass capillary can also be used)
6. Continue spooling DNA until liquid is homogeneous and clear
7. Transfer spool of DNA to eppendorf tube with 1 ml ethanol, and swirl to wash DNA
Repeat with a fresh 1 ml of ethanol, then let excess ethanol drain from DNA

Leave to drain while processing next sample, then go to step 8

8. Resuspend spool of DNA in 500 μ l TE in a fresh microfuge tube. Leave to soak in TE until all samples are processed, then scrape DNA off tip onto lip of eppendorf

At this point deal with all samples at the same time

9. Add 50 μ l 3 M sodium acetate (pH 5.2) and 400 μ l isopropanol, and invert to mix
10. Pellet at 6,000 rpm for 5 min.
11. Wash pellet with 1 ml 70% ethanol and dry pellet thoroughly
12. Resuspend DNA in 100 μ l TE
Add 1 μ l RNase (30 mg/ml) and incubate (shaking) at 45°C for ≥ 1 hr

RNase A solution (30 mg/ml) in 50% glycerol from Sigma (Cat. R 4642)

13. Leave DNA at 4°C overnight to resuspend completely.
Use 5 µl per digest for Southern blots.

ST Buffer (Autoclave. Store at RT.)	Lysis Solution (Autoclave. Store at RT.)
40 ml 5 M NaCl	40 ml 0.5 M EDTA pH 8
4 ml 1 M Tris.HCl pH 7.5	4 ml 10% SDS
156 ml H ₂ O to 200 ml	156 ml H ₂ O to 200 ml

4.2.2 Chromosomal DNA isolation (rapid, small scale method)

This method was given to me by Wan Lam (in 1991), and has been successfully used in my lab. for both Haloferax and Haloarcula species, and is probably fine for most halobacteria (except halococci- because of their tough cell walls). It allows a number of cultures to be easily handled at the same time and gives DNA suitable for Southern blotting. It does NOT give high molecular weight DNA suitable for partial enzyme digests.

1. Centrifuge 200 μ l of stationary phase culture for 2 min at 10,000 \times g in a 1.5 ml microcentrifuge tube.
2. Remove the supernatant with a micropipette; as much as possible without removing cells.
3. Squirt a 200 μ l volume of distilled water onto the cell pellet and rapidly vortex to completely disrupt and lyse the cells.
4. Add 200 μ l of buffer-saturated phenol (pH 8), and mix well to extract protein (and probably some carbohydrate) from the lysate.
5. Incubate at 65°C for 60 min, then centrifuged (10,000 \times g, 5 min).
6. Remove the aqueous top phase (carefully) to a fresh tube.

The interface material should be avoided as much as possible although this can be difficult as it tends to pull up into the pipette tip. Do not be overly concerned as the end result is usually still good enough for enzyme digestion and Southern blotting.

7. Add 400 μ l of cold ethanol (100%), mix well, and allow the DNA to precipitate on ice for 10 min.
8. Centrifuge 10 min, 10,000 \times g to collect the precipitate. Pour off the ethanol and wash the precipitate with 1ml of 70% ethanol. Centrifuge (10 min, 10,000 \times g) and carefully remove the ethanol.
9. Allow the pellet to dry out (but not completely) and dissolve in 50 – 200 μ l of water. Store at -20°C.

For restriction enzyme digestions 15 μ l samples are usually sufficient. Remember that these preparations will not have high molecular weight DNA for partial enzyme digestions.

If high quality chDNA preparations are required then use any of the common protocols, such as those in 'Current Protocols in Molecular Biology' Ausubel et al., 1988, or buy one of the many kits available.

4.2.3 Alternative rapid chromosomal DNA isolation

This method was sent by Matthias Pfeiffer (Max-Planck-Institut für Biochemie Dept. Membrane Biochemistry 82152 Martinsried Germany. Email pfeiffer@biochem.mpg.de. He comments: "I have much better and more reproducible results using the DNA isolated by this method than using other methods. Another advantage is the time of preparation: a few minutes (ca. 3 without the denaturation step). If I use the isolated DNA as template for PCR I even forego the protein denaturing step (4) because of the hot start with the PCR. I have never had problems cutting this DNA for southern blots." (MP)

1. Spin down 1 mL of a freshly grown *Hbt. salinarum* culture at exponential or early stationary phase (1 minute at max. rpm in a microcentrifuge)
2. Suck off the supernatant.
3. Add 400 µL pure water and lyse the cells by gently pipetting. The cells disrupt immediately by osmotic lysis.
4. Inactivate the proteins by heating the DNA solution up to 70°C for 10 min.

75°C for 10min is also fine (MDS)

THAT'S IT!!!

Comments

- a) DNA from freshly grown cultures (exponential or early stationary phase) give better results for further reactions than old ones.
- b) use 1 – 3 µl as a template for amplifying a DNA fragment by PCR.
- c) digest 30 µl (in 100 µl final volume) for southern blot analysis.
- d) if there is a problem using the DNA as template for PCR it could be due to much DNA. Try several concentrations or volumes e.g. 1, 5, 10 µl from stock solution.

4.2.3 *Haloferax volcanii* DNA Prep in Agarose Plugs for PFGE

1. Set up overnight culture in 5 – 10 ml.
2. Prepare 1.5% low-melt agarose in 0.5× buffered spheroplasting solution + 100 mM EDTA:
 - i. Add 0.15 g low-melt agarose to 4 ml pure H₂O + 1 ml 0.5 M EDTA, boil to melt.
 - ii. Then add 5 ml hot (> 75°C) buffered spheroplasting solution + 100 mM EDTA.
 - iii. Mix thoroughly, leave at 42°C (for 30 minutes) to cool.
3. Take 5 – 10 ml cells ($A_{650} \approx 0.5-1.0$, $\sim 0.5 - 1 \times 10^{10}$ cells/ml), chill on ice for 10 minutes.
Pellet at 6 k rpm for 8 minutes at 4°C.
4. Resuspend pellet gently in 2 ml of cold buffered spheroplasting solution, transfer to 2 ml round-bottomed microfuge tube on ice and pellet again (6000 rpm, 8 min, 4°C).
5. Gently resuspend in 150 µl of buffered spheroplasting solution. Transfer tube to 42°C.
6. Add 150 µl of 1% low-melt agarose at 42°C, and mix gently but thoroughly using blue tip.
Pipet 80 µl into plug molds (enough for 3 – 4 plugs). Leave on ice for 10 minutes.
7. Transfer plugs to 15 ml tube, add 5 ml lysis solution + proteinase K.
Equilibrate plugs at 45°C for 2 – 4 hours
8. Remove lysis solution and replace with 5 ml fresh lysis solution + proteinase K + RNAase.
Equilibrate plugs at 45°C overnight.
9. Wash plugs in 10 ml wash solution for 1 hour at 37°C.
Wash plugs in 10 ml wash solution + Phenyl Methyl Sulfonyl Fluoride (PMSF) for 1 hour at 37°C.
PMSF inactivates proteinase K. It is not necessary if no restriction digest is planned. PMSF is also TOXIC so take precautions and dispose of carefully.
10. For temporary storage leave plugs in 5 ml wash solution at 4°C.
For long term storage leave plugs in 5 ml wash solution + 50% glycerol at –20°C.
11. For PFGE, equilibrate plugs with 10 ml gel electrophoresis buffer for 1 hour at room temp.
For restriction digest, first equilibrate 1 hour in 10 ml 10 mM Tris, then twice for 1 hour in 5 ml 1× buffer.
Remove buffer, melt plug at 65°C, cool to 37°C. Add 5 – 10 µl enzyme (100 U/µl), incubate at 37°C, 4 hours.

12. Insert plugs into wells, then seal in place with molten agarose. Run PFGE.

Solutions and reagents for DNA prepn.

Buffered Spheroplasting Solution [*see* section 3.4.1.1]

Spheroplasting Solution + EDTA

14.61 g NaCl	(1 M)
0.5 g KCl	(27 mM)
12.5 ml 1 M Tris.HCl pH 8.2	(50 mM)
50 ml 0.5 M EDTA pH 8	(100 mM)
37.5 g Sucrose	(15%)
<i>pure H₂O to 250 ml</i>	

Lysis Solution

1.5 ml 3 M Tris.HCl pH 8.8	(~20 mM)
250 ml 0.5 M EDTA pH 8	(~500 mM)
2.5 g sarkosyl	(1%)
Total	~250 ml
Add RNase (30 mg/ml) to 10 µg/ml if required	
Add proteinase K (powder) to 0.5–1 mg/ml if required	

Wash Solution

6.25 ml	1 M Tris.HCl pH 7.5	(25 mM)
50 ml	0.5 M EDTA pH 8	(100 mM)
Pure H ₂ O to 250 ml		
Add 0.5 mM PMSF (100 mM in ethanol) if required		

Wash Solution + Glycerol

2.5 ml 1 M Tris.HCl pH 7.5	(25 mM)
20 ml 0.5 M EDTA pH 8	(100 mM)
50 ml glycerol	(50%)
<i>pure H₂O to 100 ml</i>	

4.2.4 PCR of haloarchaeal DNA. Recommended enzymes and conditions

Why is it that PCR's of haloarchaeal DNA templates often end with a blank gel? Strangely, in the published literature, PCRs always seem to work! Haloarchaea generally have DNA with a high mol%G+C, in the range 55-68%, making denaturation of dsDNA and stable secondary structures more difficult than with the DNA of many other organisms with lower %G+C, such as E. coli. If you are having problems, and your controls show that your reagents are working (on E. coli DNA), then altering the denaturation conditions (such as including a 'PCR enhancer', such as betaine or DMSO) or changing to a polymerase enzyme that can handle high GC templates are worth looking at first. A slight increase in the denaturation temperature often works, but see the tips below.

Tip 1. Platinum[®] Pfx DNA Polymerase from Invitrogen works satisfactorily if used with their additive ("10X PCRx Enhancer"). However, we have since found that we get **much better results** with two new enzymes by Finnzyme, Dynazyme EXT and Phusion (the latter is very high fidelity). I really recommend these enzymes, especially as they are cheaper than Platinum Pfx. Details are here: http://www.finnzymes.fi/products/products_pcr.htm

The additive for DynaZyme EXT and Phusion is DMSO, and we use it at 5%. It is important to use the GC buffer supplied with Phusion, not the standard (HF) buffer. The denaturation temperatures are as recommended by the manufacturer.

Tip from Thorsten Allers, Nottingham University, 2006.

Tip 2. Use a 1:10 mix of Deep Vent (NEB) : Taq (NEB) for PCR of GC-rich templates. Deep Vent and Taq on their own are not so impressive but the two together seem to work well just about everytime.

Tip from Brendan Russ, Univ. Melbourne, 2006

Be aware of the many other possibilities for consistently failing PCRs

i) the obvious ones - e.g. check your primer sequences are correct, the PCR machine is working correctly, etc.

ii) your organism may have a high nuclease content and degrades your DNA rapidly and extensively upon extraction.

iii) your extract from an environmental sample has inhibitors (e.g. humic acids) that will knock out PCR enzymes at vanishing concentrations!

4.3 RNA isolation and northern blotting

This can be very frustrating and most protocols are complex. We used to find the commercial RNA isolation kits extremely variable and gave them away for several years. However, the newer kits seem to be better, particularly the Qiagen kits. There are also good manual methods of RNA isolation and northern blotting that are fairly easy and safe, and one such example is given below. Remember that the usual precautions need to be taken against RNAses (i.e. DEPC treated water, clean area reserved for RNA work, wear gloves, treat all glassware, use new plasticware, etc.). Consult any of the standard protocols books on this aspect. Be paranoid, the RNAses ARE out to get you.

4.3.1 RNA isolation using a commercial kit

Of all the kits, we get the best results extracting RNA from early log phase cultures ($OD_{600} \sim 0.6$) using the Qiagen RNeasy mini kit (Cat. No. 74104). We've used this kit to prepare RNA for primer extensions, RT-PCR and northern analysis, and find that it gives reliably high quality RNA. Usually we don't bother to run our RNA on a gel if we are using it for RT or primer extension.

However, despite claims to the contrary in the company user manual, the preparation can be **laden with DNA**, so a digestion with an RNase-free DNase is required. We digest 10 μ g RNA with 2U New England Biolabs DNase [cat. #MO303S], i.e. 2 hr at 37°C in the presence of 20U RNasin RNase Inhibitor [Promega, Cat. No. N2511]. We also add RNasin to RT-PCR and primer extension reactions, and find that we have little or no trouble with RNA degradation.

4.3.2 RNA isolation using a manual method

Method based on Karumakaran and Kuramitsu (1996), Biotechniques 20:546-547, and Kevil et al. (1997) Bioch. Biophys. Res. Commun. 238: 277-279. Sen-Lin Tang used this to determine a transcription map of halovirus HF2, and C. Bath to do the same for His2, and it worked very nicely.

1. Inoculate 0.5 ~ 0.7 ml of haloarchaeal culture into fresh medium (e.g. 10 ml of 18% MGM, in a convenient bottle or tube), and shake at 190 rpm, 37°C, for 1 – 2 days, until mid-exponential phase (OD_{550} of around 0.5 – 0.8).
2. Take 0.5 – 1 ml sample into a clean 1.5ml microfuge tube and spin cells down (13,000 rpm, 1min, 4°C).

- Put the tubes on ice and remove the supernatant as completely (get the last volume out with a micropipette), then add 80 μ l of lysis solution. Pipette up and down to make sure the entire cell pellet is lysed and evenly mixed in the solution, but avoid making air bubbles.

The solution should go 'stringy', if it doesn't then the cells have not lysed properly.

- Incubate the lysed cells at 37°C for 15 min, then place the tube on ice, leave for 2 min.
- Add 30 μ l of ice-cold sodium acetate solution and vortex thoroughly. (*keep cold or on ice from now on*)
- Centrifuge the proteins down by spinning at 13,000 rpm, 30 min, 4°C.
- Remove the supernatant to a fresh tube, add 2 vol of ice-cold ethanol to precipitate the RNA, mix well.
- Centrifuge at 13,000 rpm, 15min, 4°C. Wash the pellets twice with ice-cold 70% ethanol.
- Dry the pellets in a vacuum chamber for 30min at RT, dissolve in DEPC-treated water (e.g. 50-100 μ l), and store at -70°C. You can also store at -20°C, but preparations last only a few weeks. Determine the yield of RNA by absorption at 260nm (in quartz cuvettes) using the formula $1A_{260} = 40 \mu\text{g RNA}$.

If you store the pellet in absolute ethanol, then -20°C is possible for much longer. Freeze-thawing is also a problem, and this can damage RNA, so you may prefer to store the sample in several small portions and use one at a time.

Lysis Solution (*add reagents in order below*)

Reagent	Stock Soln.	Volume for 1ml
25mM NaOH	4 M	6.25 μ l
5 μ M CDTA	(add powder)	1.7 mg
5mM EDTA	0.5 M	10 μ l
8% Sucrose	30% w/v	270 μ l
DEPC treated H ₂ O		664 μ l
0.5% SDS	10% w/v	50 μ l

**The hydroxide dissolves the CDTA powder easily, and the SDS is best added last
CDTA = diaminocyclohexane tetraacetic acid (Sigma D8928)*

4.3.3 Agarose gel electrophoresis of RNA

10. Pre-soak the electrophoresis apparatus and tray in 1% (w/v) SDS for at least 2 hours, then drain, and remove the remaining SDS solution by wiping with high quality tissues (Kimwipes).
 - i) Prepare 50 ml of 1% agarose gel in Tris-Borate-EDTA (TBE) buffer (not TAE). After melting in the agarose, cool to 65°C in a water bath or incubator.
 - ii) Make 1ml of 1M guanidine thiocyanate (MW = 118) by adding 0.118 g to 1ml of water. Mix to dissolve.
 - iii) Add 1ml of 1 M guanidine thiocyanate to the cooled agarose and mix (final concentration is about 20 mM).
 - iv) Add 1ul of 10 mg/ml ethidium bromide solution and mix.
 - v) Immediately pour about 35 ml of the agarose solution into a small gel casting tray (e.g. BioRad small electrophoresis tank gel tray) and let it set at room temperature.
11. For electrophoresis, approx. 1 – 5 µg of RNA was denatured by adding loading buffer (1/6 volume) and heating 75°C, 5 min, then IMMEDIATELY loading and running the gel. You may need a comb with thicker teeth to fit your samples on.

