M9 Medium

Minimal defined media provide no more than the simplest energy, carbon, nitrogen, phosphorus, sulfur, and metal sources for growth. Because metabolites must be made from scratch, growth is slow on minimal media. Strains that can grow without other molecules in the medium can thus be selected for growth. Additives to a medium may be needed depending on a strain's auxotrophies and growth conditions. Most minimal media are also *defined*, lacking components that are molecularly heterogeneous, thus allowing better knowledge of composition and reproducibility across labs, as heterogeneous components can vary more from different manufacturers and batches. Yeast extract, peptone/tryptone, and casamino acids provide a diverse, heterogeneous source of amino acids, cofactors, and other metabolites, and thus make a medium neither *minimal* nor truly *defined*.

M9 minimal medium is a popular formulation for bacteria including *E. coli*, with low osmolarity (0.24 Osm/L). Sodium and potassium phosphate salts provide Na, K, P. Ammonium chloride is the N source. Magnesium sulfate provides Mg and S. Calcium chloride is a common component, though probably not needed in most cases. Glucose and glycerol are commonly selected carbon sources. An alternative to M9 is M63, which has more N and K [2,3]. Non-essential growth enhancers, such as casamino acids, make it no longer minimal nor truly defined, but casamino acids is a purer source of acid-hydrolyzed casein and is thus accepted as a common additive to minimal media to improve growth, when selection for amino acid prototrophy is not important.

M9 media formulations are also common to use for their low autofluorescence compared to rich media, owing to the lack of yeast extract and digested protein source (tryptone/peptone/etc). This may have benefits for fluorescence measurements or microscopy. Casamino acids (as with any source of amino acids) adds a lower amount of autofluorescence, but is often acceptable as the growth rate improvement is of value or essential.

Materials

1. **M9 salt soln**, autoclaved
    a. 10× M9 salts, autoclaved
        i. *Neidhardt formula*: [5]
        60 g/L Na_2HPO_4 (anhydrous, dibasic), or 113.3 g/L $Na_2HPO_4 \cdot 7H_2O$ (heptahydrate); 422.65 mM.
        Commercial formula:
        67.8 g/L Na_2HPO_4 (anhydrous, dibasic); 477.60 mM.
        CSHL formula: Discrepancies. See ref 1 b, c.
        ii. **30 g/L KH_2PO_4** (monobasic); 220.44 mM.
        iii. **5 g/L NaCl**; 85.56 mM.
        iv. **10 g/L NH_4Cl**; 186.95 mM.
        v. Milli-Q water: 938.61 g or up to 1 L. See Note 2.
    b. 5× M9 salts, autoclaved
        i. 30 g/L Na_2HPO_4 (anhydrous, dibasic), or 35 g/L $Na_2HPO_4 \cdot 7H_2O$ (heptahydrate)
        ii. 15 g/L KH_2PO_4 (monobasic)
        iii. 2.5 g/L NaCl
        iv. 5 g/L NH_4Cl_2
2. **Carbon source**, either autoclaved or filter-sterilized.
    - Glycerol, typically 50%$_{V/V}$ (630.7 g/L), 50%$_{m/V}$ (500 g/L), or 5 M (460.45 g/L).
    - Glucose, typically 50%$_{m/V}$ (500 g/L) or 2.5 M (450.4 g/L). Ideally stored at 4°C.[1]
    - *50% is better than 20%, as lower water activity inhibits contamination.*
    - *Molar concentrations are preferable. Glycerol should ideally be used at same mass concentration (g/L) as glucose, but twice the molarity to account for it having half the carbon content.*
3. **$MgSO_4$**, autoclaved. Typically 1 M or 2 M.
    - *2 M is a bit more convenient for spiking into other media, too.*
    - Anhydrous MW: 120.37 g/mol. 2 M $MgSO_4$: 240.74 g/L.
    - Heptahydrate MW: 246.48 g/mol. 2 M $MgSO_4 \cdot H_2O$: 492.96 g/L.
4. **$CaCl_2$**, autoclaved. Typically 0.1 M or 1 M.
    - *Lower 0.1 M stock is better, as it is used at 0.1 mM final, and more dilute Ca^{2+} reduce chances of precipitation upon addition to phosphate or sulfate solution.*
    - ** Can be omitted; E. coli has no requirement for Ca^{2+}s upplementation; trace amounts are sufficient. MOPS medium uses 0.5 µM out of precaution [5]. Omission prevents $Ca_3(PO_4)_2$ and $CaSO_4$ precipitation.*

Protocol

	Final conc.	Stock volume
Milli-Q water	–	up to 1 L
$CaCl_2$	• 0.1 mM • *or 0.5 µM or none**	• 1.0 mL/L **0.1 M $CaCl_2$** • 0.1 mL/L **1 M $CaCl_2$**
$MgSO_4$	• 1 mM	• 0.5 mL/L **2 M $MgSO_4$** • 1.0 mL/L **1 M $MgSO_4$**
M9 salts	• 1×	• 100 mL/L **10× M9 salts** • 200 mL/L **5× M9 salts**
Glucose Glycerol	• 0.4% *m/V* • *22 mM* • 1% *m/V* • *56 mM* • 20 or 50 mM • 0.4% *m/V* • *43 mM* • 1% *m/V* • *109 mM* • 40 or 100 mM • 1% *V/V* • *137 mM*	• 8 mL/L **50% glucose (500 g/L)** • 20 mL/L **50% glucose (500 g/L)** • 8 or 20 mL/L **2.5 M glucose (450.4 g/L)** • 8 mL/L **50%$_{m/V}$ glycerol (500 g/L)** • 20 mL/L **50%$_{m/V}$ glycerol (500 g/L)** • 8 or 20 mL/L **5 M glycerol (460.45 g/L)** • 20 mL/L **50%$_{V/V}$ glycerol (500 mL/L, 630.7 g/L)**
Casamino acids	• 0.2% *m/V* (2 g/L)	• 20 mL/L **10%$_{m/V}$ casamino acids (100 g/L)**
Other	?	?
Antibiotics	?	?