6x Loading Buffer

- 0.25% Bromophenol Blue
- 0.25% Xylene Cyanol
- 30% Glycerol
- 1.2% SDS
- 60 mM Na phosphate buffer, pH 6.8

Northern Blot Hybridisation Solutions: *All stored at RT, and last indefinitely*

(1) 20% cheap SDS, filtered through Whatman #1 paper (*note SDS is an irritant, do this in a fume hood)

Take 500 g of cheap SDS and pour into 1.5 L of pure water in a large beaker

Leave on magnetic stirrer overnight. Adjust volume to 2.5 L

(2) 14% pure SDS solution. Make up a 14% solution in pure water, using high grade SDS.

This will be used in the hybridisation.

(3) Phosphate buffer, pH 7.2, 1M in Na⁺

per L 59.41g Na₂HPO₄ (anhydrous) = 0.418 M
 22.4g NaH₂PO₄ (.H₂O) = 0.163 M

Warm the H₂O and add the powders to it while stirring and they should dissolve well. If you don't warm, you may get lumps.

(4) Hybridisation solution:

1:1 mix of solutions 2 and 3 above. (have also used a 10:7:3 mix of solutions 3, 1 and water; for DNA probes)

For RNA probes, add 100 µg/ml fish DNA and yeast tRNA as blocking agents.

(5) 10x Blot washing solution

1:1 mix of solutions 1 and 3.

(6) Oligo blot washing solution

Solution 5 diluted to 1×, plus 0.5M NaCl.

The SDS may come out of solution below 25C, but doesn't seem to cause any problems.

(7) Blot stripping solution.

Solution 5, diluted to 1x, mixed 1:1 with formamide (no need to deionize).

Can reuse this many times.

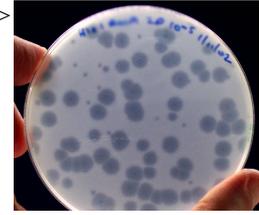
(8) 10M NaOH solution (kept in plastic container, as it reacts with glass).

(9) Blot rinsing buffer (used after the transfer)

240 ml of H₂O to 10 ml of solution 3

5.0 Haloarchaeal Viruses (Haloviruses, Halophages)

My web page (www.microbiol.unimelb.edu.au/people/dyallsmith/) has a table of reported haloviruses and their properties. Follow the links from the main page: > Research > Haloviruses > Halovirus Table (at top of page). A short review on haloviruses was published in 2003 (3) and a methods chapter (in *Methods in Microbiology*) will be published later in 2006.



Above: His1 plaques on *Halobacterium hispanica*

5.1 Isolation of haloviruses from natural waters

A lengthy discussion of this topic is provided in the *Methods in Microbiology* chapter that will be published soon {Porter and Dyall Smith, 2006, #53299}. One must be careful when looking at plaques arising from plated water samples; some may be cleared zones that mimic plaques. Some of these arise from inhibitory particulates in the sample. A low speed spin and filtration should remove most or all of these, along with cells. Residual cells from the sample can be also be a problem, as many produce halocins {Haseltine et al., 2001, #3972; Torreblanca et al., 1994, #2089}. Growth of such cells in the agar overlay may inhibit or kill the plating strain around them, forming a plaque. Use a hand lens to see the particulates and ignore any plaques with these in the centre. Any plaques picked should undergo a plaque titration to verify they are true virus plaques.

We have had best success by direct plating of samples on a variety of hosts {Bath et al., 2006, #9781; Bath and Dyall-Smith, 1998, #2692; Nuttall and Dyall-Smith, 1993, #2061; Porter et al., 2005, #4829}. Using using a few different salt concentrations {Daniels and Wais, 1990, #973; Daniels and Wais, 1998, #4830} and incubation temperatures can greatly improve plaquing (see 5.7). The general method is as follows:

1. Collect a salt lake water sample (5–10 ml is sufficient)
2. Remove cells and cellular debris by centrifugation (5000 g, 10 min, RT). Keep the supernatant.
3. Mix 100–500 µl of the cleared water sample with host cells and molten agar medium (kept at 50°C) and pour over prepared overlay plates (see below, 5.5.2).
4. Incubate aerobically 1– 4 days at 30°C and 37°C. Check each day for visible plaques.
5. Pick plaques using a glass Pasteur pipette or a sterile plastic micropipette tip.
6. Resuspend the agar plug in 500 µl of halovirus diluent (HVD; 2.47 M NaCl, 90 mM MgCl₂, 90 mM MgSO₄, 60 mM KCl, 3 mM CaCl₂, 10 mM Tris.HCl, pH 7.2).

5.2 HF1 and HF2 - examples of lytic, head-tail haloviruses

The highly related haloviruses **HF1** and **HF2** were originally isolated and described by Nuttall and Dyall-Smith (Nuttall and Dyall-Smith, 1993). Both are strictly lytic, have head-tail morphologies (myoviridae), and linear dsDNA genomes of about 78kb. We have sequenced, annotated and compared their genomes (Genbank accessions NC_004927 and NC_003345) (10, 11). The particles are sensitive to low salt concentrations (i.e. readily fall apart) but are very stable in high salt at 4°C. The methods for purification have been updated in this edition by Brendan Russ.

HF1 plaques on a wide variety of host strains, including *Hfx. volcanii*, *Hfx. lucentense* (previously “*Haloferax alicantei*”), *Har. hispanica*, and *Htg. trapanicum*. The best growth (clearest plaques, best lysis and highest titre) appears to be on *Haloterrigena trapanicum* DS67: a derivative clone of our culture collection strain of NCIMB 784 (DS11), the 16S sequence of which places it near isolate GSL-11 (Ventosa et al., 1999).

HF2 plaques on members of the *Halorubrum* genus, e.g. *Hrr. coriense* and *Hrr. lacusprofundi*. We use the former host for virus growth and purifications.

Virus stocks can be produced either by the plate overlay method (plate lysate) or by conventional liquid cultures.

Plate lysate method.

1. Mix 300 µl of actively growing (mid-exponential) host cells with sufficient virus so as to give complete lysis.

This is calculated from the (previously determined) titre of the virus stock and the approximate cell concentration such that the multiplicity of infection is about 3.

2. Allow virus to absorb for 15 min at room temperature.
3. Add 2.8 ml of molten Halovirus Top Agar (growth medium with 3 g/L agar), mix and pour onto MGM plates. Allow to set on a flat bench area for 10 min.

*Note that the MGM concentrations used are those that optimize virus growth, and have salt water concentrations below the host optimum. This is 18% MGM for *Hrr. coriense*/HF2 and 12% for *Htg. turkmenica*/HF1.*

4. Incubate plates (inverted, and in a plastic container) at 37 – 40°C until complete cell lysis (24 – 30 hours)
5. To each plate, add 500 µl of HVD and harvest the top layer using a sterile glass spreader. Transfer to a small beaker and when all the plates are harvested, pour the fluid/agar mixture into 30 ml

plastic centrifuge tubes (e.g. Sorvall SS34 tubes).

6. Pellet agar and cellular debris by centrifugation at 10k rpm for 20 min. Carefully remove the supernatant to a clean tube.
7. For **HF1**, add 100 µl of chloroform to the supernatant and mix well to lyse any remaining cells.

For **HF2**, which is sensitive to chloroform, filter the supernatant through a 0.22 µm filter (e.g. Millex GV) to remove any unlysed cells.

8. Store supernatants at 4°C (*these can last at least 7 years*)

Liquid culture lysate method for HF2

1. Add 1 ml of stationary phase *Hrr. coriense* culture to 10 ml of fresh 18% MGM medium.
2. Add 5 µl of high titred virus stock (10^{10} – 10^{11} PFU/ml), mix, and allow to adsorb for 10 min.
3. Incubate, with slow shaking (120 rpm) until lysis occurs. This usually takes 2 days.
4. Filter the lysate through a 0.22 µm membrane filter.
5. Store the lysate at 4°C. Check the titre by plaque assay. It should be 10^{10} – 10^{11} PFU/ml.

HF1 is similar but the host is Htg. trapanicum (a laboratory strain) and the medium is 12% MGM. Hfx. lucentense can also be used for HF1 (same conditions) but gives poorer yields.

NOTES:

5.2.1 Haloviruses HF1 and HF2: large scale growth and purification

HF1 and HF2 require different growth conditions to obtain high titred virus stocks ($>10^{10}$ PFU/ml).

For HF1

1. Inoculate 500 ml cultures of early exponential phase *Hfx. Aa2.2* or “*Htg. trapanicum*” DS66 cells grown in 12% MGM with between 10^6 and 10^7 PFU and incubate aerobically at 37°C in a shaker (100 – 200 rpm).
2. Clearing of the culture typically occurs after 48 hrs. Titres are usually between 10^8 - 10^9 PFU/ml.

For HF2

1. Inoculate 1 ml of late-exponential or (just) stationary phase *Hrr. coriense* culture into 10 ml of 18% MGM
2. Incubate overnight, 37°C, shaken at 180 rpm
3. Add 5 µl of HF2 stock (~10¹¹ PFU/ml) to the overnight culture, mix, and inoculate the entire contents into 500 ml of warmed 18% MGM in a 2 L flask with a cotton-wool stopper (or similar material that allows good gas exchange).
4. Incubate at 37°C with **slow** shaking (100-120 rpm).
5. Cultures should clear after 2 – 3 days.

For both HF1 and HF2

6. Add DNase I and RNase A to 1 µg/ml and 5 µg/ml, (final concentrations.), respectively. Incubate for 1 hr at 37°C.
7. Pellet cell debris and unlysed cells (Sorvall GSA/9000 rpm/30 min/4°C) and transfer the supernatant to a clean container. Keep at 4°C.

If the virus is stable to chloroform, add 100 µl of chloroform to the supernatant and mix. If the virus is sensitive to chloroform and you want to remove all unlysed cells, you can filter the supernatant through a 0.22 µm membrane.

8. Precipitate virus by adding solid PEG₆₀₀₀ to 10% wt/vol, and incubate 24 hr at 4°C.
9. Centrifuge (as in step 7 above) to pellet the PEG precipitates.
7. Carefully remove the supernatant.
8. Wash the pellet carefully with a slow stream of cold HVD (1–2 ml) to remove residual PEG so as not to dislodge the pellet. Suck out the HVD (carefully!) and discard this wash.
9. Resuspend the pellets are in 4 – 5 ml of HVD.
10. Layer the virus on CsCl (0.75 g/ml in HVD) and centrifuge to equilibrium.
Beckman SW28Ti rotor, 23000 rpm/20 hr/4°C, or for smaller volumes, Beckman 50Ti, 30000 rpm, 20 hr, 4°C
11. Collect the light-scattering band using a syringe with a long needle, and re-centrifuged to equilibrium as in step 10.

The bands are best seen if the tube is viewed in a darkened room with a small lamp placed directly above the tube and shining straight down.

10. Remove CsCl either by dialysis against HVD (2×3 hrs/500 \times volume),

or by centrifugation (Beckman 55Ti rotor, 50000 rpm, 4°C; resuspend in HVD).

The titre, protein concentration and A_{206} should be determined for each batch of purified virus. These figures can be used to assess batch-to-batch quality. Purity can also be assessed by negative stain EM.

NOTES:

5.3 Salterproviruses His1 and His2: examples of spindle-shaped haloviruses

His1 was the first spindle-shaped halovirus to be isolated (Bath and Dyall-Smith, 1998). **His2** is another spindle-shaped halovirus, distantly related to **His1**. The properties of both viruses, including their genome sequences, have been published recently (Bath et al., 2006). They are morphologically similar to *Fuselloviridae* but are genetically completely different from the latter group and have been classified into the separate virus group, *Salterprovirus* (Bath and Dyall-Smith, 2004). They have linear dsDNA genomes of about 16 kb, with terminal proteins. **His1** and **2** are both lytic and specifically infect *Har. hispanica* (Bath and Dyall-Smith, 1998).

Har. hispanica grows best at higher salt (23% MGM). It is routinely grown aerobically in either 18% or 23% MGM medium, or on MGM agar plates, at 37°C. Virus stocks are produced by infection of an early exponential *Har. hispanica* culture at a multiplicity of 1:10. For **His1**, incubation is best at 30°C, while for **His2**, growth is best at 37°C. Liquid cultures are incubated for several days, up to a week; and plates for 48 – 72 hr for visible plaques.

Virus titres are determined by serially diluting a phage sample, in HVD. Add a 100 µl sample of the appropriate dilution to 150 µl of *Har. hispanica* culture. Add molten (50°C) 18% MGM top agar (0.7%) to make 3 ml, before mixing and pouring onto 18% MGM plates (note the lower salt media, to enhance plaquing). Incubate plates at the appropriate temperatures (30 or 37°C), until plaques are clearly visible (2 – 3 days).

5.3.3 His1 and His2: Large scale growth and purification

To produce high titred stocks (>10¹⁰ PFU/ml)

1. Inoculate large (500 ml) cultures of early exponential *Har. hispanica* cells (in 18% MGM) with between 10¹⁰ and 10¹¹ PFU.
2. Incubate aerobically at either 30°C (**His1**) or 37°C (**His2**), in a shaker (100 rpm) for 3 – 5 days.

Purification proceeds even though the cultures do not clear.

3. Cell debris is pelleted (Sorvall GSA/6,000 rpm/30 min/10°C)

From this point on the purification of His1 and His2 differ because His1 particles aggregate easily and are sensitive to sucrose, while His2 does not aggregate as much, and is not sensitive to sucrose. The use of PEG to precipitate virus often causes aggregation that is difficult to reverse.

For His2

4. Collect virus from the supernatant by pelleting through a 4 ml cushion of 30 % (w/v) sucrose (in HVD) (*Beckman SW28*; 20,000 rpm, 10 hr, 10 °C)

Using a SW28 rotor means you can pellet about 216 ml of cleared virus lysate per run. The sucrose layer helps keep the virus from aggregating/sticking during pelleting, and virus pellet is much easier to resuspend. It seems a long time to centrifuge but that is the time we found necessary. Since the starting titres are so high, one run (216 ml) is usually enough starting material to get a good yield of virus.

5. Harvest the virus by removing the fluid from the top of the tube until only the bottom 1-2 ml of sucrose remains. Add 2 ml of HVD and vigorously resuspended the pellet using a pipette, then collect all the virus suspensions in a fresh, screw-cap plastic tube (of 20-30 ml capacity) and vortex intermittently for 1hr at room temperature to fully homogenize the virus.
6. Load the concentrated virus on a linear 30 – 70 % (w/v) sucrose-HVD gradient and centrifuge (*Beckman SW28*; 23,000 rpm, 2 hr, 10 °C)

A simple way to prepare such gradients is to prepare sucrose solutions of 70, 60, 50, 40 and 30%, then layer equal volumes (from most to least dense) so as to fill the centrifuge tube (leaving sufficient space for the sample to be loaded on top). For a large, SW28 tube, this is about 4-5 ml per solution. Then store the step gradient overnight at 4°C, load the next morning and centrifuge.