1. Calculate ingredient volumes and combine:
To a sterile bottle, add water. Then add $CaCl_2$ and $MgSO_4$ in

- Anhydrous MW: 110.98 g/mol. 0.1 M $CaCl_2$: 11.098 g /L.
- Dihydrate MW: 147.01 g/mol. 0.1 M $CaCl_2 \cdot 2H_2O$: 14.701 g/L.

5. (opt.) **Casamino acids**, typically 100 g/L (10% $_{m/V}$) autoclaved or (preferred) filter-sterilized
   - *Greatly increases growth rate and alleviates gene expression stress.*
   - *Autoclaving will greatly darken yellow color, increase autofluorescence, and reduce quality. Filter-sterilize and keep frozen or at 4° to minimize its initial autofluorescence and its darkening over time.*
   - *Has all amino acids except tryptophan, glutamine, and asparagine, which get degraded during casein acid hydrolysis. Auxotrophs of these need supplementation.*
6. (opt.) **Other Nutrients**: Vitamins, Amino Acids, Trace Metals. Required for certain auxotrophic species/strains or growth conditions, in which a certain type of metabolism might require certain trace metals. See genotype for auxotrophies.
   - *e.g.* TG1/Turbo and DH5 require thiamine, usually supplemented with 0.1 mg/L 0.3 mM; DH10B requires leucine (present in casamino acids).
7. (opt.) **Antibiotic(s)**, 1000×
   - Use to maintain selection of plasmids or genomic marker, with secondary benefit of counterselecting contaminants.
8. **Milli-Q water**, autoclaved

that order, closing the bottle and swirling *each time* to mix. Then in any order, add antibiotics, casamino acids, carbon source, and M9 salts, and swirl at the end.
   a. *Note: everything added is sterile, so with proper aseptic technique the final solution is sterile.*
   b. *Color should be clear to slightly yellow. Casamino acids adds yellow tint, which darkens with age and heat.*
2. Cold 4°C, dark storage preserves labile nutrients (amino acids, vitamins) the best, though a dark cabinet is sufficient for usage over a few weeks.
   - *Precipitates may form during storage over months. Omitting $CaCl_2$ may solve this, as $Ca_3(PO_4)_2$ and $CaSO_4$ precipitation are thought to be the primary cause, though $Mg_3(PO_4)_2$ might also precipitate. Thorough mixing of Ca and Mg salts in water before adding PO_4 from M9 salts prevents precipitation during preparation. As these salts exhibit exothermic dissolution, cold preparation and storage might benefit their solubility.*

Note 1: autoclaving should be done for a minimal time to minimize reaction/degradation of labile nutrients. A 15 min liquid cycle is sufficient for volumes 1 L or less.
Phosphate and other salts are best autoclaved separately from agar to minimize H_2O_2 (4).

Note 2: Calculation of 10× M9 salts volume:
Na2HPO4: 67.8 g, 1.7 g/mL (anhydrous)
KH2PO4: 30 g, 2.37 g/mL
NaCl: 5 g, 2.16 g/mL
NH4Cl: 10 g, 1.53 g/mL
112.8 g total
$V_{salts,soln}$ = (67.8/1.7)+(30/2.37)+(5/2.16)+(10/1.53) = 61.39 mL
V_{water} = 1000–61.39 = 938.61 mL

1. http://cshprotocols.cshlp.org/content/2010/8/pdb.rec12295.full
   b: https://cshprotocols.cshlp.org/content/2006/1/pdb.rec614.full
   c: https://cshprotocols.cshlp.org/content/2009/10/pdb.rec11973.full
2. M63 medium "is similar in composition to M9 medium base by compound type. However, it has increased amounts of phosphate and ammonium relative to M9, and nearly an order of magnitude more sulfate and potassium. M63 also adds iron, which is not among the nutrients added to make M9." https://biocyc.org/ECOLI/NEW-IMAGE?type=Growth-Media&object=MIX0-48
3. Escherichia coli K-12 substr. MG1655 M63 medium base (biocyc.org)
4. Reguera, Gemma. "The Great Plate Count Anomaly" that is no more." *Small Things Considered*, https://schaechter.asmblog.org/schaechter/2014/12/the-great-plate-count-anomaly-that-is-no-more.html. https://doi.org/10.1128/aem.02741-14
5. Neidhardt, Frederick C., Philip L. Bloch, and David F. Smith. "Culture medium for enterobacteria." *Journal of bacteriology* 119.3 (1974): 736-747. http://doi.org/10.1128/jb.119.3.736-747.1974