7. Collect the red band containing the intact virus is collected and centrifuged to equilibrium in a CsCl-HVD solution of 1.3 g/ml (*Beckman 70.1Ti*; 60,000 rpm, 20 hr, 10°C)
8. This produces one distinct red band that is harvested, diluted in HVD and pelleted (*Beckman SW55*; 35,000 rpm, 75 min, 10 °C) to remove the CsCl
9. The pellets are re-suspended in a small volume (about 1 ml) of HVD

For His1

We currently do not have a good purification method for His1. The scheme used for His2 does not work well for this virus, even though the particle density and dimensions are similar. His1 is sensitive to dense solutions of sucrose but stable in glycerol and CsCl. Replacing sucrose with glycerol still doesn't give good bands in the CsCl or equivalent glycerol gradients. Particles appear to aggregate strongly to flagella (and perhaps other cell debris). Virus bands are white, not the red bands formed by His2. We hope to find a good purification soon.

NOTES:

5.4 SH1: an example of a round halovirus

SH1 is a round, lipid-membrane containing (and chloroform sensitive) halovirus that was isolated in 1998 from salt water samples (Serpentine Lake) collected in Rottnest Is., Western Australia (Dyall-Smith et al., 2003). SH1 is lytic and infects *Har. hispanica* as well as *Halorubrum* isolate CSW 2.09.4 (Porter et al., 2005). SH1 displays a two-layer, spherical morphology with an average diameter of ~55 nm. The genome is linear dsDNA of about 30 kb, and has been fully sequenced (Bamford et al., 2005). The virus is unstable at low salt, but is stabilized by 0.14 M MgSO₄.

5.4.1 Small scale stocks of SH1

1. To a 10 ml cultures of early exponential phase *Har. hispanica* (~ OD₅₅₀ 0.1 – 0.3)
2. Infect with virus at a multiplicity of 0.05.
3. Incubate aerobically at 30°C, with low speed shaking (100 rpm).
4. Cultures usually don't lyse, but harvest at 3 days, remove unlysed cells and cell debris by a low speed centrifugation (e.g. 6000 rpm, 30 min, 10°C) and store the supernatant at 4–10°C.
5. Check the titre by plaque assay at 30°C on 18% MGM plates. You should expect titres of around 10¹⁰ PFU/ml.

5.4.2 Large scale growth of SH1

This is essentially a scaled-up version of the previous method.

1. Infect a 200 ml culture of *Har. hispanica* (18% MGM, early exponential phase, OD₅₅₀ 0.1 – 0.3) with virus (MOI 0.05).
2. Incubate aerobically at 30°C, with low speed shaking (100 rpm).
3. Cultures usually don't lyse, but harvest at 3 days, remove unlysed cells and cell debris by a low speed centrifugation (Sorvall GSA rotor, 9000 rpm, 30 min, 10°C) and store the supernatant at 4–10°C.
4. Check the titre by plaque assay at 30°C on 18% MGM plates. You should expect titres of around 10¹⁰ PFU/ml.

5.4.3 Purification of halovirus SH1

This was described in the 2005 study by Porter *et al.* (Porter et al., 2005).

1. Collect virus from the supernatant by pelleting through a 4 ml cushion of 30% (w/v) sucrose (in HVD) (Beckman SW28 rotor, 23,000 rpm, 18 hr, 10°C)
2. Harvest virus as for His2
3. Load onto a 10-70 % (w/v) sucrose gradient (in HVD) and centrifuge (Beckman SW28, 23,000 rpm, 2 hr, 10°C)
4. Collect the white light-scattering band
5. Load onto a CsCl solution (1.3 g/ml CsCl in HVD) and centrifuge to equilibrium (Beckman 70.1 Ti rotor, 60,000 rpm, 20 hr, 10°C)
5. Harvest the white, light-scattering band, dilute in HVD sufficiently to fill the volume of one or more SW55 centrifuge tubes. Pellet the virus to remove the CsCl (Beckman SW55, 35,000 rpm, 75 min, 10°C).
6. Resuspend virus in a small volume of HVD and store at 4°C.

5.5 Titrations of Haloviruses

5.5.1 Simple Method of titrating virus.

This uses very few plates and allows estimates of titres from a number of phage preparations at the same time. Accurate titrations can then be obtained using the standard plate titration method given below.

1. Mix 150 µl of the appropriate haloarchaeal host strain with 2.8 ml of MGM top layer agar (7 g/L) and pour on top of a MGM plate. Allow to set (10 min, room temp.).
2. Prepare dilutions of virus solutions in HVD, e.g. ten-fold dilutions from 10^{-3} to 10^{-7} .
3. With a felt pen, rule a grid on the underside (agar containing plate) of the MGM plates prepared in part 1 above. Label the grid with the preparations and dilutions to be assayed.
4. Spot duplicate 1 – 5 µl samples of virus dilutions onto the MGM lawns (in their correct positions). Incubate in an inverted position at 37°C.
5. Individual plaques are countable after 24 hr.

While only small numbers of plaques can be counted in any one spot, the results will give the titre to within a power of 10. With judicious ruling of grids on the agar plate, about five different phage preparations can be determined per agar plate.

5.5.2 Standard plate titrations.

Precise virus titres can be determined as follows:

1. Dilutions of virus are made in HVD.
2. 100 μ l of each dilution is added to 150 μ l of host cells (early exponential phase) mixed with 2.8 ml of MGM top layer agar (kept molten at 50°C) and poured on MGM plates. Each dilution is plated in duplicate.

Try and keep the agar volume the same for all samples as the thickness of the top layer can affect the plaque size and clarity. The minimum volume of agar solution is about 2.8 ml, but anywhere between 3–4 ml is fine.

3. Allow to set on an even bench and then incubate 37°C (inverted position).
4. Plaques should be able to be counted after 24 hr. Only count plates that have between 30 and 300 plaques.

Average the values of duplicate plates and determine the concentration in the original virus preparation by the formula below:

Average plaque count \times 1/dilution \times 1/(plated volume) = number of plaque forming units per ml (PFU/ml)

e.g. say the 10^{-8} dilution had counts of 50 and 60 plaques (on the duplicate plates). To calculate the titre

a) average the two counts: $(50+60)/2 = 55$

b) multiply by the dilution factor, which is: $1/10^{-8} = 10^8$

c) multiply this by 1/(plated volume) to get the virus concentration per ml.

i.e. since 100 μ l (0.1ml) of phage dilution was plated, then $1/0.1 = 10$.

d) So the titre would be $55 \times 10^8 \times 10 = 5.5 \times 10^{10}$ PFU/ml

5.6 Virus DNA Preparation

*This method came from Steven E. Kotsonis and Ian B. Powell, and was a personal communication to Katrina Ngui (Dept. Biochemistry, Univ. Melb.). It is a simple method for preparing HF1 or HF2 dsDNA that doesn't involve virus purification, and relies on digestion of protein and nucleic acids outside the virus capsid and then extraction and precipitation of the virus genome. It can give excellent yields of virus DNA (e.g. HF2), suitable for direct sequencing and cloning. We do **not** use it for viruses with terminal proteins (His1, His2, SH1) as the DNA sticks to the columns.*

1. Grow host O/N then inoculate culture 1/100 into fresh MGM

NB: I have diluted 1/1000 (200 µl into 200 ml MGM) successfully.

2. Inoculate a 2nd flask in the same manner.
3. Infect one flask with phage (add approx. $10^5 - 10^6$ PFU in total).

NB: I added 10^8 PFU in total

4. Incubate both flasks at 37°C, approx. 48 hrs or until infected culture clears.

NB: I added another 10^8 PFU of phage at this point, and culture cleared approx. 6 hrs later

5. Mix cleared culture with uninfected culture and incubate a further 30 hrs.

Culture should be mostly cleared at this point.

6. Centrifuge to remove cells (Sorvall GS-A, 9000 rpm for 30 min at 4°C).
7. Discard pellet and to the S/N add 1/4 volume (approx. 100 ml) 50% PEG₈₀₀₀. Stir 24 – 48 hrs at 4°C.
8. Centrifuge to pellet phage (Sorvall GS-A, 10000 rpm for 30 min at 10°C) and resuspend pellet in 10 ml of phage resuspension solution.
9. Add DNase I to 40 µg/ml and RNaseA to 20 µg/ml and incubate 37°C for 30 min.
10. Add Proteinase K to 50 µg/mL and incubate for 10 min at 37°C.
11. Add 1/4 volume (approx. 2.5 ml) of 5× proteinase K buffer then heat to 80°C for 10 min then allow to cool to RT unassisted.
12. Adjust Proteinase K concentration to 100 µg/mL and leave at 50°C for 2 hr.

5.6.2 HVD. Used for dilution and storage of haloviruses

[18% SW (v/v), 10mM Tris.Cl, pH 7.2]

per Litre:

1. In a 1L graduated cylinder, add

30% Salt Water (see sectn. 2.2)	600 ml
1M Tris.Cl buffer (pH 7.2)	10 ml
Pure water	390 ml

2. Mix and dispense into 100ml volumes in suitable (e.g. 200 ml) glass bottles.
3. Sterilize by autoclaving at 101 kPa (15 psi) for 15min.

If diluted virus loses infectivity with time, the addition of 10% glycerol may be useful

5.7 Notes on plaque assays and virus growth in halobacteria.

The two viruses **HF1** and **HF2** have different host ranges, and the growth medium must be suitable for the host strain being used. The **salt concentration** greatly affects virus replication with highest efficiencies being achieved at salt concentrations lower than the optimum for the host, but not too low so that the host cannot grow. For example, for **HF1**, best growth is achieved in *Hfx. volcanii* at about 12 – 15% SW, whereas if plating on *Hbt. salinarum* about 20% SW is required.

Another variable we have found in growing haloviruses is the incubation **temperature** (sometimes 30°C is better than 37°C, temperatures higher than 40°C don't give us as good plaques.)

The **shaker speed** is another factor. Some haloviruses do not like their hosts shaken at high speed and you will get much larger yields at about 30 – 50 rpm rather than 150 – 200 rpm. **HF2** prefers low speed.

6.0 Whole Cell Methods

6.1 Measuring cell growth using Absorbance (optical density)

What wavelength do you use given that most halobacteria are coloured various shades of red-orange? Reading the literature you will find various wavelengths used by different laboratories, ranging from 550 nm up to 620 nm. Cells absorb strongly in 400 – 500 nm range anyway, but if you pick a wavelength that is absorbed significantly by the carotenoids (bacterioruberins) then you may find problems associated with variation in pigment production by cells. Carotenoid production is known to vary with salt concentration, media composition, and at different stages of growth (see *The Biology of Halophilic Bacteria* (1992), Chpt 2, p35), and this variation can make absorbance measurements for cell growth unreliable. Looking at an old paper by Soliman and Truper (Soliman and Truper, 1982), they show an absorption spectrum of two halobacteria. They are very similar, showing major absorption peaks (due to carotenoids) at 475, 505 and a lesser one (really a bump on the way down) at 545 nm. Cell absorbance drops away after the 505 peak and is relatively very low by 600 nm. These authors used 620 nm as it was well away from any carotenoid peaks.

So, if you then restrict your range to something past 545 nm, how do you choose? Reading p265 of *Methods of General Molecular Bacteriology* (ASM) is instructive here. The author recommends that, in the absence of pigments, you should consider that lower (i.e. shorter) wavelengths give higher turbidities. The advantage of lower wavelengths is that lower cell turbidities can be measured (i.e., more sensitive). Alternatively, you may want a low sensitivity, say to measure dense cultures without having to use a dilution to get an accurate reading. 660 nm would then be useful because the medium absorbs about the same as a water blank.

Some halobacteria have little or no pigment (e.g. *Natrialba*, or colour negative mutants of *Halobacterium*), so 550 nm may be quite reasonable, but most strains are obviously red, and if you want to measure growth accurately, you should move up closer to **600nm**.

6.2.1 Making a cell lysate of *Hfx. volcanii* for enzyme assays

Most halobacteria have no rigid cell wall, just a thin protein S-layer. They will lyse by dilution in water (not recommended for the preservation of enzyme activity!), or treatment with a small amount of detergent. Known exceptions are *Halococcus* species which have thick polysaccharide cell walls (and can stain Gram positive), and thick cell wall forms (resting stages?) that develop after prolonged culture (several weeks) of certain species, e.g. *Har. hispanica* (Cline and Doolittle, 1992). While lysis by a non-ionic detergent (e.g. 0.001% TritonX-100) is quick and easy, some prefer a physical breakage, such as sonication (below).

Sonication method:

1. Take 100 ml of culture, centrifuge 6,000 rpm, Sorvall SS34 tubes, 15 min, 4°C
2. Pour off the supernatant carefully (pellet should be tight), and allow to drain
3. Resuspend in 2 M NaCl or 2 M KCl with 50 mM pH 7.5 buffer (Tris or phosphate) and 2 mM EDTA.

The EDTA will chelate out magnesium ions, which will weaken the S-layer. The salt concentration is usually lower than that in the growth medium, and cause a bit of cell swelling, but is high enough to preserve the activity of haloarchaeal enzymes.

4. Transfer suspension into a 1.5 ml microcentrifuge tube and place on ice
5. Sonicate for 3 x 15 sec (low setting, thin probe), cooling on ice between each sonication.

There will be an obvious difference in clarity and colour between lysed and untreated cell suspensions

6. Centrifuge 10,000 ×g for 5 min to pellet cell membranes and unbroken cells
7. Transfer supernatant to a fresh tube and keep on ice (store at -20°C)

You may wish to analyse the membranes as well (wash well and store). You may need to remove a sample for protein estimations.

NOTES:

6.2.2 Cell Free Extract (CFE) of *Hbt. salinarum*

Method supplied by Matthias Pfeiffer (16th July 2000). Address: Max-Planck-Institut für Biochemie Dept. Membrane Biochemistry 82152 Martinsried Germany; pfeiffer@biochem.mpg.de) Reference: Schinzel, R., and Burger, K. (1986). *FEMS Microbiol. Lett.*, 37, 325 - 329.

Solutions: CFE buffer = 50 % (w/v) PEG₆₀₀₀ in CFE (see next page)

1. Harvest cells of a 700 ml *Hbt. salinarum* culture in late exponential growth by centrifuging e.g. GS3 Sorvall rotor at 8000 rpm / 7 min / 4°C).
2. Resuspend 1 g of the wet pellet in 1 ml CFE buffer.
3. Disrupt the cells by sonication (ca. 10 sec in Cell Disruptor B15 from Branson).

settings: Frequency: continuous

Cycle Duty: 50 %

Output Control: 5

4. Spin down the debris in a 1.5 ml reaction tube at maximum rpm / 3 min / 4°C.
5. Transfer 800 µL from the supernatant to a 1.5 mL reaction tube
6. Add 200 µL of a 50 % PEG₆₀₀₀ (in CFE buffer) solution for a final concentration of 10% PEG₆₀₀₀.
7. Incubate for 60 min on ice.
8. Centrifuge the extract at maximum rpm / 10 min / 4°C. transfer the supernatant to a sterile reaction tube
9. Store the cell free extract (CFE) at 4°C for activity experiments (or -20°C if activity is no longer required).

NOTES:

Solutions

CFE buffer

Component	Concentration	Make up from solid/stock (per L)
KCl	3 M	223.68 g
NaCl	1 M	58.44 g
NH ₄ Cl	0.4 M	21.39 g
MgCl ₂ •6H ₂ O	0.04 M	8.13 g
Tris/HCl, pH 7.5	0.01 M	5 ml (2M)
		Add water to 1000 ml

50% (w/v) PEG₆₀₀₀ in CFE

PEG₆₀₀₀ 25 g

CFE buffer, add 50 ml

6.3 Making Gene Knock-Out Mutants of *Hfx. volcanii*

Protocol and diagram contributed by Dr Thorsten Allers, Nottingham Univ., UK. See:- Allers, T., Ngo, H., Mevarech, M., and Lloyd, R. G. (2004). Development of additional selectable markers for the halophilic archaeon *Haloferax volcanii* based on the *leuB* and *trpA* genes. *Appl Environ Microbiol* 70(2), 943-953.

Marker	Hv-YPC	Hv-Ca	Hv-Min
<i>pyrE2</i>	OK	+ura	+ura
<i>trpA</i>	OK	+trp	+trp
<i>leuB</i>	OK	OK	+leu
<i>hdrB</i>	+thy	+thy +hypox	+thy +hypox + met + gly +panto

1. Make a deletion construct (by PCR, or restriction digest if convenient sites are available) containing regions of flanking homology of gene. The regions should be equal in size and >500 bp (you can go smaller, but the efficiency drops off).
2. Clone this in *pyrE2*-marked plasmid pTA131 or pGB70. If the gene of interest is important or essential, mark the deletion construct by inserting the *p.fdx::trpA* construct (from pTA106) at the site of the deletion.
3. Before transformation, passage plasmid through *E coli dam⁻* host (e.g. strain JM110)
4. Transform H26 (or H53, H66, H98 or other *pyrE2* strain) with 2 µg of DNA. After regeneration resuspend cells in transformant plating solution (18% SW + 15% sucrose) to remove uracil in regeneration solution. Dilute in same buffer and plate 100 µl at 10⁰, 10⁻¹ and 10⁻² on Hv-Ca agar (+ trp for Δ *trpA* strains such as H53, unless deletion is marked with *trpA*).
5. Restreak transformants on Hv-Ca agar, then set up 5 ml liquid cultures in Hv-YPC broth for genomic DNA minipreps.
6. Prepare genomic DNA from 1 ml by miniprep method, check integration at correct locus by restriction digest and Southern. Ideally, the restriction enzyme should cut only once in the plasmid, in one of the regions of flanking homology. This will allow the orientation of the integration to be determined, which can be important.
7. Set up 5 ml liquid culture with single colony. When this has reached late exponential phase ($A_{650} = 1$), dilute 1/500 into a fresh 5 ml Hv-YPC broth (non-selective growth for pop-out). Repeat dilution once this culture is at late exponential phase (ie 3 × growth in liquid).

8. Plate 100 μ l of 10^{-4} dilution on Hv-Ca +5-FOA (note this contains 1/5 usual amount of uracil), and 10^{-6} dilution on Hv-Ca + ura (positive control plate).

You should find that around 1% of the viable cells are 5-FOAR (pop-outs). If using a *trpA*-marked deletion, also plate on Hv-Ca +5-FOA +*trp*; the 5-FOAR *trp*⁺ cells should carry the deletion, whereas the *trp*⁻ ones will have reverted to wild-type.

9. Restreak 5-FOA^R transformants on Hv-YPC and test for loss of function if possible. If there is not an obvious phenotype, patch out 40-120 of the 5-FOA^R colonies on Hv-YPC, grow for a few days and take a colony lift with a nylon filter. Use a probe corresponding to the deleted portion of the gene, and obviously, choose colonies that do NOT hybridise with this probe. Restreak these on Hv-YPC.
10. Set up 5 ml liquid cultures in Hv-YPC for genomic DNA minipreps, check deletion by restriction digest and Southern (as before, in step 6). Use a probe to the flanking regions (not the probe used for the colony lift in step 9).
11. Set up 7.5 ml liquid culture with single colony. When $A_{650}=1$, add 2.5 ml 80% glycerol, 6% SW and mix thoroughly. Divide into 2 aliquots of 5 ml, freeze on dry ice and store at -80°C .

6.3.1 Characteristics and requirements of *Hfx. volcanii* mutants

Strain	Genotype	Hv-YPC	Hv-Ca	Hv-Min
H26	Δ pyrE2	+	Ura-	Ura-
H37	Δ leuB	+	+	Leu-
H77	Δ trpA	+	Trp-	Trp-
H66	Δ pyrE2 Δ leuB	+	Ura-	Ura- Leu-
H53	Δ pyrE2 Δ trpA	+	Ura- Trp-	Ura- Trp-
H98*	Δ pyrE2 Δ hdrB	Thy-	Ura- Thy-	Ura- Thy-
H119	Δ pyrE2 Δ leuB Δ trpA	+	Ura- Trp-	Ura- Leu- Trp-
H100*	Δ pyrE2 Δ leuB Δ hdrB	Thy-	Ura- Thy-	Ura- Leu- Thy-
H99*	Δ pyrE2 Δ trpA Δ hdrB	Thy-	Ura- Trp- Thy-	Ura- Trp- Thy-
H133*	Δ pyrE2 Δ leuB Δ trpA Δ hdrB	Thy-	Ura- Trp- Thy-	Ura- Leu- Trp- Thy-

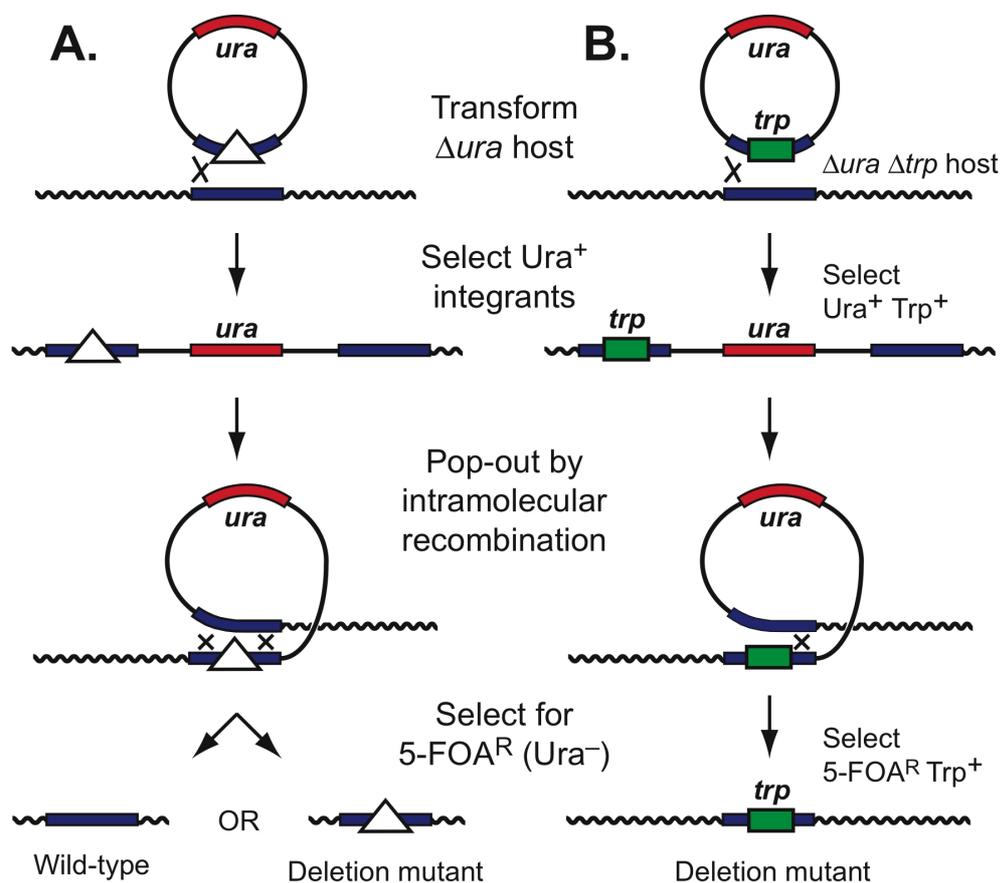
*In addition to thymidine, Δ hdrB strains should be supplemented with hypoxanthine in Hv-Ca, and hypoxanthine, methionine, glycine and pantothenic acid in Hv-Min (Ortenberg, R., Rozenblatt-Rosen, O., and Mevarech, M. (2000). *Haloflex volcanii* has two different dihydrofolate reductases. Mol Microbiol 35, 1493-1505.) Use 50 μ g/ml uracil, leucine, tryptophan, methionine, glycine, pantothenic acid, 40 μ g/ml thymidine, hypoxanthine. Uracil should be dissolved in DMSO, and hypoxanthine in 50 mM NaOH; for all others, use water.

6.3.2 Plasmids used with *Hfx. volcanii* mutants

Selectable marker	Source†	Integrative plasmid (pBluescript II-based)	Shuttle vector (pHV2 origin)
pyrE2	pGB70	pTA131	pTA230
leuB	pTA105	pTA133	pTA232
trpA	pTA106	pTA132	pTA231
hdrB	pTA187	pTA192	pTA233

†The selectable marker on fragment flanked by convenient restriction sites

6.3.3 Diagram of plasmid-chromosome recombination and selection method for creating mutants.



6.4 Restriction Enzyme Activity of *Hbt. salinarum*

*Method supplied by Matthias Pfeiffer (16th July 2000). Address: Max-Planck-Institut für Biochemie Dept. Membrane Biochemistry 82152 Martinsried Germany; pfeiffer@biochem.mpg.de.
References: Schinzel, R., and Burger, K. (1986). FEMS Microbiol. Lett., 37, 325 - 329. Blaseio, U. and Pfeifer, F. (1990). PNAS, 87, 6772 - 6776.*

Solutions: Phenol/Chloroform solution (for standard phenol extraction of protein/DNA solutions)

Materials: PEG₆₀₀₀ in CFE buffer

Cell Free Extract (see section 6.2.2 above for both PEG and CFE).

1. Add 50 µL of a fresh cell free extract (CFE, see 6.2.2 above) with 5 µg vector DNA
Any well known vector/plasmid can be used e.g. pBR322, pBLUESCRIPT, pGEM, pKS, etc.
2. Incubate for 2 hr at 37°C.
3. As a negative control, incubate the same amount of DNA with a mixture of 10 µl 50 % PEG₆₀₀₀ in CFE buffer and 40 µl H₂O.
4. At the end of the incubation, add 120 µl of water (to stop the reaction and increase the extraction volume).

Extract with 100 µl phenol/chloroform (standard protocol) and ethanol precipitate as usual.

5. Resuspend the DNA pellet in 10 - 20 µL H₂O and separate on a 1.2 % agarose gel to see if the DNA has been digested (e.g. specific digestion sites will give discrete fragment patterns).

From the size of the fragments, and the known sequence of the vector, you may be able to identify the likely recognition site (see 3.2.3)

NOTES:

6.5 Reporter genes in haloarchaea

There have been a number of reporter genes developed for measuring gene expression in haloarchaea. The first was a yeast tRNA gene, with a northern blot hybridization used to assay transcription (Palmer and Daniels, 1995). A halophilic β -galactosidase (BgaH) was developed in this laboratory (Holmes and Dyll-Smith, 2000; Holmes et al., 1997), and can be used in much the same way LacZ is used in *E.coli* and other organisms (Gregor and Pfeifer, 2001; Gregor and Pfeifer, 2005; Hofacker et al., 2004; Patenge et al., 2000; Sartorius-Neef and Pfeifer, 2004; Zhu et al., 2006; Zimmermann and Pfeifer, 2003). Drug resistance genes have also been used, notably trimethoprim resistance, with assay being the level of resistance. The most recent has been the application of certain derivatives of green fluorescent proteins (GFP) for use in haloarchaea. Methods for the use of *bgaH* and GFP reporters are given below.

6.5.0 Measuring haloarchaeal β -galactosidase activity

See my web page (<http://www.haloarchaea.com>) for up-to-date details on *bgaH* gene and vectors for using it with foreign promoters. The enzyme (BgaH) was described in 1997 (Holmes et al., 1997). The *bgaH* gene was described in 2000 (Holmes and Dyll-Smith, 2000). It has been used to assay promoters of haloarchaeal genes (see refs. above) and extensively in our lab to assay halovirus promoters (Brendan Russ, unpublished data).

BgaH is a member of glycosyl hydrolase family 42. The enzyme hydrolyses ONPG, p-nitrophenol-D-Fucose and lactulose, but not lactose. The crystal structure for a member of the glycoside hydrolase family 42 has been determined (Hidaka et al., 2002) and, surprisingly, is a homo-trimer! We thought originally that BgaH was probably a dimer. We don't know what the natural substrate is (and ditto for the other family 42 glycohydrolases). No-one seems to know this. It may be an oligomer of galactose.

6.5.1 Protocol for β -galactosidase assays in Halobacteria

β -galactosidase specific activity from natural halobacteria or from haloarchaeal transformants (eg. *Hfx. volcanii* transformants) can be quantitated by following the hydrolysis of ONPG (ortho-nitro-phenol β -D-galactoside) to ONP (a bright yellow colour) at 405 nm. All assays are performed in duplicate. BgaH appears to be stable and active in *bgaH* buffer solution for at least 24 hours at room temperature.

6.5.2 Strain purification and growth of *Hfx. volcanii* DS52 transformants (*bgaH*⁺)

When using the reporter system to analyse promoters, it is best to make sure your construct, transposon

mutant or transformant, is both stable and a pure culture. We routinely go through the following procedure before doing quantitative tests for bgaH activity. Mostly our constructs are fine, but occasionally you find some problems and this method almost always overcomes it. We use the radA- (recombination deficient) host of *Hfx. volcanii*, strain DS52 (from our lab). It avoids problems with recombination, but is a bit slower growing than wt *Hfx. volcanii*. This organism has no endogenous BgaH activity.

1. Grow transformants on selective plates and spray resulting colonies with X-gal (using a perfume atomiser /nebuliser containing X-Gal at 10 mg/ml dissolved in dimethyl formamide)
2. Follow the colour production at RT or 37°C over 24 hours.
3. Select the most typical colonies and streak on fresh (selective) plates to purify on 18% MGM. You may need to do this a second time if you believe there is some instability (varying colours, sectoring).
4. Inoculate into 10 ml of liquid medium (e.g. 18% MGM with selective agent, such as 0.3 µg/ml Novobiocin) and shake at 37°C until culture reaches stationary or late exponential phase.
5. From stationary cultures, subculture using 1.8 ml of culture into a fresh 10 ml of fresh, selective medium (e.g. 3 µl of 1 mg/ml Novobiocin stock), and grow to an OD₆₀₀ 0.45 – 0.7 (Approx 30 hr). Record the OD₆₀₀ of all test cultures for later use in β-galactosidase assays.

6.5.3 Assay for BgaH specific activity in *Hfx. volcanii* strain DS52 (*radA*)

1. Dispense 700 µl volumes of *bgaH* buffer into clean, labeled plastic spectrophotometer cuvette (*this is the most convenient, or use 1.5 ml microfuge tubes*)
2. Add 100 µl of test cells to the cuvette and mix using the micropipettor.
3. Add 100 µl of 2% triton X-100 to lyse the cells. Seal the cuvette with Parafilm or similar plastic, and vortex mix for 10 seconds.

If using supernatants or pure enzyme, there is no need to include the TritonX-100 lysis step. Use lysates as soon as possible, preferably immediately, as enzyme activity may decrease with time.

4. **Start** the reaction by adding 100 µl of 8 mg/ml ONPG solution, recap with Parafilm, and vortex mix for approximately 3 seconds.
5. Quickly transfer each mixture to a separate, labeled plastic spectrophotometer cuvette.

6. Follow the release of *o*-nitrophenol from ONPG (catalysed by the BgaH) by recording the increase in absorbance at 405 nm in a spectrophotometer. Blank against a mixture containing ONPG but with 100 µl of MGM added instead of culture.

If activity is strong, the tubes go visibly yellow within seconds to minutes. Take readings every minute, or if very weak activity, every 5-10 minutes. Stop when A405 reaches about 0.7 – 1. Weaker activity can be followed every 10 min for 1 hr.

7. Control reactions to include with every experiment are:
- Negative control: use a strain with no active *bgaH* gene, preferably also carrying a plasmid and grown under the same selective conditions as your tests.
 - Positive control contained BgaH buffer, Triton X-100, ONPG and a culture of a known positive strain (e.g. *Hfx. volcanii* DS52 containing the natural *bgaH* gene in plasmid pVA513).

The enzyme is quite stable under these conditions and you should see a linear increase in absorbance for many hours (unless it is so strong as to break down most of the substrate!).

8. The linear gradient of A_{405} / time (minutes), cell volume and optical density (600 nm) of initial cultures were used to calculate β -galactosidase specific activity (see formula below).

The absorbance values for ONP are linear only up to about 1.2. Don't go much above this. Graph A_{405} with time (min) and find gradient over the linear part. Use the gradient ($\frac{DA_{405}}{Dt}$) in the formula below.

Formula: β -galactosidase specific activity (SA)

$$SA = \frac{DA_{405}}{Dt} \cdot \frac{1000}{\text{cellvolume} \cdot OD_{600}}$$

ΔA_{405} = change in absorbance at 405nm,

Δt = change in time (minutes)

OD_{600} = optical density of the culture at 600 nm

cell volume = volume, in ml, of culture added to the assay

We find this formula the best in terms of accuracy and reproducibility. It is similar to the formula of Miller for assaying LacZ in *E.coli*. The units arising from this formula are not standard enzyme kinetic units (e.g. µmole/min/mg), but they are convenient! Negative cultures give values of ≤ 1 whereas

positive constructs give values in the 100's up to around 1200 U (e.g. strains with plasmid pVA513).

If you want to convert these to more standard activities, the molar extinction coefficient of ONP was determined to be $3.3 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 405 nm under the assay conditions used.

COMMENTS:

- a) Occasionally, the ONPG reaction turns cloudy-white about 10 minutes after beginning the assay. This completely interferes with absorbance readings. We discovered the problem was the 2% Triton X-100 solution. A new solution made from a fresh batch of detergent eliminated the problem.
- b) Without drug selection, or with poor selection, you may well see sectored colonies on X-gal spraying. Also, you can check the homogeneity of your assayed cultures afterwards by doing viable counts using selective and non-selective media, and spraying the resulting colonies with X-gal.
- c) For promoter studies, some fusions are better done about 5 codons downstream of the start codon of the gene being investigated, i.e. translational fusions. This problem was noted by Gregor and Pfeifer (2001) (Gregor and Pfeifer, 2001), and has also been found by us recently for some (very few) halovirus promoters. We have developed better and easier vectors for promoter studies (B.Russ, unpublished), such as pRV1 and pRV2, which include a transcriptional terminator to block outside transcription. Contact us for the latest versions.

SOLUTIONS

***bgaH* buffer :** 2.5 M NaCl,
50 mM Tris pH7.2,
10 μ M MnCl₂,
0.1% 2-mercaptoethanol (added fresh before use).

Stored at 4°C without the 2-ME. Once added, the 2-ME does not last more than about 2 weeks.

ONPG solution : 8 mg/ml in *bgaH* buffer
ortho-nitro-phenyl- β -D-galactoside (Sigma N 1127; MW = 301)
Stored frozen at -20°C

2% Triton X-100 : 2ml of Triton X-100 in 98 ml pure water. Store at RT.
See note above about cloudy assays.

6.5.4 Protein Assays using the BioRad Coomassie Blue kit

1. Spin down $2 \times 100 \mu\text{l}$ sample of culture at 13,000 rpm, 5 – 10 minutes

Assays need to be done in duplicate.

2. Resuspend cell pellet in $50 \mu\text{l}$ of 0.01% TritonX-100 (in water) which should lyse your cells, but is low enough not to interfere with the assay.

Samples may be stored frozen (-20°C) at this stage.

3. Set up 5- to 8- point standard curve using the Bovine Serum Albumin (BSA, 1.36 mg/ml stock) protein standards supplied with the kit.

You should get approx. $10 \mu\text{g}$ in your samples so you should aim to have a standard curve in the range of $2 - 40 \mu\text{g}$. Set up protein standards in duplicate. Also set up one negative control comprising 2.5 ml dye and $50 \mu\text{l}$ of your diluent (no protein) i.e. 0.01% Triton X 100. The assay is sensitive to the amount of detergent which should be below 0.1%.

BSA (μg)	Volume BSA soln (1.36 mg/ml)	Volume H ₂ O	Volume 0.1% TritonX-100	Volume CBB Dye
0 (Blank)	$50 \mu\text{l}$ 0.01% TritonX-100	$0 \mu\text{l}$	0	2.5 ml
1.36	* $10 \mu\text{l}$	$35 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml
2.72	* $20 \mu\text{l}$	$25 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml
4.08	* $30 \mu\text{l}$	$15 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml
6.8	$5 \mu\text{l}$	$40 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml
13.6	$10 \mu\text{l}$	$35 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml
27.23	$20 \mu\text{l}$	$25 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml
34	$25 \mu\text{l}$	$20 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml
40	$30 \mu\text{l}$	$15 \mu\text{l}$	$5 \mu\text{l}$	2.5 ml

** use a 10 x dilution of BSA stock in .01% TritonX-100 for these additions, so = 0.136 mg/ml*

4. Add sample, dye and vortex 10 sec. Leave 5 min at room temperature.
5. Read Absorbance at 595 nm against blank (i.e. subtract value from blank value).

I set up the standards first and my first sample in duplicate. I then read the standards and make sure that the values are within 15% of each other, otherwise set up the particular duplicates again. Once you are satisfied with the standard curve set up the rest of the samples and read the absorbance at 595 nm. Read your protein concentration off the standard curve.

6.6 Pulse-chase and Immunoprecipitations with *Hfx. volcanii*

This method was supplied by Dr M. Pohlschroder, University of Pennsylvania, who is working on the secretory pathway in halobacteria. Fax: +1 215 898-8780, e-mail: pohlschr@sas.upenn.edu, web: <http://www.sas.upenn.edu/biology/faculty/pohlschroder/>.

It works well for Hfx. volcanii, and I would be interested to hear comments by people who use it for other halobacteria.

1. Grow cells (shaking, 40°C) in minimal medium, with 0.5 mg/ml methionine-free medium (Difco), until mid- exponential phase, i.e. OD₆₀₀: approximately = 0.4. Takes about 2 days.
2. Dilute mid- to late-log culture 1:40 into fresh (prewarmed) minimal medium with above ingredients.
3. Grow to mid- exponential phase, (shaking, 40°C); OD₆₀₀: approximately = 0.4

Labeling cell proteins:

4. Dispense into large disposable tubes equilibrated to desired labeling temperature. Use 50 µCi ³⁵S-Met per ml of cells to be labeled.
5. Add cells and pulse for appropriate length of time (5-15 min)
6. At end of pulse time add 2% stock solution of unlabelled methionine to a final concentration of 0.2% and immediately remove 1 ml of labeled *Archaea* to a 1.5 ml microfuge tube, pre-chilled in an ice-water bath containing 1/10 volume 100% TCA.
7. Leave on ice for 15 min. At the appropriate time points remove additional 1 ml samples to chilled microfuge tubes with TCA. Leave samples on ice for at least 15min.
8. Spin for 20 minutes at 4°C. Discard supernatant and wash pellet TWO times with 1 ml 80% cold acetone. (you need two washes to eliminate residual TCA)
9. Dry in vacuum or leave at room temperature for ~ 30 min.
10. Resuspend in 50 µl SDS-PAGE sample buffer and heat to 90°C for 5 minutes.
11. Cool to R.T.

Samples are now stable at 4°C. The SDS concentration is too high for antibody binding to occur so the next steps prepare the sample for immunoprecipitation.

12. To dilute out the SDS, add 800 μ l chilled TX buffer. Vortex, and put on ice 15 min. (this is just a dilution step, the SDS does not come out of solution)
13. Spin samples in microfuge at 4°C for 10 min. (this pellets insoluble TCA precipitates).
14. Carefully remove 700 μ l of **supernatant** to a new microfuge tube. To test for adequate incorporation of label, remove a 5 μ l sample of this supernatant, and check its activity. It should give > 20,000 counts/min.

If you're getting lots of non-specific absorption to the IgGSorb you can add 100 μ l of IgGSorb per 800 μ l of TX buffer and incubate on ice for 1 – 2 hours, then spin at 4°C for 15 min.

IgGSorb should be rehydrated in water (to resuspend it well it can be sonicated, or leave at 4°C overnight for complete rehydration). These samples should be stable for at least 4 weeks – the expiration dates on the bottles don't usually mean that you can't use it after this date (approximately 6 months). The company continuously tests the batches – you can call them and ask for the results – I've had a batch for nearly 2 years!

15. Add antibody to samples. The samples can be left on ice for 1 hour to overnight.
16. Add IgGSorb: For < 3 μ l serum, use 100 μ l IgGSorb; For 3-10 μ l serum, use 200 μ l IgGSorb
17. Then incubate on ice for 20 minutes, mixing occasionally.
18. Spin for 20 sec at room temperature. Aspirate the supernatant.
19. Wash twice with 1 ml of High Salt buffer. Wash once with 10 mM Tris, pH 8.0.
20. Resuspend in 50 μ l of (Laemmli) SDS-PAGE sample buffer (2% SDS, 125 mM Tris pH 8.0, 15% glycerol, bromphenol blue). Heat to 90°C for 2 minutes to release the antibody and antigen from the IgGSorb.
21. Spin 5 minutes in microfuge.
22. Load 15 μ l of each sample per lane onto Laemmli gel for SDS-PAGE. The polyacrylamide concentration will depend on the expected sizes of your proteins, e.g. 10 – 12% gel will separate mid-range proteins (50 kD) satisfactorily. Samples can be stored after heating at 4°C, but should be reheated prior to loading gel.

The TCA precipitation and washing steps eliminate any salt from the protein samples. If salt from the halobacterial culture remains in your protein sample you will experience strange electrophoretic behaviour (typically a very reduced mobility).

After electrophoresis, process the gel for autoradiography and expose to x-ray film. If you used pre-stained protein Molecular Weight markers, you may not need to stain the gel first before drying for autoradiography.

REAGENTS and SOLUTIONS

SDS Buffer

High Salt Buffer	TX Buffer
1% Sodium Dodecyl Sulfate (SDS)	2 % Triton X-100
1% Triton X-100 (in water)	50 mM Tris.Cl pH 8.0
10 mM Tris.Cl pH 8.0	150 mM NaCl
50 mM Tris.Cl pH 8.0	1 mM EDTA
1 mM EDTA	
1 M NaCl	
1 mM EDTA	

IgGSorb (Staph A)

Add 10 ml sterile H₂O to lyophilate and refrigerate O/N. Wash once with TX buffer and resuspend in 1 TX buffer. As the name suggests, this is sepharose-protein A preparation. Our source is “The Enzyme Center” in Malden Massachusetts Tel: 781 322 4885, but any other Sepharose A should be O.K.

SDS PAGE Loading Buffer (LB) for TCA precipitates

1. Make up the following two solutions separately.

Solution 1	Solution 2
0.2 M Tris base	8.3% SDS (4.0 ml of 25% soln)
0.02 M EDTA, pH 7-8	83.3 mM Tris base (1.0 ml of 1 M soln)
	29.2% glycerol (3.5 ml of 100%)
	0.03% Bromophenol Blue (3.5 ml of 0.1% soln)

2. Mix together the following volumes of these solutions to make 1ml. Store at room temperature.

5 parts solution 1 = 0.5 ml
4 parts solution 2 = 0.4 ml
1 part 1 M DTT = 0.1 ml

NOTES:

6.6.1 Purification of tagged *Haloferax volcanii* proteins

- 1 Grow large-scale cultures (2 liter) of the *Hfx. volcanii* strain expressing the tagged protein of interest, with shaking in liquid MGM to late log phase.
- 2 Pellet cells at 6,500 ×g for 10 min at 4°C.
- 3 Resuspend the cell pellet in 20 ml of PB(2M)S containing 10 mM EDTA.
- 4 Freeze cell suspension once at -80°C and thaw at 37°C (optional).
- 5 Pass cell suspension through a French press twice at 20,000 psi.
- 6 Remove cell debris and unbroken cells from the lysate by centrifugation at 18,900 ×g for 10 min at 4°C.
- 7 Transfer supernatant to precooled ultracentrifugation tubes. Pellet membranes from the cleared lysate by ultracentrifugation at 50,000 RPM (184k ×g average RCF) for 1hr at 10°C in a Beckman Type 70Ti rotor.
- 8 Collect the supernatant as the cytoplasmic fraction. If interested in membrane proteins, resuspend membrane pellets in a total of 5 ml PB(2M)S buffer by gentle trituration through an 18 gauge needle. Wash once with PB(2M)S and recentrifuge at 50,000 RPM as described. (If interested in cytoplasmic proteins, recentrifuge this cytoplasmic fraction to get rid of residual membrane).
- 9 Resuspend membrane pellets in a total of 5 ml PB(2M)S buffer.

6.6.2 Purification of His-tagged membrane proteins by Ni-NTA metal affinity chromatography

- 1 Solubilize *Hfx. volcanii* membranes for 1 hour at room temperature with continuous inversion on a rolling platform, by the addition of 1 volume of NiNTA Native Adjustment buffer (PB(2M)S, 20% glycerol, 80mM imidazole, 0.4% dodecyl maltoside, (DDM)).
- 2 Pellet NiNTA resin from 0.5 ml of a 50% slurry, wash three times with Native Column binding buffer (PB(2M)S, 10% glycerol, 40 mM imidazole, 0.2% dodecyl maltoside).
- 3 Add resin to solubilized membranes and allow batch binding of the His tag to the resin for 1 hour at room temperature, with continuous inversion.
- 4 Apply to a 2 ml column that's closed on the bottom and allow the beads to settle onto a glass wool frit, then open the column and collect the flow-through.

- 5 Wash the NiNTA beads with 2 ml of Native Column binding buffer (see above) and perform a second wash with 2 ml of native column wash buffer (PB(2M)S, 10% glycerol, 100 mM imidazole, 0.2% dodecyl maltoside).
- 6 Elute bound proteins with Native elution buffer (PB(2M)S, 10% glycerol, 250 mM imidazole, 0.2% dodecyl maltoside) in 4 fractions of 0.25 ml each.

Note: The imidazole concentration can be changed in order to make the purification conditions more or less stringent.

6.6.3 Purification of His-tagged cytoplasmic proteins by Ni-NTA metal affinity chromatography

To purify proteins from the cytoplasmic fractions the protocol only differs from the one described above in that no dodecyl maltoside (or other detergent) is added to the buffers.

Note: *Hfx. volcanii* expresses a cytoplasmic, approximately 60 kDa protein with a cluster of consecutive histidines that also purifies with NiNTA. Thus, if the protein of interest is about this size, his-tagging might not be the way to go. Moreover, this highly abundant co-purifying band prevents loading of a concentrated amount of the sample as the highly abundant histidine-rich protein distorts the gel. We have recently started using Strep tags which results in fewer non-specific bands from both, cytoplasmic and membrane fractions. Like the NiNTA purification, this purification method can be carried out at high salt concentrations (apparently up to 5 M, we've only gone up to 2M).

BUT: There may be a better way to purify your his-tagged protein from *Haloferax* cells. This tip was contributed by Andrew Large, University of Birmingham, UK. The method will be published in Kapatai *et al.* 2006 (*in press*) Mol. Microbiol.

1. His-tagged proteins were purified from cell lysates in 2 M sodium chloride, 20 mM sodium phosphate, pH 7.9 using a Nickel-sepharose column with a gradient (60 ml) of 0–125mM imidazole in 2 M sodium chloride, 20 mM sodium phosphate, pH 7.9 for elution, using a BIORAD Econo-pack.

Other tagged proteins may require a higher Imidazole concentration (up to 500 mM), determine the best range by experiment, but the shallowness (small range) is the key to separating the tagged protein from the other stuff.

6.6.4 Purification of cytoplasmic and membrane proteins using Strep-Tactin Superflow affinity chromatography.

- Use the protocol described in the Quiagen or IBA handbooks (IBA is a bit cheaper, both methods are more expensive than NiNTA purification), except that 2M NaCl (or up to 5 M is possible) has to be added to all buffers. In case of membrane fractions add 0.2% DDM to each buffer.

Note: The protocols include an optional step where prior to purification the membrane or cytosolic preparations are dialyzed over night against the lysis buffer at 4 °C to eliminate biotin. We have always included this step.

6.7 EMS mutagenesis of *Hfx. volcanii*

Published by (Rosenshine and Mevarech, 1991), and see also (Rosenshine et al., 1989), and (Tchelet and Mevarech, 1994)

1. Dilute a stationary culture of *Hfx. volcanii* 1:10 in complete (i.e. rich) medium (e.g. H medium, 2.5.2)
2. Grow for 16 – 20 hours at 37°C
3. Take 3 ml and wash twice in SMT buffer (3.5M NaCl, 0.15M MgSO₄, and 10mM Tris.Cl pH7.2) and resuspend in 8ml of SMT.
4. Add 120 µl of EMS (Merck) and shake vigorously for 30 sec.
5. Incubate with gentle shaking at 37°C and take samples (2ml) at 0, 10, 20 and 30min.
6. Wash samples twice with SMT and resuspend in complete medium.
7. Dilute a sample of the total mixture 1:10 and grow overnight for phenotypic expression.

The usual survival frequencies were reported to be 1-50%. Auxotrophic mutants were isolated by plating dilutions of the final culture on minimal medium (MM, 2.4 - 2.5) with 0.02% yeast extract added. Auxotrophs grew slowly and formed small colonies. When the small colonies were tested approximately 30-50% were true auxotrophic mutants.

NOTES:

6.8.1 Concanavalin A-Sepharose binding

This and the following methods(6.8.1 - 6.8.4) were contributed by Dr J. Eichler, Dept. of Life Sciences, Ben Gurion University of the Negev, P.O. Box 653, Beersheva 84105 ISRAEL. email: jeichler@bgumail.bgu.ac.il web: www.bgu.ac.il/life/Faculty/Eichler/index.htm

Reference: (Eichler, 2000)

1. Radiolabelled *Hfx. volcanii* cells or membranes (10^6 cpm) are added to 100 μ l of concanavalin A-Sepharose beads (prewashed in 1 ml lectin buffer) and lectin buffer, in a final volume of 1 ml.
2. Reactions are nutated (rocked) overnight at 4°C.
3. The beads are then washed 4 times in lectin buffer without BSA and heated (3 min, 95°C) in the presence of 35 μ l sample buffer.
4. The beads are pelleted in a microfuge (5, 000 \times g, 3 min) and the supernatant transferred to a clean microtube

Solutions

Lectin buffer: 1 mM Ca^{2+} , 1 mM Mn^{2+} , 1 mg/ml BSA, 1 % Triton X-100, 50 mM Tris-HCl, pH 7.9

SDS-PAGE Sample buffer: 4% SDS, 20% (v/v) glycerol, 0.02% bromophenol blue, 1.5% β -mercaptoethanol, 125 mM Tris-HCl, pH 6.8

NOTES:

6.8.2 Preparation of inverted membrane vesicles (IMVs)

[Contributed by Dr. J. Eichler]. **Reference:** (Ring and Eichler, 2001)

Note: Menadione-dependent NADH dehydrogenase serves as a marker of the inner *Hfx. volcanii* membrane surface. The method for this assay is below the IMV preparation method.

1. *Hfx. volcanii* cells (5 L) are grown to mid- exponential phase and harvested by centrifugation (30 min, 8, 000 g).
2. The cells are resuspended in 50 ml of buffer A to an OD₅₂₀ of approximately 40 and ruptured with a French Press pressure cell (3 passes at 8, 000 psi each).
3. Cell debris is removed by centrifugation (8, 000 g, 20 min).
4. The supernatant is centrifuged in a Beckman L7-80 ultracentrifuge (60 Ti rotor, 50K rpm, 60 min, 4°C).
5. The pellet is resuspended in 5 ml of buffer A and 1 ml aliquots are applied to each of four step gradients prepared with 0.9, 1.1, 1.2, 1.3, 1.4 and 1.5 M sucrose in buffer B (2 M NaCl, 50 mM Tris-HCl, pH 7.2).

Upon centrifugation (SW-28 rotor, 18, 000 rpm, 40 h, 4°C), four distinct, red-colored bands appear.

6. Each gradient band is collected, diluted with 20 ml buffer B, centrifuged (60-Ti rotor, 45, 000 rpm, 1 hr, 4°C) and the resulting pellets are resuspended in 0.5 ml buffer B, aliquoted and frozen in liquid N₂.
7. The aliquots are stored at -70°C until assayed.

The membrane fractions retain their enzymatic activities for up to 3 months under these storage conditions.

SOLUTIONS

Buffer A: 1.75 M NaCl, 50 mM Tris-HCl, pH 7.2

Buffer B: 2 M NaCl, 50 mM Tris-HCl, pH 7.2

NOTES:

6.83 Menadione-dependent NADH dehydrogenase activity

[Contributed by Dr. J. Eichler]

*Menadione-dependent NADH dehydrogenase serves as a marker of the inner *Hfx. volcanii* membrane surface.*

1. *Hfx. volcanii* membrane preparations (0.2 mg/ml) are suspended in :
2 M NaCl, 0.5 mM MES, 0.5 mM PIPES, pH 6.5.
2. Menadione (0.5 mM) is added to the sample
3. The reaction is initiated upon addition of NADH (0.5 mM).
4. Follow the OD₃₄₀ at 10 sec intervals and calculate the enzyme activity

In some cases, the membranes are incubated with 0.01% (w/v) Triton X-100 (60 min, RT) prior to addition of menadione and NADH.

NOTES:

6.8.4 Cellulose purification of CBD-tagged proteins

[Contributed by Dr. J. Eichler]

Fusion of the cellulose-binding domain (CBD) of the *Clostridium thermocellum* cellulosome (Morag et al., 1995) to the genes encoding haloarchaeal proteins allows for salt-insensitive cellulose-based purification.

Reference: (Irihimovitch et al., 2003)

To capture CBD-fused proteins, transformed *Hfx. volcanii* cells are first metabolically [³⁵S] radiolabeled as follows:

1. Cells are grown aerobically at 40°C to mid-exponential phase in *Hfx. volcanii* medium, harvested and resuspended in minimal medium (Ortenberg et al., 2000) containing all standard amino acids, each at a final concentration of 40 µg/ml, to an OD₅₅₀ = 0.6, and grown for 24 hr.
2. Transfer the cells to minimal medium lacking methionine and cysteine for 1 h and then radiolabel with 15 µCi [³⁵S] labelling mixture per ml for 90 min.
3. One ml aliquots of the labeled cells are harvested (3,000 rpm in a microfuge, 3 min, 4 °C), the supernatants are removed and the pelleted cells are resuspended in 1 ml solubilization buffer (1% Triton X-100, 1.8 M NaCl, 50 mM Tris-HCl, pH 7.2) containing 3 µg/ml DNase.
4. Gently rock the solubilized mixture for 10 min at room temperature (RT)
5. Add 50 µl of a 10% (w/v) solution of cellulose.
6. After a 60 min nutation at RT, the suspension is centrifuged (3,000 rpm, 3 min), the supernatant is discarded and the cellulose pellet is washed with 2 M NaCl, 50 mM Tris-HCl, pH 7.2.
7. Repeat the washing procedure twice.
8. Centrifuge the cellulose beads (5,000 rpm, 3 min), remove the supernatant and resuspend the cellulose pellet in 40 µl SDS-PAGE sample buffer (4% SDS, 20% (v/v) glycerol, 0.02% bromophenol blue, 1.5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8).
9. Boil the samples for 5 min, centrifuge to pellet the cellulose (5,000 rpm, 5 min). Analyse the released proteins (in the supernatant) by 15% SDS-PAGE and fluorography using Kodak X-Omat x-ray film.

NOTES:

6.9 Storage of halobacteria

The simplest method is to just leave the cultures on the bench or in the refrigerator (not the -20C freezer!). Cultures in well sealed glass bottles, or plates sealed with plastic, will last a long time just left on the bench. You can tell a halobacterial lab by the number of cultures just left lying about. These eventually lyse (1-2 yr), so it is best to play safe and store them properly. Storing in 20-50% glycerol at -20C is not to be recommended as they tend to lyse even more quickly than if left on the bench. Frozen solid in glycerol/sucrose at -70C (in spheroplasting solution) appears good for at least 2 years. Liquid nitrogen storage (15% glycerol added to growth medium) is excellent and very simple, but requires a convenient and well-maintained storage tank. They preserve for at least 15 years.

6.9.1 Freezing cells in liquid nitrogen.

1. Late exponential phase growth in liquid medium
2. Label a cryotube (e.g. 1ml Nunc #366656) containing some sterile glass beads. Put the plastic insert in the top of the lid and label with the strain number
3. Transfer 500 μ l of culture to the cryotube and add 120 μ l of 80% glycerol
4. Mix well to blend glycerol, and to get liquid into holes of glass beads
5. Place in liquid nitrogen tray
6. Fill in the details in the culture collection !

To recover cells from liquid nitrogen:

1. Sterilise forceps (straight ones, not bent at ends), by dipping in ethanol and flaming
2. Allow to cool, keep sterile by placing upside down in rack
3. Go to liquid nitrogen storage tank and carefully open tray and remove tube
4. Using the sterile end of the forceps, grab one or two of the glass beads from the cryotube and immediately drop them into culture medium (labeled).
5. Put cryotube back, and incubate culture appropriately. Should take at least 3 days to grow up fully.

The methods above work for both E.coli and halobacteria. Halobacteria do not freeze-dry at all well, and even the refinement called 'L-drying' (see below) is not as good as freezing (in my experience- MDS).

6.9.2 Storage at -80°C

See section 3.1 for freezing competent *Haloferax* spp. and *Haloarcula hispanica* cells. The method below was contributed by Thorsten Allers (2004) and is a simple way of storing laboratory strains of haloarchaea.

For storage of *Hfx. volcanii* stocks

1. To 7.5 ml of fresh overnight culture ($\text{OD}_{600} = 0.8-1.0$)
2. Add 2.5 ml 80% glycerol, 6% SW (recipe below)
3. Mix and snap-freeze on dry ice. Works well.

80% Glycerol, 6% SW solution

For 100 ml mix

Glycerol	80 ml
30% SW	20 ml
<i>Autoclave</i>	
When cool, aseptically add	200 μl of (sterile) 0.5 M CaCl_2

6.9.3 L-Drying (liquid drying)

Contributed by M. Kamekura (Noda Inst., Japan). A more complex method is L-drying (L is for liquid, as you dry the culture directly from the liquid state). The advantages of this are that cultures can be kept at room temperature, or in a 4°C refrigerator, they can be mailed to others very simply, and they keep a very long time. If a large number of skim milk ‘sponges’ or ‘plugs’ are made first, then the rest is very simple, only requiring a freeze-drier. It works, but freezing in liq. nitrogen is far easier.

1. Make a 20% skim milk suspension (Difco Bacto), i.e. dissolve 10 g in 30 ml water. Then make to 50 ml.
2. Dispense into cotton-plugged small tubes (previously sterilised by heating at 160°C for 2 hr) to about 5mm depth
3. Autoclave
4. Snap freeze the tubes in ethanol-dry-ice (leave 10 min) and transfer to a freeze-drying machine. Leave under vacuum in freeze-drier overnight.

5. Store in a desiccator.

To store a culture:

1. Harvest cells from 20 ml of culture, and resuspend in 1ml of the following suspension solution:

per 100ml

Monosodium glutamate (.1H ₂ O)	10 g
Adonitol (Adonite)	1.5 g
D-Sorbitol	2.0 g
Sodium Thioglycollate	0.05 g
NaCl	20 g

Made up in 0.1M phosphate buffer, pH 7. Sterilized by filtration (0.45µm).

2. Place 1 – 2 drops of the resuspended culture on a skim milk 'sponge' and place in a freeze-drier. (Drying should take very little time - 30 – 60 min)
3. Put the small tube inside a larger test tube and heat seal,

To open a culture:

1. Open the outer tube by heating the top in a bunsen burner, then put a drop of water on the glass.
2. Take out the inner glass tube and add a small amount of fresh growth medium to the skim milk sponge. Resuspend using a Pasteur pipette.
3. Transfer the contents to a shake flask with medium and incubate standing at 37°C until turbid

7.0 Reagents, Solutions, Drugs

7.1 Purification of Polyethylene Glycol PEG₆₀₀ for transformations.

Polyethylene glycol (average MW of 600) is purchased from Sigma, and purified by the method of (Klebe et al., 1983), as outlined by (Cline et al., 1989a). This involves large volumes of toxic organic solvents (both flammable and toxic), and should be performed in a fume hood. Store the purified material at 4°C (where it is a white solid). When required it is warmed (40-50°C) to convert it to a clear viscous liquid.

***NOTE:** at the 2003 GRC meeting on Archaea, an informal discussion on this point was almost unanimous that PEG purification wasn't worth the effort and danger. Maybe the PEG sold nowadays is of higher purity. In any case, check your PEG without purification. If it is OK for your purposes, just use it (but store it carefully, frozen and sealed. You don't want any oxidation products).

Example of purification protocol as described by Klebe et al. (1983).

Perform all steps under good ventilation in a fume hood. Benzene is toxic, Iso-octane and diethyl ether are highly flammable. Discard solvents appropriately.

1. Weigh out 100 g of PEG₆₀₀ into a 1 or 2 L vacuum flask
2. Add 200 ml of benzene to dissolve the PEG. Mix well.
3. Add 200 ml of iso-octane (2,2,4-trimethylpentane), mix and put on ice for at least 1 hour.

The liquid will separate into two phases. On ice, the lower phase will become like a gel, and the top phase (which is just solvent) will be able to be poured off and discarded (appropriately; i.e. in an organic solvent discard in the fume hood).

4. Pour off and discard the liquid supernatant.
5. Take flask off the ice and add 100 ml of benzene to dissolve the PEG layer.
6. Estimate the volume and add 5 volumes of diethyl ether, stir and let mix overnight at 4°C to precipitate the PEG completely (a white, flocculent precipitate). Put a Buchner funnel, 1-2 L vacuum flask and about 500 ml of diethyl ether in the -20°C freezer to cool down.

Work fast, and use cold ether with the cold Buchner funnel and Whatman type 4 filter paper. This is because the PEG will become liquid when it warms up to room temperature.

7. Set up the vacuum flask (on ice) with the funnel and filter paper and hook up the vacuum line. Filter the precipitate and wash with cold ether.
8. Let go under suction until all the ether has been removed, or until the PEG begins to melt. Scrape the PEG into a small beaker and remove most of the residual ether in a vacuum desiccator for several hours.

The final fluid should be clear and viscous, and smell only slightly of ether. Yields vary but should not be less than 50%. Store at 4°C in tightly capped containers. For use, warm to 30-40°C to melt.

There is probably a simpler method to purify PEG: please let me know if you find one! I know Ford Doolittle's lab simply asked one supplier for a number of samples from various batches, and happened to find one batch that worked fine without further purification.

Other labs tell me they just don't bother and put up with reduced transformation rates. The safety issues involved in handling ether and benzene (both FLAMMABLE and TOXIC) are now quite onerous. I recently paid a chemistry graduate student to do the purification; they couldn't find a better method in the literature. Alternatively, just see how the PEG works out of the package. You may not need to purify.

7.2 DRUGS for use media

DRUG	MW	STOCK	STORAGE	Final Concentration
Novobiocin	634	1mg/ml in water	-20°C	0.3 µg/ml
Mevinolin Simvastatin	404	20 mg/ml in ethanol	-20°C	2-4 µg/ml ^b
Trimethoprim	290	20 mg/ml in water	-20°C	2µg/ml ^c
Bleomycin SO ₄	1414	Stock in water	4°C	4 µg/ml ^d
Pseudomonic Acid ^f	485	10 mg/ml in water	-20°C	5 µg/ml ^e

^a based on selection of *Hfx. volcanii* transformants in top agar.

Note a 10mM solution of Simvastatin = 4.04 mg/ml (but 3.41 mg/ml for Mevinolin).

^b 2-3 µg/ml for *Ha hispanica*, 5 µg/ml for *Hfx. volcanii*.

^c see below for more details

^d MIC for *Hfx. volcanii* is 1.25µg/ml. (Nuttall *et al.*, 2000).

^e MIC of pseudomonic acid for wild-type *Hfx. volcanii* is approx. 0.1µg/ml

^f Mupirocin

Mevinolin (lovastatin) and **Simvastatin** (zocor), are cholesterol-lowering drugs (for human use), and are sold by Merck (MSD) in tablet form. They inhibit the enzyme HMG CoA reductase (hydroxy methyl glutaryl coenzymeA reductase). The active drug can be readily extracted from the tablets by crushing and dissolving in ethanol, warming to 37°C (10 min) and then centrifuging the insoluble filler material (e.g. 10,000 g for 5 min). The clear supernatant can be stored at -20°C for months. I haven't tried the new versions, e.g. Fluvastatin

Often the drug will come out of solution at -20°C, but will dissolve again when warmed up to 37°C for several minutes. It is possible to ask the company for a small amount of pure drug (as a gift), but this takes a lot of effort and time (and they require you to sign a highly restrictive form for the privilege), and it is much easier to buy the drug. **NOTE** a 10 mM solution of Simvastatin = 4.18 mg/ml (but 3.88 mg/ml for Mevinolin). A simple google search will give you more details about these compounds.

Novobiocin (inhibits DNA gyrase) (from Sigma Chemical Co., N1628). It is very cheap and easy to obtain in pure form. Soluble in water. Stable at elevated temperatures. MW of the sodium salt is 634

Trimethoprim (inhibits dihydrofolate reductase). Cheap and easy to obtain. Works even in rich media. A trimethoprim-resistance gene has been developed by Rosenshine *et al.* (Mol Gen Genet (1987) 208:518-522). Many halobacteria are **insensitive** to trimethoprim so its applicability is limited (but it also works for *Hfx. mediterranei*). See, (Ortenberg *et al.*, 2000) and references cited within.

Pseudomonic acid/Mupirocin, from Beecham Research Labs. The tablets are sold under the brand name

Bactroban, but it is better to get the pure drug. Contact the company representative for this but be prepared to fill in a form and wait several weeks. Soluble in water.

Heat resistance of antibiotics - see the study (Peteranderl et al., 1990) by Peteranderl et al. (1990) Appl. Env. Microbiol. 56:1981-1983. They give degradation constants for novobiocin, trimethoprim, bacitracin and several others, but not for Mevinolin. Novobiocin is very heat resistant.

7.3 Molecular Weights of salts and buffers commonly used in media etc.

Compound	MW	Compound	MW
Ca	40	MgCl ₂ .6H ₂ O	203.3
CaCl ₂ .2H ₂ O	147	MgSO ₄ .7H ₂ O	246.5
KCl	74.55	NaCl	58.5
K ₂ HPO ₄	174.18	Tris buffer	121.1
Mg	24.3		

7.4 Codon usage for halobacteria

The following web site keep codon usage for many organisms; including halobacteria.

<http://www.kazusa.or.jp/codon/>

The genome sequences of a few haloarchaea are now available. As can be seen by the example of the *Halobacterium salinarum* data below, the high GC content of this genome (66%) produces a heavy bias towards GC rich codons.

Halobacterium sp. NRC-1 [gbbct]: 2605 CDS's (732624 codons)

fields: (2) [amino acid] [fraction] [frequency: per thousand] ([number])

UUU	F	0.08	2.4	UCU	S	0.03	1.8	UAU	Y	0.12	3.1	UGU	C	0.45	3.4
UUC	F	0.92	28.9	UCC	S	0.22	11.8	UAC	Y	0.88	22.7	UGC	C	0.55	4.2
UUA	L	0.01	1.1	UCA	S	0.06	3.0	UAA	*	0.23	0.8	UGA	*	0.49	1.7
UUG	L	0.08	7.0	UCG	S	0.34	18.3	UAG	*	0.28	1.0	UGG	W	1.00	11.0
CUU	L	0.05	4.6	CCU	P	0.04	1.6	CAU	H	0.11	2.5	CGU	R	0.08	5.2
CUC	L	0.48	40.7	CCC	P	0.36	16.9	CAC	H	0.89	19.8	CGC	R	0.47	30.9
CUA	L	0.02	1.6	CCA	P	0.10	4.6	CAA	Q	0.18	4.9	CGA	R	0.09	6.1
CUG	L	0.36	30.4	CCG	P	0.50	23.4	CAG	Q	0.82	22.9	CGG	R	0.32	20.6
AUU	I	0.09	3.5	ACU	T	0.06	3.7	AAU	N	0.10	2.2	AGU	S	0.06	3.5
AUC	I	0.88	33.0	ACC	T	0.44	29.7	AAC	N	0.90	20.1	AGC	S	0.29	15.4
AUA	I	0.03	1.1	ACA	T	0.07	5.0	AAA	K	0.36	6.4	AGA	R	0.02	1.2
AUG	M	1.00	17.3	ACG	T	0.43	29.3	AAG	K	0.64	11.5	AGG	R	0.02	1.4
GUU	V	0.08	7.6	GCU	A	0.05	6.6	GAU	D	0.14	12.0	GGU	G	0.10	8.3
GUC	V	0.52	48.3	GCC	A	0.42	52.2	GAC	D	0.86	76.6	GGC	G	0.56	46.0
GUA	V	0.03	2.6	GCA	A	0.08	9.5	GAA	E	0.27	18.8	GGA	G	0.07	5.9
GUG	V	0.37	34.0	GCG	A	0.45	55.3	GAG	E	0.73	51.0	GGG	G	0.27	21.9

Coding GC 66.88% 1st letter GC 69.34% 2nd letter GC 45.96% 3rd letter GC 85.35%

See also the web sites listed in the introductory **section 1**, which have links to the haloarchaeal genome sequence projects that are being undertaken, or completed.

A good web site for analysing codon usage, comparing codon usage between organisms etc is the GCUA site below. It gives very nice graphical outputs.

<http://gcu.schoedl.de/index.html>

8.0 Microscopy

Wet preparations are performed as usual, just make sure you seal the edges of the coverslip to prevent evaporation, which causes salt crystallization rather quickly. Gram staining is more difficult, as most halobacteria have only thin protein coats (S-layer) and will change shape upon drying or worse, lyse in low salt. However, salt needs to be removed as upon drying, the salt crystals that form will occlude everything. Most publications have used phase-contrast microscopy to show the morphology of halobacteria. Scanning EM gives beautiful results; see figs 25.25 and 25.31 in the latest Bergey's Manual, 2001, from (Grant and Larsen, 1990). Transmission EM of subcellular structures and some (not all) haloviruses can also be difficult because of the salt issue e.g. haloviruses **HF1 and HF2** (Nuttall and Dyall-Smith, 1993). Note that pure cultures of many halobacteria can have different shapes; for example *Haloarcula* can display geometric forms (triangles) as well as short rods.

8.1.1 Fixing and Staining halobacteria for light microscopic examination

This is a very old method, but is quick and still widely used. Since peptidoglycan is not found in haloarchaea, and most species have a simple S-layer, the use of Gram staining is not very useful as a distinguishing characteristic. For routine use, such as checking culture purity, phase contrast of wet preparations, or fluorescence staining, are better.

Ref: H.P. Dussault (1955) An improved technique for staining red halophilic bacteria. J. Bact. 70:484-5.

“The organisms are first suspended in sterile 20 per cent brine by mixing thoroughly in order to break up the culture, which usually is slimy. One large loopful of this suspension is thinly spread on a clean slide. After being air dried, the slide is fixed and desalted simultaneously by immersing in 2 per cent acetic acid for 5 minutes. Then it is removed and dried unwashed. This is important since washed slides have shown poor results. The smear is covered with 0.25 per cent aqueous solution of crystal violet for 3 minutes. After washing and drying the slide is ready for examination. Gram staining is also successful using basic fuchsin as the counter-stain.”

8.1.2 Phase contrast light microscopic examination on agarose coated slides.

Contributed by Thorsten Allers, Univ. Nottingham, UK, 2005.

Note (MDS) This is an adaptation of the normal use of agar/agarose coated microscope slides (usually dried on before use). The trick being that adding culture fluid with bacteria leads to the agar swelling up as it absorbs water and trapping live cells between slide and coverslip so they cells held still long enough to get clear photographs. I found the usual method for bacteria didn't work, as the high salt culture fluid didn't wet or get absorbed well by the agarose, and even after waiting a considerable time the cells were not tightly trapped. On top of all this, the agar slides gave me less contrast (under phase contrast) than wet preparation on plain glass slides. Thorsten's method is a great improvement.

This sticks cells of haloarchaea to a flat surface, so they end up in the same focal plane.

1. Make up the 1% agarose in 18% SW as follows
 - i. Melt 0.1 g agarose in 4 ml water
 - ii. Add 6 ml of hot 30% SW to make up to 10 ml 18% SW

Both of these steps can be done, very carefully, in a microwave oven. You may find a safer method (please let me know). I do each tube separately, and with the cap loosely sitting on top of the 10 ml plastic tube. You are talking about seconds here, usually about 10 secs for the first heating, then 4 second bursts until the agarose is melted or the SW is hot (not boiling, just hot). I wait until the end of a short burst or when the cap is knocked off by the steam. Sometimes you can't get the last bit of agarose at the bottom of a conical plastic 10ml tube to melt; just use the 90% that is molten and mix with the SW. It works.

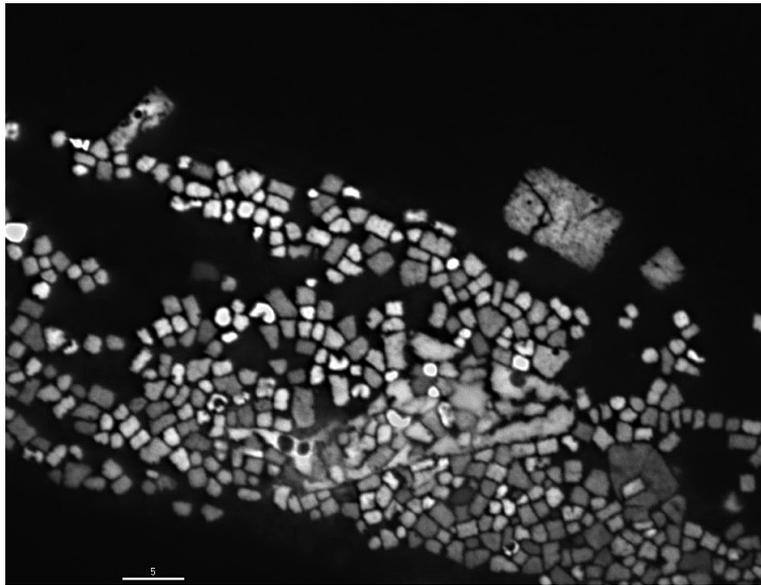
2. Mix the two solutions and pour ~500 µl evenly across one or more glass microscope slides. A glass pipette or a micropipette are both fine to use, just make sure it is an even spread, so it will set with a perfectly even surface.
3. Allow the agarose to set on a level surface. This only takes a minute. Use within 15 – 30 min (or they will dry out).

You can prepare a number of slides and keep them for later on the same day by storing them in a humid chamber (such as a plastic box with a wet tissue stuck on the inside of the lid). I've used slides up to 2 days later. Just touch a tissue to the extruded water drops at the edges of the agarose. If you use 2 day old slides, just beware of salt precipitates. Maybe a SW mix without Mg/Ca would be better. You can also store the molten agarose in a 50C oven for at least a week.

4. Place a 10 µl drop of culture fluid and a cover slip as normal. Only press *lightly* on the cover slip

(or gas vesicles will break, or the coverslip!).

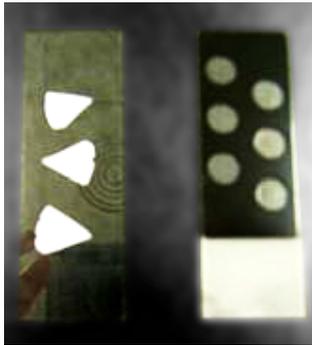
*Be careful when focusing down on the cells or the objective lens may stick through the coverslip and into the agarose. Most microscopes have the objectives set so they can't smash through a normal glass slide, but the agarose layer on your slides sits up higher than a normal slide, so take extra care. Phase contrast is good, as are DNA stains like Acridine Orange and Hoechst 33342 (both in the presence of PPD as antifade). DAPI doesn't work well at high salt. A typical picture is shown below (a novel *Haloquadratum* isolate, Bajool9, AO stained. MDS, 2008).*



8.2 Fluorescent in-situ hybridisation (FISH)

Contributed by Josefa Antón and Fernando Santos, (Division de Microbiología, Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, 03080 Alicante, Spain) in July 2006, and updated in Sept 2007. Email: anton (AT) ua.es

There are basically two ways to do the fixation and the hybridization, on filters or on slides. There are advantages and disadvantages for each method:



On filters:

Pros: good for counting, fast preparation of the samples for fixation

Cons: worse pictures, the processing of many samples/probes is more cumbersome

On slides:

Pros: easier, faster, nice pictures of the squares, good for processing different samples

Cons: not appropriate for counting, preparation of samples takes longer

Below is the protocol that was developed at the MPI (Max-Planck Inst) of Marine Microbiology in Bremen, so most of the steps of the protocol (and tricks) are the ones they use there.

8.2.1 FISH ON FILTERS

Fixation:

1. Add 22 μ l of formaldehyde (37%, good quality) to 100 μ l of sample and incubate o/n at 4°C.

This volume of sample is appropriate for crystallizers with around 10^7 cels/ml. If you don't know the cell concentration, fix 1 ml and filter different volumes (e.g. 50, 100, 200 and 450 μ l plus PBS, as explained below, in different filters).

2. Add 1 \times PBS up to 10 ml and filter on GTTP 02500 Millipore (0.2 μ m pore size, 2.4 cm diameter) filters.
3. Wash with 10 ml of PBS (on the same filtration device) and air dry. Filters can be kept at -20°C for a long time (at least months, but we do not know exactly how much longer).

Hybridization:

1. Prepare 2 ml of the hyb buffer according to the probe to be used.

See recipes at the end of the protocol). Normally new probes for halophiles need a lot of formamide.

2. Prepare the probe. It can be diluted when you receive it and stored at -20°C .

At MPI they check probe concentration by spectrophotometry, which is the right thing to do... we do not do it). We normally use probes labeled with Cy3. When we do doubles, we use also fluoresce.

3. Cut the filter in pieces (the size depends on the number of hybs you want to do with the same sample).

It is important to keep to filter upside (cells on top) and to "label" the pieces of filter (by doing small marks with the scissors, see picture, needs steady hands and small scissors). Put the filters on slides. From now on keep flat all the time!!

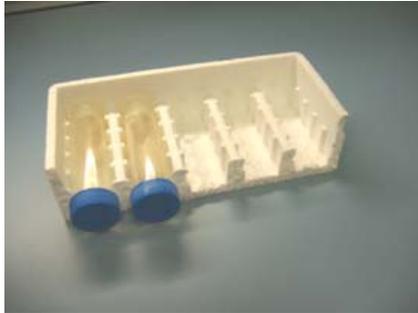


4. Overlay 18 μl of hyb buffer on top of the pieces of filter, add 2 μl of probe (50 ng/ml), mix very carefully.

5. Prepare the isotonic humid chamber: a 50 ml Corning plastic tube (or similar) tube with a piece of toilet paper (see picture) inside. Soak the paper with the rest of the hyb buffer (you only use a few μl but prepare 2 ml). Then, carefully, introduce the slide with the pieces of filter plus hyb buffer and probes on top of the paper. Keep flat!



- Put the tubes on a “device” made with the boxes that normally come with the disposable spectrophotometer cuvetes (see picture below).



- Incubate tubes for 2 h at 46°C. We use an hyb oven.
- Prepare 50 ml of washing buffer (according to the formamide concentration used in the hyb) in a plastic tube. Get the filter with forceps and put it in the buffer.

You can put several pieces of filter in the same tube (make sure that every piece has different marks). Do not mix filters that have been hybridised with different formamide concentrations, of course.

- Wash 2x 15 minutes at 48°C.
- Take the filter from the tubes (can be a bit tricky, just have to practice), put them flat (cells up) over filter paper and allow to dry.
- Do DAPI staining. On a petri dish put one drop (or as many as you need) of DAPI (0.5-1.0 µl/ml), put the filter on top of the drop (cells facing down) for 1 min. Then take the filter with forceps, and wash in water and ethanol (you can have two 50 ml tubes, one with water and the other with ethanol prepared in advance). Watch out, don't lose the filter in the tube.
- Air dry.
- Put the filter/s on a slide, add one small drop of citifluor on top of the filter, put a cover slide and seal. Go to the microscope and see... !

8.2.2 FISH ON SLIDES

1. Prepare agarose 2% in filtered water, NaCl 29% filtered, PBS (10 mM phosphate, 0.130 M NaCl) filtered, PBS-high salt (29% NaCl, 10 mM phosphate) and FA in PBS-high salt (10 ml PBS-high salt plus 2 ml of 37% FA).
2. Mix 500 μ l agarose with 1500 μ l PBS-high salt. Keep at 50-55°C
3. Mix 1 vol of sample (for instance 1 ml of crystallizer water) with 1 vol of the agarose with salt. This mixture must be very homogeneous and with no bubbles.
4. Add 15 μ l to each “well” of the slide (see reference below and picture at the beginning). Allow to dry for half an hour or less if the sample is getting too dry (you will see the salt crystals).
5. Put 100 μ l of FA with salts on top of the spot of sample. Put the slide inside a petri dish and incubate o/n at 4°C.
6. Drain the FA from the slide, and wash in PBS (for instance, drop the slide in a beaker with 500 ml of PBS, and shake carefully).
7. Air dry, and dehydrate in EtOH (3 min in 50%, 3 min 80%, and 3 min in 100% EtOH)
8. Keep at RT until needed.
9. Hyb, wash, etc. as explained above. (Do DAPI directly on the slide).

8.2.3 Reagents, solutions, etc. for FISH

All the solutions must be filtered

Hybridization buffer

360 μ l of 5M NaCl

40 μ l of 1M Tris-HCl, pH 8.0

x μ l formamide (it depends on the probe)

fill to 2 ml with mQ water

Washing buffer

1 ml of 1M Tris-HCl, pH 8.0

y μ l of 5M NaCl (see table)

500 μ l 0.5 M EDTA

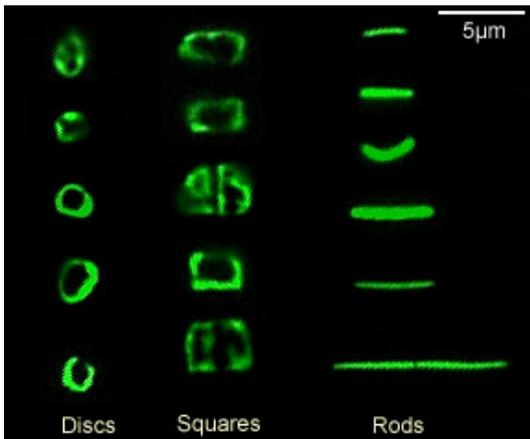
fill to 50 ml with mQ water

Slides:

Marienfeld Superior 12151301 (Made in Germany). Microscope slide 75 \times 25 \times 1 mm, half-white, twin-frosted 20 mm at the left side, 6 fields of 8 mm diameter, in two rows, staggered (Amann type), colour black.

% formamide in hyb buffer	y μl of 5M NaCl
0	9000
5	6300
10	4500
15	3180
20	2150
25	1490
30	1020
35	700
40	460
45	300
50	180
55	100
60	40
65	-

8.3 Acridine Orange Fluorescent staining of halobacteria



This is a quick and simple way of looking at unfixed halobacterial cells. Morphology seems to be retained well and the fluorescence is very bright. Check out the results on my web site (e.g. picture at left. A collage of discs, squares and rods. Oil immersion, 100× obj. MDS 2001).

The method below is based on one that is published on the web site of J. Fuhrman

(www.usc.edu/dept/LAS/biosci/faculty/fuhrman.html). He cites (Noble and Fuhrman, 1998). If you want a nice PDF protocol for AO staining of soil bacteria, see <http://hrcweb.lv-hrc.nevada.edu/qa/IP/IPLV-006.pdf>

Tip one: you can't use DABCO as an anti-fade compound, it precipitates at the high salt concentrations required for halobacteria. I found PPD (para-phenylene diamine) to be fine. A comparison of antifade agents for confocal microscopy (J. Histochem. Cytochem. (2001) 49:305-12) found that PPD was the second best antifade agent, with the top being the molecular probes agent, ProLong.

The recipe for the PPD solution is:

Antifade mounting solution:

- 50% glycerol
- 50% HVD (18% SW, 10mM Tris 7.2)
- 0.1% p-phenylenediamine*

**The PPD should be made fresh DAILY from a frozen 10% stock of the p-phenylenediamine (made in water). I just thaw out a frozen stock, remove what I need, then freeze it again. Seems to last a long time (>1 yr). You will know if it has been left too long on the bench, it goes brown on exposure to light.*

1. Centrifuge 1ml of salt lake water sample to concentrate the cells (2 min, max speed in microfuge).

This may break gas vesicles and cell clusters (e.g. square bacteria). Use non-centrifuged material if possible.

2. Take out 900 μl of the liquid and resuspend the cells in the remaining 100 μl
3. Add an equal volume of anti-fade mounting fluid and mix.

I have also got good results by adding 10 μl of 10% stock PPD (dissolved in water, or 50% glycerol) to the sample and mixing quickly.

4. Add Acridine Orange stain (2–5 μl of 1 mg/ml in water), mix and leave for 1-2 min.

Just enough so that the sample is faintly orange. AO stocks gradually aggregate and may need to be filtered or replaced or the aggregates will make even staining of cells difficult.

5. Spread 5 μl on an agarose-coated glass slide and cover with a cover slip. Seal edges with mounting solution.

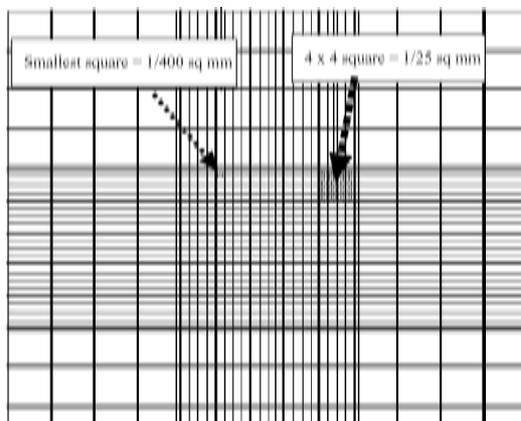
Actually, the dry agarose-coating doesn't always work that well. It is supposed to soak up water and help immobilize cells, but in high salt it doesn't take up much, if any water, and doesn't seem to immobilize cells much. Be very careful when pressing down on the coverslip, as you may burst any gas vesicles. Thorsten's method, using freshly prepared platforms of wet agarose/salts, 8.1.2., works much better, and is worth the trickiness of slide preparation. You get beautiful, flat cells that can be readily photographed.

6. Examine by epifluorescence using the correct filter set. Confocal microscopy is also possible.

8.4 Direct counts of halobacteria using a standard (Neubauer) haemocytometer.

There is a nice web site to help with calculations of direct cell counts using haemocytometer slides at:

<http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html>



I use a Neubauer slide with the smallest squares being 0.05×0.05 mm (i.e. 50 μm each side). It is important to stain the cells with acridine orange so that you can easily count all the cells. Phase contrast is not sufficient as you miss the very thin squares and even though most cells are single, some exist in small clumps, and you can't count the number of cells in clumps without fluorescence.

Follow the usual instructions for counting cells over a number of squares (sufficient for a total of over 30, but

around 100 is better for statistical reasons). You would expect a visibly turbid salt lake sample (e.g. a light pink, moderately turbid crystallizer pond sample) to have at least 10^7 cells per ml.

To use oil immersion with your 100× objective you need to keep the coverslip stable. I use a small dab of quick setting mounting solution on each side of the coverslip (and rub it off with a tissue immediately after counting). The coverslip to slide depth in this counter is 0.1mm, and each smallest square is 0.05mm (or 1/20 mm) each side (= 1/400 mm²). If you count several (smallest) squares worth of cells, and get an average number of cells per smallest square (N), then the actual cell concentration in the sample is N x 400 x 10,000 cells/ml. For example, say you counted all the cells in 10 smallest squares and the average number of cells per smallest square is 5.3, then the cell concentration is:

$$5.3 \times 400 \times 10^4 = 2120 \times 10^4 = 2.12 \times 10^7 \text{ cells/ml.}$$

Now, you need to know how many cells you are missing! Using fluorescence, rack the fine focus up and down to see how many cells are above the focus of the grid. I've found the total number of cells over a grid square is about 7 fold higher than you can see when counting with the grid in focus. Check it out and compensate for this, then do the calculation above. If you find this does not work for you, there are other methods, for example, staining with DAPI and spreading out a known volume on a slide and counting several fields. Or, filtering onto the cells onto gridded filters and counting the cells by epifluorescence.

8.4 Electronmicroscopy of halobacteria and halovirus particles

The main difficulties are:

- a) disruption of particles due to low salt, e.g. HF1 (and possibly low Ca²⁺)

We have overcome this problem by glutaraldehyde fixation (1-2%) for 1-2 hours (may need longer).

Others have used 10mM CaCl₂ to stabilize cell S-layers.

- b) poor adsorption to plastic coated grids (e.g. with *Fuselloviridae* isolate **His1**, see (Bath and Dyall-Smith, 1998).

Overcome by pretreatment of plastic-coated grids with a solution of poly-L-Lysine (0.1mg/ml).

We prefer 2% uranyl acetate as a negative stain.

8.4.1 A simple negative-staining method.

Contributed by Dr Valery Tarasov (March, 1999) for looking at Hbt. salinarum. He used it to look at flagella (and mutants in the fla genes) and I was surprised it worked so well considering there is no fixation of the cells. Cells and flagella were remained intact. Have a look at the results in (Tarasov et al., 2000).

1. A sample of the culture (OD₆₀₀ 1 – 1.2) is diluted 5-9 fold in basal salt solution.
2. The (plastic coated) grid is placed at the top of a drop of diluted culture medium and left for 1.5 – 2 min.
3. The grid is then placed on top of a drop of 2% uranyl acetate and left for 1-1.5 min.
4. Excess stain is removed by touching the grid to filter-paper, and the grid is air dried.

Some **EM** studies of haloarchaea.

a) S-layer preparations examined by various EM methods. Fortunately the S-layers were stable in 10 mM CaCl₂. The introductions to both papers give useful background. (Kessel et al., 1988b)(Kessel et al., 1988a)

b) Flagellar bundles of *Hbt. salinarum*. (Kupper et al., 1994).

This study also shows negative stain preps of disrupted cells (after 2% glutaraldehyde fixation they were dialyzed against tap water).

c) See the scanning EM pictures in Bergey's Manual, 2nd ed, 2001. They are very good.

9.0 Contributors, Phylogenetic Tree, and References

CONTRIBUTORS

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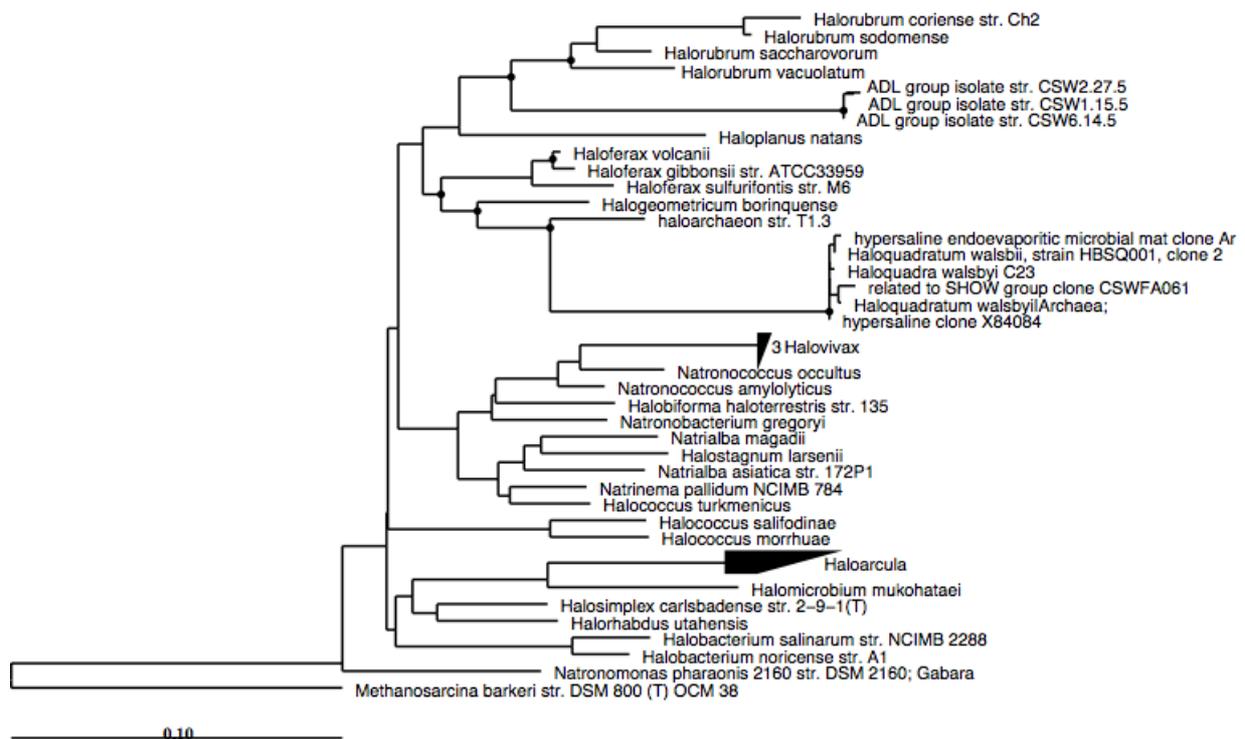
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Phylogenetic reconstruction of Halobacteriaceae



M.D-S., 2006. ML tree with bootstrap values above 75% indicated by closed circles.

The latest review on haloarchaeal taxonomy is in Bergeys Manual, 2nd Edn., 2001.

The 3-letter abbreviations of genera are: Haloarcula (Har.), Halobacterium (Hbt.), Halobaculum (Hbl.), Halobiforma (Hbf.), Halococcus (Hcc.), Haloferax (Hfx.), Halogeometricum (Hgm.), Halomicrobium (Hmc.), Haloquadratum (Hqr.), Halorhabdus (Hrd.), Halorubrum (Hrr.), Halosimplex (Hsx.), Haloterrigena (Htg.), Natrialba (Nab.), Natrinema (Nnm.), Natronobacterium (Nbt.), Natronococcus (Ncc.), Natronomonas (Nm.), Natronorubrum (Nrr.)

For the ADL (Deep Lake) clone group, see (Bowman et al., 2000) and (Burns et al., 2004a). The isolates of the ADL group will be published soon.

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